Characterization & identification endophytic bacteria from *Sorghum bicolor* (L.) Moench as plant growth promoting bacteria

Charlie Ester de Fretes, Donny Widianto, Yekti Asih Purwestri, Tri Rini Nuringtyas

Abstract— This study aims to characterize the ability of endophytic bacteria isolates in fixation N, phosphate solubilization, production of IAA and ACC-deaminase, and antagonists against fungal pathogens to be developed as biological fertilizer agents. Twenty isolates of endophytic bacteria from sweet sorghum were tested for their ability to promote plant growth. Isolates capable of N fixation were grown in the LGI medium and showed a change in medium color. In addition, nifH gene detection was also performed to determine the diazotroph endophytic bacteria. Screening of isolates for ability to produce IAA was carried out by adding of Salkowski reagent in bacteria culture and was measured quantitatively by spectrophotometer λ 530 nm. Testing of isolates capable of producing ACC-deaminase was performed using the LGI medium with the addition of ACC as the source of N. Bacteria isolates that capable to dissolve inorganic phosphate tested with Pikovskaya medium. Antagonism mechanism of endophytic bacterial isolates against pathogens was shown by inhibition of Fusarium fungus growth on PDA medium. Identification of potential endophytic bacteria as PGPB was carried out by sequencing the nucleotide of the 16S rRNA gene and aligned with the reference strain in the GenBank database. The results showed that five endophyte bacterial isolates were proven as diazotroph, six isolates were able to dissolve phosphate, two isolates were able to produce IAA, six isolates were able detection for ACC-deaminase activity and one isolates successfully inhibited the growth of pathogenic fungi. There are seven isolates of the genus *Beijerinckia*, *Achromobacter*, *Bacillus*, *Paenibacillus*, and *Staphylococcus* which have several plant growth promoting mechanisms. Endophytic bacterial isolates can potentially be used as biological fertilizer agents in solving environmental degradation problems due to the use of chemical fertilizers and pesticides.

Index Terms — endophytic bacteria, plant growth promoting bacteria, sequencing, 16S rRNA gene

1 Introduction

Groups of bacteria that can enhance plant growth and development include non-symbiotic bacteria, symbiotic bacteria, endophytic bacteria and cyanobacteria (Glick, 1995). Endophytic are microbes that part or all of their life cycles are in plant tissues but do not have a negative effect on host plants (Schulz and Boyle, 2006). Endophytic bacteria can be found in roots, stems, leaves, seeds, fruit, tubers, ovules, and legume nodules (Hallmann et al., 1997; Benhizia et al., 2004).

Beneficial interactions between endophytic bacteria and plants include bacteria getting nutrients, protection against environmental stress, and passive spread between hosts or vectors (Schulz and Boyle, 2006). Therefore, endophytic bacteria are better protected from biotic and abiotic stresses compared to rhizosphere bacteria (Hallmann et al., 1997). The benefits obtained by host plants are resistance to pathogenic microbes to be induced and increased growth in the presence of nitrogen fixation and fitohormon production (Schulz and Boyle, 2006). Symbiosis that occurs between plants and endophytic bacteria is that plants get nutrients and growth hormones that are needed to grow and protection from pathogens, while endophytic bacteria get nutrients from plant metabolism and protection against environmental stress. Secondary metabolites produced by endophytic bacteria and rhizobacteria in their host plants can affect the physiological development of plants and provide resistance to disease (Khan and Doty, 2009).

In this study endophytic bacterial isolates used were isolated from sweet sorghum plants. Globally, sweet sorghum since it is used for grain, forage, syrup, fodder, and bioethanol production (Almodares and Hadi 2009). Several studies have shown the association of sweet sorghum with several endophytic bacteria from the genera Herbaspirillum, Azospirillum, Klebsiella, Enterobacter, Burkholderia, Paenibacillus (Olivares et al., 1996; Budi et al., 1999; Zinniel et al., 2002; Gönemeyer et al., 2011), *Rhizobium*, *Achromobacter*, *Herbaspirillum*, *Ralstonia*, *Acinetobacter*, *Enterobacter*, *Pantoea*, *Pseudomonas*, *Serratia, Stenotrophomonas, Kocuria, Streptomyces, Brevibacillus, Bacillus, Paenibacillus, Staphylococcus*, and *Chryseobacterium* (Mareque et al., 2015).

2 Material and Methods

2.1 Endophytic bacteria from Sorghum bicolor cv. FS105

Twenty endophytic bacterial isolates used in this study were isolated from the tissues of sweet sorghum plants cv. FS501 is planted in two types of land from Piyungan and Playen, Gunung Kidul, Yogyakarta. Isolation of endophytic bacteria was carried out by the method by de Fretes et al. (2018).

2.2 Screening of biofertilization activities

N fixing endophytic bacteria test

Endophytic bacterial isolates were grown on semi-solid LGI medium (N-free medium) at 30°C. After 7 days of incubation,

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observations were made on the formation of the membrane/pellicle and discoloration of the tube as a marker of the occurrence of fixing N (Kirchhof et al., 1997). Diazotrophic detection isolates are made to target nifH PCR amplification using the PolF primers (5'-TGCGAYCCSAARGCBGACTC-3') and PolR (5'-ATSGCCATCATYTCRCCGGA-3'). The PCR mixture was 12.5 µL GoTaq reaction buffer, 9.5 µL ddH_2O, 0.8 µM of both sets of primers and 1 µL DNA template, in a final reaction volume of 25 µL. The PCR conditions are as follows: one cycle at 95°C for 5 minutes; 30 cycles at 95°C for 45 seconds for denaturating; 58°C for 45 seconds for annealing, 72°C for 30 seconds for extension, and a final cycle at 72°C for 5 minutes. The amplification products were analyzed by 1% (w/v) electrophoresis agarose gel in TBE buffer and stained with SYBR (Invitrogen).

Solubilization phosphate test

Testing of phosphate soluble by inoculating endophytic bacteria isolates in Pikovskaya medium (Afzal and Asghari, 2008). After 72 hours incubation, observations were made on the growth of isolates. In a medium containing phosphate solvent endophytic isolates there will be a halo clear area around the colony.

2.3 Enzyme production for phytostimulants

Test for indole-3-acetic acid producing

Testing of endophytic bacteria producing IAA was carried out quantitatively by the colorimetric method (Sarwar and Kreme, 1995). Endophytic bacterial isolates were inoculated in a test tube containing 5 mL TSB medium containing 1 mg / mL of L-tryptophan at pH 7.0 and incubated at room temperature for 24-48 hours by shaking. Then the culture was centrifuged at 13,000 rpm for 15 minutes. One milliliter of supernatant is mixed with 2 mL of Salkowski reagent (50 mL 35% $HClO_4$ and 1 mL 0.5 M $FeCl_3.6H_2O$) and left for ± 30 minutes for color development, the presence of IAA production is indicated by the appearance of pink. Quantitative analysis of IAA concentration was carried out by a spectrophotometer at λ 530 nm.

Test for acc-deaminase activity

Testing of endophytic bacteria producing ACC deaminase was carried out by growing bacteria on LGI + N medium for 48 hours. After that, the culture was centrifuged 10,000 rpm for 20 min. the supernatant was removed and the pellet was washed 2 times with the LGI medium. Washed cells were resuspended in 25 mL LGI. After that, 150 µL of the suspension was inoculated into a solid LGI medium with the addition of 30 mmol ACC as a source of N. The bacterial isolates that were able to grow on the medium were considered positive resulting in ACC deaminase.

2.4 Antagonism test for endophytic bacteria isolates

Antagonism test was carried out using pathogenic fusarium sorghum fungi. Endophytic bacterial isolates were grown in medium TS broth at 28°C for 24 hours. Antagonism activity was tested using PDA (Potato Dextrose Agar) media. Pathogenic fungi that were 5 days old were taken with a 5 mm diameter drill and placed in the middle of the Petri dish and incubated at 25°C for 72 hours. Then 100 µL of endophytic bacteria isolates were grown with pathogenic fungi and incubated at 25°C for 48 hours. Endophytic bacterial isolates capable of inhibiting the growth of pathogenic fungi will form clear zones around the isolates.

2.5 Identification of potential endophytic bacterial isolates with 16S rRNA gene sequences

The isolated endophytic bacteria were cultured in TS at room temperature for 24 h and centrifuged at 11000 g for 5 min. The genomic DNA was extracted from the pellet by using the FavorPrepTM Genomic DNA Isolation Kit. Amplification of the 16s rRNA gene was carried out using 27f forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492r (5'-TACGGHTACCTTGTTACGACTT-3'). DNA was amplified with the Biorad Thermal Cycler program as follows: denaturation at 94°C for 5 minutes, 30 cycles of denaturation (1 minute at 94°C), annealing (for 45 seconds at 55°C) and extension (for 2 minutes at 68°C) with the final extension of 10 minutes at 72°C. All amplified products were repaired using gel electrophoresis with 1% agarose in 1x TBE buffer for 30 minutes with a voltage of 100 V and colored with Sybr. PCR products are analyzed by order of their nitrogen bases using BigDye® Terminator v3.1 (1st Base Pte.Ltd, Singapore). The results of the analysis of the nitrogen base sequence of the 16s rRNA gene were then used as queries in BLAST at NCBI web site (McGinnis and Madden, 2004). Phylogentic analysis was conducted using the MEGA 5.0 software (Tamura et al., 2007). The neighbor-joining method was used to infer the tree topology and the reliability of the tree was tested by bootstrap with 1000 replicates.

3 Results & Discussion

Endophytic bacteria have been isolated from several tissues on sweet sorghum var. FS 501 which are sterilized on the surface. Then, the tissue pieces are macerated with a NaCl solution and planted on the TSA medium. Testing the ability of endophytic bacteria as plant growth promoting bacteria is expected to be used as biological fertilizer for the growth of graminae plants. The results of this research in Table 1 show that isolates of endophytic bacteria from sweet sorghum have the ability as plant growth promoting mechanism.

The results of this study indicate that there are endophytic bacterial isolates that have the ability to N fixing and phosphate solubilization. Of the 20 isolates tested, 5 and 6 isolates were able to fix N and soluble phosphate, respectively (Table 1). Thus, many PGPB can supply plants with substances that are essential for the proper growth of the plant. Some PGPB can fix atmospheric nitrogen and provide it to plants (Compant et al., 2005 and Watanabe et al., 1979). Research by Koomnok et al., 2007 reported that several genera of bacteria capable of fixing nitrogen include *Beijerinckia*.

The mechanism of phosphate solubilization by endophytic bacteria involves several enzymes, namely, C-P lyase, phosphatase and phytase. However, most microbial genera soluble phosphate through the production of organic acids such as

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gluconate, ketogluconate, acetate, lactate, oxalate, tartaric, succinate, citrate, and glycolate (Stella and Halimi, 2015). The type of organic acid produced to dissolve phosphate can depend on the source of carbon used as the substrate. Research shows that the highest solubilization P mechanism has been observed when using glucose, sucrose, or galactose as a single carbon source in the medium (Khan and Doty, 2009; Park et al., 2010). Solubilization and mineralization of phosphorus by PGPB enhances the bioavailability of soluble phosphorus for the plants and is considered an important plant growth-promotion mechanism under field conditions (Verma et al., 2001). Endophytic bacteria *Lysinibacillus fusiformis*, *Bacillus cereus* and *B. megaterium* isolated from ginseng plants also showed high P soluble activity (Vendan et al., 2010).

TABLE 1. PLANT GROWTH PROMOTION FEATURES OF ENDOPHYTIC BACTERIAL ISOLATES FROM SWEET SORGHUM CV. FS501.

Isolates	Plant growth promoting fiture				
	Diazo-troph	Solubiliza-tion phos-pate	IAA pro-duction (µg/mL)	ACC-deaminase activity	Antagonism *Fusarium*
PiA2	+	++	44,083	+	-
PiA30	+	-	-	+	-
PiA31	-	+	-	+	+
PiA35	-	+	-	+	-
PlA4	+	++	4,379	-	-
PlA10	+	+	-	-	-
PlA13	+	+	-	-	-

The results of this study indicate that there are endophytic bacterial isolates that have the ability to produce IAA, ACC-deaminase activity, and are able to inhibit the growth of pathogenic fungi *Fusarium*. Of the 20 isolates tested, 2 isolates were able to produce IAA between 4-44 µg/mL, 4 isolates had ACC-deaminase activity, while 1 isolate was able to inhibit the growth of *Fusarium* pathogenic fungi.

Some endophytic microorganisms have the potential to synthesize IAA. This may be a reason for the increased growth promotion of some plants when the plant is colonized with endophytes (Shi et al., 2009). For the microbial synthesis of IAA in tryptophan-dependent route, tryptophan is used as the precursor. There are different pathways that can lead to tryptophan-dependent microbial production of IAA. The various pathways for IAA biosynthesis include tryptophol, tryptamine, indole-3-pyruvic acid and indole-3-acetamide pathways (Gravel et al., 2007).

All isolates were screened for the production of ACC deaminase on LGI medium amended with ACC as nitrogen source. PiA2, PiA30, PiA31, and PiA35 was found to be positive for ACC deaminase production as indicated by its growth in the media. PGPB may also promote plant growth as a consequence of the action of bacteria expressing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (E.C. 4.1.99.4). ACC is the immediate precursor of ethylene in all higher plants. ACC deaminase is a multimeric enzyme that cleaves ACC to α-ketobutyrate and ammonia and thereby decreases ethylene levels in host plants (Sessitsch et al., 2005; Sun et al., 2009; Penrose et al., 2001; Glick, 2005). Glick et al. (2007) suggests that some microbes can utilize the ACC as nitrogen source from the exudates of roots or seeds. This decrease in the levels of ACC and ethylene may prevent the ethylene-mediated plant growth inhibition. Endophytic microbes with these capabilities residing inside the host plants can benefit the host by reducing the stress and increasing the plant growth (Hardoim et al., 2008).

Isolation of the 16S rRNA gene with a size of 1500 bp was successfully amplified in the DNA of 7 isolates endophytic bacteria (Figure 1). The results of sequencing and BLAST analysis of the 16S rRNA gene indicated that the endophytic bacteria belonged to the phyla Alphaproteobacter (*Beijerinckia*), Betaproteobacter (*Achromobacter*), and Firmicutes (*Bacillus*, *Paenibacillus*, *Staphyloccocus*). Phylogenetic tree analysis on the basis of 16S rRNA gene sequences of the 7 isolates that had PGP features are shown in Figure 2. Bacteria from the genus *Achromobacter*, *Paenibacillus*, *Bacillus*, and *Staphylococcus* are endophytes in sweet sorghum plants (Mareque et al., 2015). Koomnok et al. (2007) reported bacteria from the genus *Beijerinckia* as endophytic diazotroph in rice.

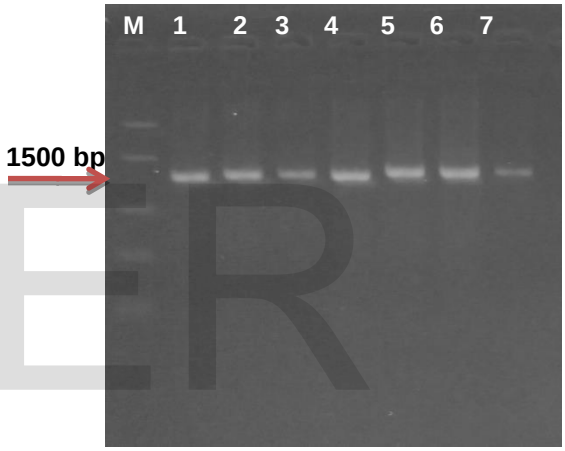

Figure 1. Visualization of the 16S rRNA gene from endophytic bacterial isolates with PGP. Lanes M. FastRuler DNA leader, 1. isolate PiA2, 2. isolate PiA30, 3. isolate PiA31, 4. isolate PiA35, 5. isolate PlA4, 6. isolate PlA10, 7. isolate PlA13

Endophytic bacteria *Lysinibacillus fusiformis*, *Bacillus cereus*, and *B. megaterium* isolated from ginseng plants showed high P soluble activity (Venden et al., 2010). Bacteria *Staphylococcus* isolated from the roots of maize is also reported to be capable of dissolving organic phosphate (Correa-Galeote et al., 2018). Research by Park et al. (2010) showed that *Bacillus fusiformis* was able to produce IAA and nitrogen fixation to improve plant growth. Endophytic bacteria *Bacillus* (Verma et al., 2001) and *Staphylococcus* (Correa-Galeote et al., 2018) were able to produce ACC-deaminase. Previous research also revealed that *Paenibacillus polymyxa* is an endophytic bacterium that produces active metabolites with antimicrobial and antifungal activity (Strobel et al., 2003).

In this study, the potential of endophytic isolates for the promotion of plant growth indicated by assessing factors such as nitrogen fixation, phosphate solubilization and IAA production. Although the isolates showed all the capabilities of growth-promoting, were positive for most of these characteristics, suggesting their role in promotion the growth of sweet

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sorghum. Their potential to be important to further study the growth-promoting microbial inoculants for growing sweet sorghum.

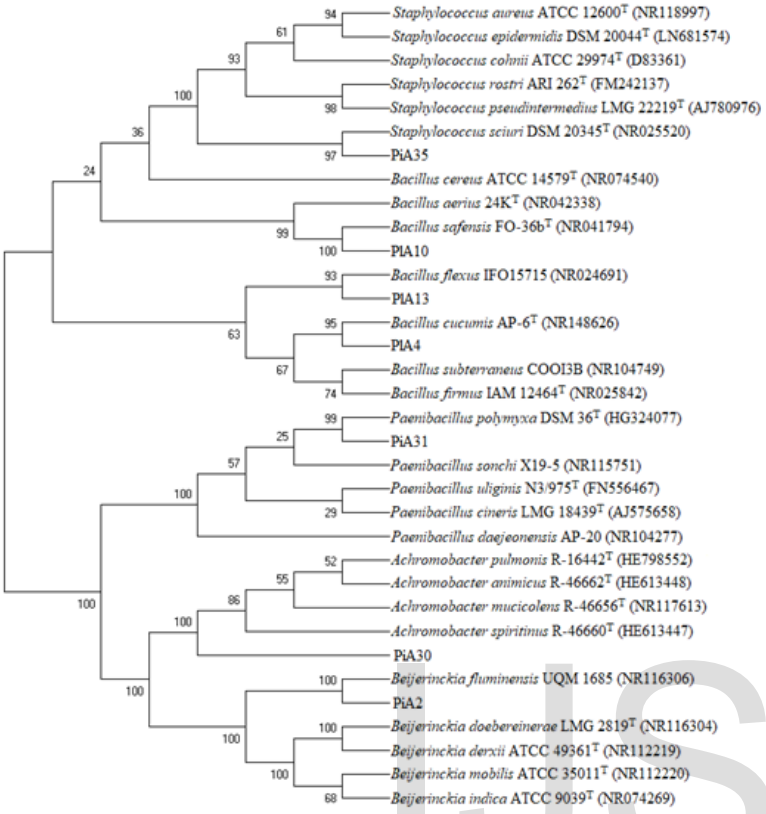

Figure 2. Phylogenetic tree based on 16S rRNA gene sequences of the endophytic bacteria of sweet sorghum cv. FS501 and other related genera using the neighbor-joining method. Bootstrap from 1000 replicates is indicated at the node.

4 Conclusions

This research demonstrated endophytic bacteria isolated from sweet sorghum cv. FS501 as plant growth promoting bacteria. There are seven isolates of the genus *Beijerinckia*, *Achromobacter*, *Bacillus, Paenibacillus*, and *Staphylococcus* which have several plant growth promoting mechanisms. Some of these bacteria have shown combined abilities for nitrogen fixation, phosphate solubilization, IAA production, ACC-deaminase activity and antagonism, which are some of the most important mechanisms required for the promotion of plant growth. These particular strains would be of commercial interest to biotechnology companies with a goal of producing an inoculant for sweet sorghum cultivars.

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