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Molecular Identification of Endospore-Forming Rhizobacteria from Organic Cabbage Farm Potential as Biocontrol against Phytopathogen *Xanthomonas campestris*

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Rhizobacteria are rhizosphere competent bacteria that colonize and proliferate in all the ecological niches found on the plant roots at all stages of plant growth, in the presence of a competing microflora. These bacteria are potential as biological control agent to inhibit the growth of phytopathogen. The aim of this study was to isolate endospore-forming rhizobacteria from cabbage farm and determine its ability as biocontrol against *Xanthomonas campestri*, a pathogen causing black rot on cabbage. The methods used consisted of isolation, antibacterial activity test, biochemical characterization and molecular identification. Fourteen isolates of endospore-forming rhizobacteria were obtained from cabbage farming. Isolate K.9 had the highest ability to inhibit the growth of *X. campestri.* Based on molecular characterization by sequence analyses of 16S rRNA, isolate K9 had 97% homology with *Bacillus cereus* strains BF15.

Key words: *Bacillus cereus,* biocontrol, endospore-forming rhizobacteria*, Xanthomonas campestris*

Rhizobakteri merupakan bakteri yang hidup disekitar perakaran tanaman, yang mampu mengkoloni dan berkembang disemua tahap pertumbuhan akar tanaman dan berkompetisi dengan bakteri lainnya. Bakteri ini berpotensi sebagai agen biokontrol untuk menghambat pertumbuhan pathogen tanaman. Penelitian ini bertujuan mengisolasi rhizobakteri pembentuk endospora dari tanaman kubis serta menguji kemampuan isolat tersebut untuk menghambat pertumbuhan patogen *Xanthomonas campestris* penyebab penyakit busuk hitam pada kubis. Metode yang digunakan meliputi isolasi bakteri, karakterisasi isolat bakteri secara morfologi, uji antibakteri, identifikasi secara molekuler dengan 16S rRNA, dan uji biokimia. Hasil isolasi diperoleh empat belas isolat rhizobakteri dan terdapat empat isolat yang memiliki potensi sebagai agen bakterisida terhadap patogen *X. campestris.* Isolat K.9 memiliki daya hambat terbesar terhadap *X. Campestris* yaitu 12,6 mm. Identifikasi secara molekuler berdasar analisis 16S rRNA menunjukkan isolate K.9 mempunyai homolog 97% dengan *Bacillus cereus* BF15.

Kata kunci: *Bacillus cereus,* biokontrol, rhizobacteria pembentuk endospora, *Xanthomonas campestris*

Cabbage has an important nutritional value as a source of vitamin A and C, protein, lipid, carbohydrates and fiber needed by human body (Sastrosiswojo *et al.* 2005). Production of cabbage in Indonesia had been steadily increasing from 1 323 702 ton/Ha in 2008 to 1 432 318 ton/Ha in 2012 (Directorate General of Horticulture 2013). However, cabbage production is hampered by diseases and pests that reduce the quality and quantity of the cabbage.

One of the major diseases of cabbage in Indonesia and other Asian countries is black rot which is caused by *Xanthomonas campestris.* The leaves of cabbage attacked by *X. campestris* shows black spots and in short time the plant will die simultaneously (Semangun 1989).The use of pesticides has been done to eradicate the problems of disease in plants, as it is considered effective and fast. However, increasing use of chemicals causes several negative effects, e.g. development of pathogen resistance to the applied agents as well as other environmental impacts (Compant *et al*. 2005).

The harmful effects of chemical pesticides has forced the search for environmentally friendly biocontrol to eliminate the diseases in plants. Extensive research has demonstrated that plant growth promoting Rhizobacteria (PGPR) could have an important role in agriculture and horticulture in improving crop productivity. While diverse microbes may contribute to the biological control of plant pathogens, most research and development efforts have focused on isolates of three genera, *Bacillus*, *Trichoderma*, and

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Pseudomonas (McSpadden Gardener and Driks 2004).

Rhizobacteria are rhizosphere competent bacteria that colonize and proliferate in all the ecological niches found on the plant roots at all stages of plant growth, in the presence of competing microflora (Antoun and Kloepper 2001). Endospore-forming rhizobacteria is one of the successful biocontrol agent commercially produced and used against plantpathogens. Ryu *et al.* (2005) elucidated the benefit of endospore-forming rhizobacteria that is more stable under unfavorable condition. In addition, endospore makes the rhizobacteria easier to be applied and commercialized as biopesticide with long duration of storage. The advantage of endospore-forming rhizobacteria as biopesticide led to research towards the finding of new isolates potential as biocontrol agents. The aims of this study were to isolate endospore-forming rhizobacteria from organic cabbage farm, determine its ability against *X. campestri* and identify based on molecular methods.

MATERIALS AND METHODS

Isolation of Endospore-Forming Rhizobacteria. Endospore-forming rhizobacteria were isolated from organic cabbage farm in Banyubiru, Semarang, Central Java. Isolation method was modified from Ryu *et al.* (2005). Five grams rhizosphere soil was placed in 100 mL flask containing of 45 mL sterile distilled water, and then shaken and heat-treated at 80 ^{O}C for 30 m. Afterwards, the suspension was 10-fold serially diluted in sterile aquadest and plated in tryptic soy agar (TSA) medium. After 24-48 h of incubation at room temperature, single-colony isolates were characterized. Bacteria was selected based on the morphological characteristics including color, form, elevation, and margin of the colony. The pure cultures were kept in slant agar**.**

Antibacterial Activity Test. *X. campestri* was obtained from Laboratory of Pest and Plant Disease Brawijaya University. Isolate of endospore-forming rhizobacteria and *X. campestri* were grown in 5 mL tryptic soy broth medium overnight at 30 ^{O}C on rotary shaker at 120 rpm. One mL of the *X. campestri* was then used to inoculate 100 mL TSA (40 °C), and evenly shaken. Fifteen mL of the inoculated TSA was poured into petri dish and cooled down, and 5 paper discs were placed on the dish. Fifteen µL of endospore-forming rhizobacteria isolate was dropped on the paper disc and the dish was incubated at 30 °C for 48 h. The antibacterial test was determined by the formation of clear zone.

DNA Extraction. DNA extraction was done by Chelex method (Walsh *et al.* 2013). Three loops overnight culture of endospore-forming bacteria were placed in a tube containing 100 µL ddH_2O. 1 mL 0,5 % saponin was added. Then, it was incubated overnight at 4 °C before being centrifuged for 10 m at 12.000 rpm. The supernatant was removed, and 100 µL ddH_2O and 50 µL chelex 20% was added to the pellet. The mixture was then boiled for 10 m with vortexing every 5 m, and centrifuged. The DNA would be in the supernatant and kept at 4 °C.

DNA Amplification. DNA amplification was performed using the following program: pre denaturation at 95 °C for 3 m, denaturation at 94 °C for 1 m, annealing at 55 °C for 1 m, extention at 72 °C for 1 m, and the final extention at 72 °C for 7 m. Primers used were 27F (5'-AGAGTTTGATC**M**TGGCTCAG-3') and 1492R (5'-GG**Y**TACCTTGTTACGACTT-3') (Osborne *et al.* 2005). The PCR mixture contained 3 µL DNA template, 1.5 µL of each primer, 25 µL KAPA 2GFAST Kits, and 19 µL ddH_2O. Visualization of the PCR product was done using gel electrophoresis with 1 % agarose concentration run at 100V for 30 m. The band of PCR product was seen on gel documentation.

Sequences Analyze. Sequencing of PCR product was done at PT. Genetika Science, Indonesia. The sequence was analyzed by base alignment using Basic Local Alignment Search Tool (BLAST) to establish the percentage of base pair similarity with reference isolates from the GenBank.

Phylogenetic Tree. Phylogenetic tree was constructed using MEGA 5 software. Phylogenetic tree was constructed using Neighbour-joining tree and Bootstrap method.

RESULTS

Seventeen endospore-forming rhizobacteria has been isolated from the rhizosphere of cabbage grown on organic farming. Each isolate was characterized based on colony characteristic, morphology of the cell and Gram stain. Table 1 shows the characteristic of colony and cell of the isolates of endospore-forming rhizobacteria. All of the isolates were then tested their antagonistic activity against *X. campestris*, a causative agent of black rot disease. Out of seventeen isolates tested, two isolates demonstrated their antagonistic activity showed by inhibition zone toward *X. campestris*. Amongst those two isolates, isolate K-9 showed the highest antagonistic activity (Table 2),

hence, will be preceded or further molecular identification.

DNA extraction of K-9 isolate was done using Chelex method. The DNA obtained was then used for molecular identification based on 16S rRNA. Figure 2 shows visualization of the amplified 16S rRNA gene of K-9 isolate on 1% agarose gel. The PCR product of 16S rRNA gene was sequenced and obtaining sequence of 1360 bp length. The sequence of K-9 was analyzed using Basic Local Alignment Search Tool (BLAST) program. The result showed that the sequence had 97% homology with *Bacillus cereus* strain BF15. Phylogenetic tree of the K-9 isolate showing appropriate affiliation is depicted in Fig 3.

DISCUSSION

Finding potential rhizobacteria that have the ability to eliminate plant pathogens is a great interest for the farmers and agricultural industries. The consumers demand agricultural products containing less pesticide residues. Therefore, the discovery of potent biocontrol agents against pythopathogens is highly needed. One of the sources of biocontrol agents is rhizobacteria, especially endospore-forming bacteria. Endopsoreforming bacteria have advantages in term of application and formulation compared to non endospore-forming bacteria (Ryu *et al.* 2005). Slepecky and Hemphill (2006) stated that heating the sample at 80 °C for 30 m was enough to isolate endospore-forming bacteria, as vegetative cells of bacteria generally die at heat treatment of 60-70 °C.

The highest ability of isolate K-9 in suppressing the growth of phytopathogen *X. campestri* amongst other isolates showed that this isolate has unique secondary metabolites. McSpadden Gardener, 2004 stated that many strains of endospore-forming *Bacillus* and *Pseudomonas* showed ability to suppress pests and pathogens or otherwise promote plant growth. Allabouvette *et al*, 2006 supported the idea that antibiosis is a mechanism used by biocontrols, like *Bacillus* spp., *Trichoderma* spp., *Pseudomonas* spp., and *Streptomyces* spp., due to the production of antibiotic, bacteriocin, degradation of cell wall enzyme, and other volatile compounds.

Based on 16S rRNA molecular identification, the

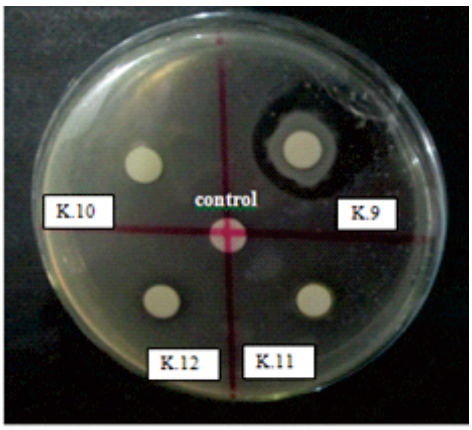

Fig 1 Antagonistic activity of endospore-forming rhizobacteria isolates against *X. campestris*.

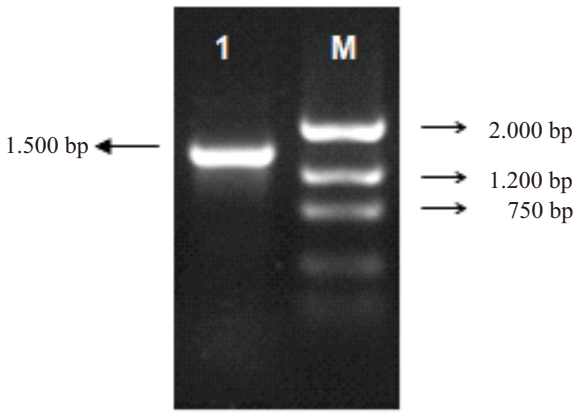

Fig 2 Visualization of amplified 16S rRNA gene from K-9 isolate in 1% agarose gel using *low molecular mass ladder* (1 = K-9, M = *marker*).

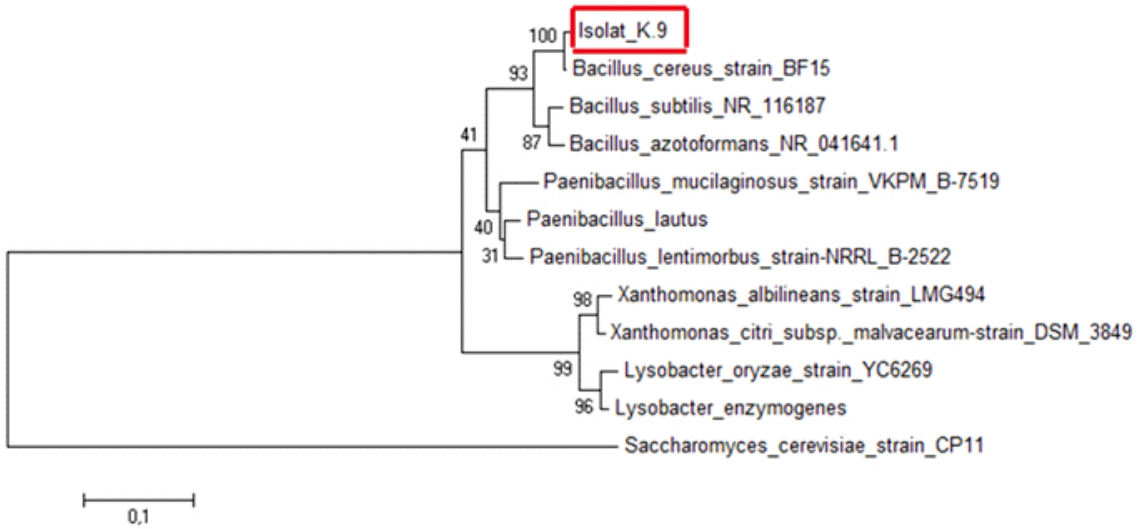

Fig 3 Phylogenetic tree of isolate K-9.

Table 1 Colony and cell morphology of endospore-forming rhizobacteria from rhizosphere of cabbage

Isolate	Colony characteristic				Cell form	Gram
	Color	Form	Margin	Elevation		
K-1	Ivory	Irregular	Lobate	Flat	Rod	Positive
K-2	Cream	Circular	Entire	Raised	Rod	Positive
K-3	White	Filamentous	Filiform	Flat	Rod	Positive
K-4	Ivory	Circular	Serrate	Raised	Rod	Positive
K-5	Ivory	Circular	Entire	Raised	Rod	Positive
K-6	Cream	Rhizoid	Filiform	Flat	Rod	Positive
K-7	Transparent	Circular	Entire	Flat	Rod	Positive
K-8	White	Circular	Undulate	Flat	Rod	Positive
K-9	Cream	Circular	Entire	Raised	Rod	Positive
K-10	Orange	Circular	Entire	Raised	Rod	Positive
K-11	Yellow	Circular	Entire	Raised	Rod	Positive
K-12	Ivory	Irregular	Undulate	Flat	Rod	Positive
K-13	Ivory	Circular	Serrate	Raised	Rod	Positive
K-14	Ivory	Circular	Entire	Flat	Rod	Positive
K-15	White	Circular	Undulate	Flat	Rod	Positive
K-16	Yellow	Circular	Entire	Raised	Rod	Positive
K-17	Yellow	Circular	Entire	Raised	Rod	Positive

Table 2 Inhibition Zone produced by endospore-forming rhizobacteria isolates against *X. campestris*

Isolate	Inhibition zone (mm)
K-9	12.6
K-12	2.05

K-9 isolate was *Bacillus cereus* strain BF15. The 16S rRNA gene was used for molecular identification because this gene is present and conserved in all bacteria (Janda and Abbott 2007). Phylogenetic tree analysis of the K-9 isolate compared to other antibiotic-producing rhizobacteria, such as *Paenibacillus* and *Bacillus*, shows that K-9 isolate lies in the same clade with *B. subtilis* and *B. azotoformans* (Fig 3). This means that K-9 isolae is more closely related to these bacteria rather that to the genus *Paenibacillus*, which is in different clade.

Foldes *et al.* (2000) explained that many members of genus *Bacillus* produced various antibacterial compounds. The compounds include the lipopeptide antibiotics iturin A and surfactin, which could suppress damping-off in tomato, and zwittermicin A, which si correlated to suppression of damping-off in alfalfa. Hassi *et al* 2012, showed that *Bacillus* produces variety of antibiotics able to suppress the growth of gram positive and gram negative bacteria. However, *B. cereus* is well known for its pathogenicity in animals and human (Zhang *et al.* 2016). Hence, it will limit the application as biocontrol in agricultural systems.

In conclusion, endospore-forming rhizobacteria isolated from cabbage farm is potential as antibacterial compound against phytopathogen *X. campestris*. Based on 16S rRNA molecular identification, the isolate is *Bacillus cereus* strain BF15.

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