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Plant growth-promoting activity of endophytic bacteria from sweet sorghum (*Sorghum bicolor* (L.) Moench)

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ABSTRACT Application of high levels of chemical fertilizers for optimal growth of sweet sorghum causes environmental degradation. Plant growth-promoting bacteria have biotechnological importance because they can improve the growth and health of important agronomic plants. This study aimed to isolate, characterize, and identify endophytic bacteria associated with sweet sorghum (cv. KCS105), and also to study the inoculation effects of selected isolates on sorghum growth. In this study, 35 isolates were evaluated for their ability to support plant growth. The results showed that seven isolates were diazotrophic, six were capable of dissolving phosphate, six produced IAA and could detect ACC-deaminase activity, and three inhibited the growth of pathogenic fungi. Nine isolates exhibiting mechanisms for promoting plant growth from the Alphaproteobacteria (*Devosia*), Firmicutes (*Bacillus*, *Paenibacillus*, *Staphylococcus*), and Actinobacteria (*Microbacterium*, *Brachybacterium*) phyla were identified. In addition, the *Paenibacillus* sp. BB7, *Bacillus* sp. PIB1B, and *Bacillus* sp. PLB1B isolates showed increasing effects on plant growth in greenhouse tests. Endophytic bacterial isolates which display plant growth-promoting features can potentially be employed as biofertilizer agents. They may also address environmental damage problems resulting from the use of chemical fertilizers and pesticides.

KEYWORDS Endophytic bacteria; plant growth-promoting bacteria; sweet sorghum; 16S rRNA gene

1. Introduction

Globally, sweet sorghum is widely utilized for grain production for food, syrup, animal feed, and bioethanol (Almodares and Hadi 2009). However, the increasing demand for sweet sorghum has led to the excessive use of chemical fertilizers, which may cause unavoidable deleterious effects on the environment, including the groundwater. Therefore, innovation in sustainable agricultural technology by employing biological agents for chemical fertilizers and pesticides substitution is required (Pretty and Bharucha 2014).

Most endophytes are microbes that are living in the plant tissue but do not negatively affect the host plant. Some endophytes promote plant growth through plant growth stimulation mechanisms, including providing plants with needed resources/nutrients and modulating plant growth (Santoyo et al. 2016). It has been reported that endophytic bacteria are found in roots, stems, leaves, seeds, fruit, tubers, ovaries, and legume nodules (Hallmann et al. 1997). Several studies have reported that the sweet sorghum has interaction with several endophytic bacteria, such as genus *Rhizobium*, *Herbaspirillum*, *Enterobacter*, *Paenibacillus*, *Achromobacter*, *Ralstonia*, *Azospirillum*, *Acinetobacter*, *Klebsiella*, *Burkholderia*, *Pantoea*, *Pseudomonas*, *Streptomyces*, *Staphylococcus*, and *Chryseobacterium* (Grönemeyer et al. 2012; Mareque et al. 2015).

The host plants may benefit from increased growth by nitrogen fixation and phytohormone production and resistance to pathogenic microbes (Compant et al. 2010). In addition, the secondary metabolites produced by endophytic bacteria and rhizobacteria on their host plants affect the physiological development of plants and provide resistance to disease (Afzal et al. 2019). This symbiosis may allow the endophytic bacteria to obtain nutrients from plant metabolism and protect from environmental stresses. Therefore, the endophytic bacteria are better to protect from biotic and abiotic stress than the rhizosphere bacteria (Hallmann et al. 1997).

In this study, the endophytic bacterial were isolated from sweet sorghum KCS 105. Previous research showed that sweet sorghum var. KCS105 grown in dryland farming areas has the highest production of fresh biomass, dry

biomass, sugar stems, and ethanol compared to other varieties (Gusti et al. 2013). The use of high fertilizers follows the increased productivity of sweet sorghum. Therefore, the determination of biological agents is expected to reduce the use of chemical fertilizers. The previous report showed that the growth of sweet sorghum was significantly increased by the addition of potential bacterial supernatant plus humic acid compared to the use of chemical fertilizers (Afifi et al. 2014). This study aims to obtain endophytic bacteria capable of nitrogen fixation, phosphate dissolution, indole-3-acetic acid (IAA) and ACC-deaminase production, and antagonistic tests against pathogenic fungi so that they can be utilized as biofertilizers.

2. Materials and Methods

2.1. Isolation of endophytic bacteria from sweet sorghum

In this study, the endophytic bacteria were isolated from the roots, stems, and shoots of sweet sorghum cv. KCS105 and carried out using the improved method of de Fretes et al. (2018). Ten grams of plant parts were sterilized by soaking for 5 min in 70% EtOH, then immersed for 20 min in 4% sodium hypochlorite solution. Next, the plant material was rinsed four times with sterile deionized water. To ensure that the disinfection process is successful, the final rinse water was spread on Tryptic Soy Agar (TSA) and incubated at 28 °C for 5 d. The sterilization was considered successful if there was no bacterial growth. The sterilized plant material was macerated aseptically in 0.9% NaCl solution and inoculated on TSA medium followed by incubating at 28 °C for 5 d. The different colonies that grew on the medium were further purified by the quadrant streak technique on the TSA medium. The 50% glycerol stock from the pure culture of isolates was prepared and stored at -80 °C for preservation.

2.2. Nitrogen fixation ability and detection of nifH gene of endophytic bacteria

The endophytic bacterial isolates were cultivated on semi-solid LGI (N-free medium) at 30 °C (Mareque et al. 2015). After 7 d of incubation, observations were made on the formation of membrane/pellicle and color change in the tube as a marker of N fixation. Isolates that were positive on testing with LGI medium were subjected to test for diazotrophic properties by targeting nifH gene using primers of PolF (5'-TGCGAYCCSAARGCBGACTC-3') and PolR (5'-ATSGCCATCATYTCRCCGGA-3'). The PCR conditions were as follows; one cycle for 5 min at 95 °C; 30 cycles for 45 s at 95 °C for denaturation; for 45 s at 58 °C for annealing, for 30 s at 72 °C for an extension, and final cycle for 5 min at 72 °C. The amplification products were analyzed with 1% (w/v) electrophoresis agarose gel in TBE buffer and stained with SYBR (Invitrogen).

2.3. Phosphate solubilization activity of endophytic bacteria

The phosphate solubility test was conducted by inoculating endophytic bacterial isolates in the Pikovskaya medium. After 72 h of incubation, observations were made on the growth of the isolates. A clear halo area around the colony would be observed in a medium containing phosphate endophytic isolates.

2.4. IAA production of endophytic bacteria

The test for endophytic bacteria in producing IAA was quantitatively tested using the colorimetric method (Jasim et al. 2014b). The isolates were inoculated in a test tube containing 5 mL of Tryptic Soy Broth (TSB) media containing 1 mg/mL L-tryptophan at pH 7.0 and were incubated for 24-48 h at room temperature. Next, the culture was centrifuged at 13,000 rpm for 15 min. As much as 1 mL of supernatant was mixed with 2 mL of Salkowski reagent (50 mL of 35% $HClO_4$ and 1 mL of 0.5 M $FeCl_3.6H_2O$ solutions). The mixture was allowed to stand for ± 30 min for color development in a dark environment. The development of pink color indicated the production of IAA. The quantitative analysis of the IAA concentration was carried out using a spectrophotometer at λ 530 nm.

2.5. ACC deaminase activity of endophytic bacteria

The test for endophytic bacteria in producing ACC deaminase was carried out by growing the bacteria on LGI + N media for 48 h (Mareque et al. 2015). Next, the cultures were centrifuged at 10,000 rpm for 20 min. The supernatant was removed, and the pellets were washed twice with LGI media. The washed cells were again suspended in 25 mL of LGI. As much as 150 µL of suspension was inoculated into solid LGI media with addition of 30 mmol of ACC as a nitrogen source. The growing isolated bacteria were considered capable of producing ACC deaminase.

2.6. Antagonism test against pathogenic fungi

The antagonism test was conducted using the pathogenic fungus *Fusarium*. First, the bacterial isolates were grown in TSB media for 24 h at 28 ºC, and the antagonistic activity was evaluated using PDA media. The pathogenic fungi aged 5 d were taken with a 5 mm diameter plate, placed in the center of a Petri dish, and incubated for 72 h at 25 ºC. Next, the endophytic bacterial isolates were streaked on PDA plates and incubated for 48 h at 25 ºC. The endophytic bacterial isolates that could inhibit pathogenic fungi' growth would form a clear zone around the isolate.

2.7. Plant growth-promoting activity test of endophytic bacteria

The growth response of sweet sorghum cv. Numbu inoculated with endophytic bacteria was studied under greenhouse conditions. The surface-sterilized seeds were put into Erlenmeyer containing potential endophytic bacterial cultures (OD 600 nm = 1) and were incubated for 45 min

along with slow agitation. The inoculated seeds were germinated in 0.8% water agar for 2 d, transferred to a single-use Petri dish (Ø 20 cm) containing 250 g of sand as a substrate, and maintained in greenhouse conditions with a photoperiod of 8/16 h light/dark. The test had five treatments with six replications in a completely randomized design. The isolates tested as inoculants were *Paenibacillus* sp. BB7, *Bacillus* sp. PIB1B, and *Bacillus* sp. PLB1B. The treatment containing plants without inoculation was used as the negative control. Moreover, the plants inoculated in fertilized media were used as the positive control. At 28 experimental days, the plants were harvested and stem and root lengths were measured. In addition, the plants were dried at 60 °C to constant weight, then the dry weight was determined.

2.8. Identification of potential of endophytic bacteria with the sequence of 16S rRNA gene

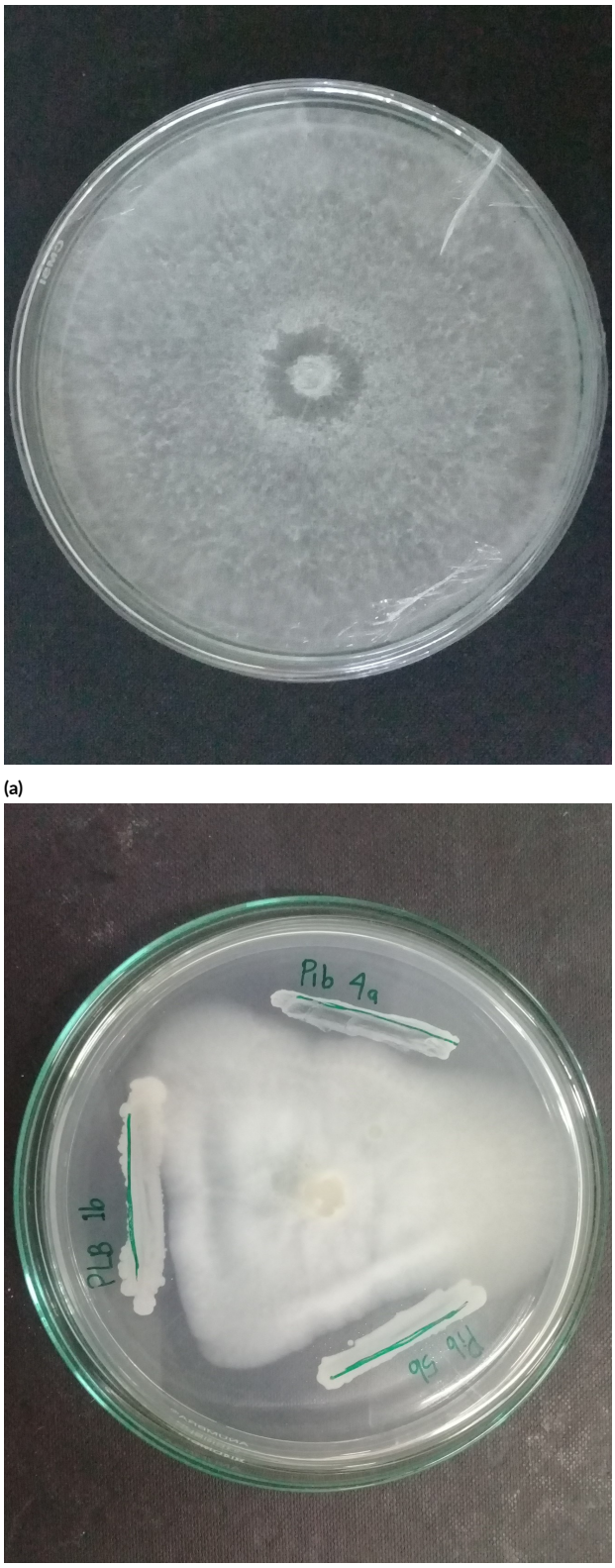
The potential endophytic bacteria were cultured in TS medium for 24 h at room temperature and centrifuged at 11,000 rpm for 5 min. The genomic DNA was extracted from pellets using FavorPrepTM Genomic DNA. The amplification of the 16S rRNA gene was performed with 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGHTACCTTGTTACGACTT-3'). DNA was amplified with the Biorad Thermal Cycler program as follows: denaturation at 94 °C for 5 min, 30 cycles of denaturation (at 94 °C for 1 min), annealing (at 55 °C for 45 s), and extension (at 68 °C for 2 min) with a final extension at 72 °C for 10 min. All amplified products were visualized using gel electrophoresis with 1% agarose in 1x TBE buffer and stained with SYBR. The PCR products were analyzed based on their nitrogen base sequence using BigDye® Terminator v3.1 (1st Base Pte. Ltd, Singapore). The results of the analysis of nitrogen base sequences of the 16S rRNA gene were then used as queries in the BLAST on the NCBI website. The phylogenetic analysis was performed using MEGA X software. The neighbor-joining method was used to construct the phylogenetic tree and the tree reliability was tested using bootstrapping with 1000 replications.

2.9. Data analysis

Analysis of variance was performed using SPSS 15 program, where the significant differences at $p < 0.05$ were followed by the Duncan test.

3. Results and Discussion

Thirty-five endophytic bacterial isolates were obtained from the roots, stems, and shoots of sweet sorghum cv. KCS105 consisted of 13, 11, and 11 isolates, respectively. Previous studies reported that higher population density and diversity of endophytic were found in plant roots than above-ground tissue and the endophytic bacterial migration would increase from root to leaf (Rosenblueth and Martínez-Romero 2006). It is indicated that the root is the main entry point for endophytic microbes from the soil and their distribution in the tissue above. The micro-ecosystems in the root area are widely recognized as the source of endophytic bacteria colonialization. Moreover,



(a)

(b)

FIGURE 1 Inhibition of the growth of *Fusarium* pathogens (a) by endophytic bacteria PIB4A, PIB5B, and PLB1B (b)

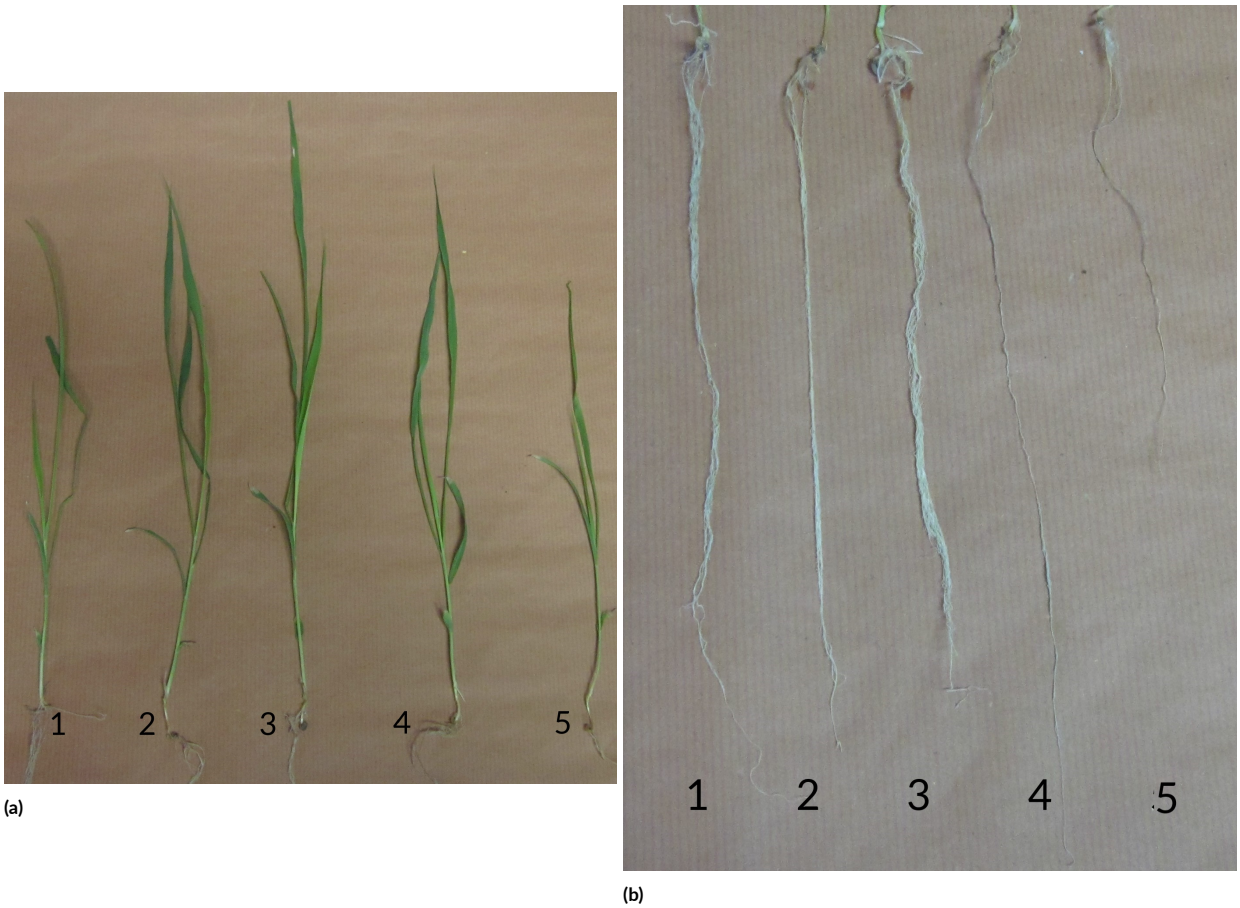


FIGURE 2 Growth of sweet sorghum cv. Numbu which was inoculated by potential endophytic bacteria after 30 days of planting; 1. positive control, 2. PIB1B isolates, 3. BB7 isolates, 4. PLB1B isolates, 5. negative control.

the diversity of endophytic bacteria can be thought of as part of the rhizosphere and/or the bacterial population in plant roots (Hallmann et al. 1997).

The ability of endophytic bacteria, a plant growth-promoting bacteria, functions as natural fertilizers were investigated by evaluating the mechanism of plant growth-promoting bacteria indirectly promoting plant growth, including nitrogen fixation, phosphorus dissolving, production of auxins, ACC deaminases, cytokinins, and gibberellins, as well as iron sequestration by siderophore bacteria. The results in Table 1 indicated that the endophytic bacterial isolates from sweet sorghum display the ability to promote plant growth.

The results demonstrated that of the 35 tested isolates, seven isolates could perform the nitrogen fixation and dissolve phosphate. The nitrogen fixation is catalyzed by the nitrogenase group enzyme, consisting of two proteins, namely MoFe dinitrogenase (EC 1.18.6.1) and Fe nitrogenase reductase (EC 1.19.6.1). The former is encoded by the *nifD* and *nifK* genes, while the latter is encoded by the

TABLE 1 Plant growth-promotion features from endophytic bacteria.

No.	Isolate	Fixation N		Solubilization P	IAA (µg/mL)	ACC-deaminase	Antagonism
		LGI	*nifH*				
1	BB5B	+	+	+	25,335	-	-
2	BB7	+	+	+	33,887	+	-
3	PIB4A	-	-	-	-	+	+
4	PIB1B	+	+	+	28,964	+	-
5	PIB5B	+	+	+	4,182	+	+
6	PIB6U	-	-	-	2,595	+	-
7	PLB4A	+	+	+	-	-	-
8	PLB1B	+	+	+	16,887	+	+
9	PLB22	+	+	-	-	-	-

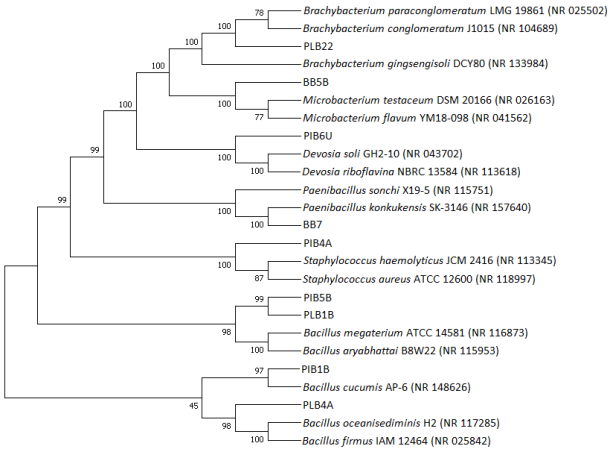


FIGURE 3 Phylogenetic tree based on 16S rRNA sequences of the endophytic bacteria from sweet sorghum cv. KCS105 and other related genera using the neighbor-joining method. Bootstrap values from 1000 replicates are indicated at each node.

nifH gene. Previous studies reported that the *nifH* gene was detected in isolates of endophytic bacteria from sweet sorghum belonging to the genera *Ralstonia*, *Staphylococcus*, *Bacillus*, *Rhizobium*, and *Paenibacillus*. (Mareque et al. 2015). The phosphate dissolution mechanism by endophytic bacteria involves several enzymes, namely C-P lyase, phosphatase, and phytase. However, most of the microbes from the phosphatase family are soluble through the production of organic acids such as ketogluconate, gluconate, lactate, acetate, succinate, tartaric, oxalate, glycolic, and citrate (Behera et al. 2017) The mechanism is affected by the bacterial strain, environmental conditions, plant, and soil conditions (Gupta et al. 2015). The endophytic bacteria of *Lysinibacillus fusiformis*, *Bacillus cereus*, and *B. megaterium* isolated from the ginseng plant also showed high solvent P activity (Vendan et al. 2010).

As depicted in Table 1, six endophytic isolates can produce 2.595 to 33.887 µg/mL of IAA. Jasim et al. (2014a) reported that the IAA produced by five endophytic bacteria isolated from *Piper nigrum* averaged 35 µg/mL and could increase if there was the induction of the endophytic IAA biosynthetic pathway by host plant metabolites. The growth of some plants can be promoted when the plants are colonized with endophytic microbes with the ability to generate IAA (Jasim et al. 2014b). The synthesis of IAA by microbes in each pathway is highly dependent on tryptophan as the precursor. Several pathways for the IAA biosynthesis by microbes have been reported, including the indole acetamide (IAM), the indole pyruvic acid (IPyA), the indole acetaldoxime (IAOx)/indole acetonitrile (IAN) pathway (Duca et al. 2014), the indole acetaldehyde (IAH) and the tryptamine pathway (Olanrewaju et al. 2017).

TABLE 2 Effect of endophytic bacterial inoculation on the growth of sweet sorghum cv. Numbu.

Treatment	Stem length (cm)*	Root length (cm)*	Dry weight (g)*
Negative control	24.33^{b}	14.32^{c}	0.03c
Isolate BB7	34.08^{a}	24.53^{b}	0.06^{a}
Isolate PIB1B	30.85^{a}	22.90^{b}	0.06^{a}
Isolate PLB1B	31.33^{a}	15.23^{c}	0.05^{ab}
Positive control	30.63^{a}	29.68^{a}	0.04^{bc}

*Means that two treatments that have different letters have a significant difference with the Duncan test of 0.05

All isolates were screened for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production on LGI media, at which ACC was used as a nitrogen source. Six isolates of endophytic material were found to have ACC-deaminase activity (Table 1). 1-Aminocyclopropane-1-carboxylic acid is a direct precursor of ethylene produced by plants. Therefore, the decrease in ACC levels may prevent ethylene-mediated inhibition of plant growth. The ACC deaminase is a multimeric enzyme that converts ACC into α-ketobutyrate and ammonia, thereby reducing ethylene levels in the host plant (Sun et al. 2009). Endophytic bacteria with the ability to reside in the host plant can benefit the host by increasing plant growth and reducing stress (Hardoim et al. 2008). Endophytic bacteria from the genus *Bacillus* and *Staphylococcus* can produce ACC-deaminase (Mareque et al. 2015; Correa-Galeote et al. 2018).

The results showed that three isolates of sweet sorghum plant-bacteria could inhibit the growth of Fusarium, namely PIB4A, PIB5B, and PLB1B (Figure 1). In general, bacteria possessing antibiotics have compounds that interfere with fungi's morphological or physiological growth. Additionally, several biocontrol bacteria produce enzymes such as cellulase, chitinase, protease, lipase, and β-1,3 glucanase, which lyse many pathogenic fungi's cell walls (Hayat et al. 2010). The screening of endophytic microbial extract showed a large structural diversity of natural compounds with a broad spectrum of biological activities, such as antiviral, antimicrobial, antitumor, and immunosuppressive activities (Sansinenea and Ortiz 2011). Endophytic bacteria from the Actinobacteria and Bacillus may produce lipopeptides, polysaccharides, aromatic compounds, plant hormones, and several enzymes associated with phenylpropanoid metabolism, thus representing the high potential for PGP and plant management strategies (Ek-Ramos et al. 2019).

Bacterial isolates that were positive for the features of PGP in vitro were used as inoculants in sweet sorghum. Twenty-eight days of the post-inoculation plants were harvested and measured for biometric parameters (Table 2). Sweet sorghum cv Numbu inoculated with endophytic bacterial isolate *Paenibacillus* sp. BB7, *Bacillus* sp. PIB1B, and *Bacillus* sp. PLB1B showed a significant difference compared to negative controls for stem length and dry weight of sweet sorghum. In the case of root length, the isolates of *Paenibacillus* sp. BB7 and *Bacillus* sp. PIB1B showed a significant difference from the negative control (Figure 2) but not for the plants inoculated with *Bacillus* sp. PLB1B, which did not give a significant

difference from the negative control.

The isolation 16S rRNA gene with a size of 1500 bp was successfully amplified from the DNA of potential endophytic bacterial isolates. The sequencing and analysis of the BLAST 16S rRNA gene showed that these endophytic bacteria belong to the phylum Alpha Proteobacteria (*Devosia*), Firmicutes (*Bacillus, Paenibacillus, Staphylococcus*), and Actinobacteria (*Microbacterium, Brachybacterium*). The phylogenetic tree analysis based on 16S rRNA gene sequences from isolates with PGP characteristics is displayed in Figure 3. The 16S rRNA gene sequence is stored in GenBank with the following accession numbers of MW666782, MW666784, MW667585, MW667586, MW683242, and MW683305. Bacteria from the genus *Paenibacillus, Bacillus, Staphylococcus,* and *Microbacterium* are endophytes in sweet sorghum plants (Mareque et al. 2015; de Fretes et al. 2018). Previous studies reported that bacteria from the genus *Devosia* were endophytes in the roots of *Nitraria* (Xu et al. 2017) and *Brachybacterium* were endophytes in rubber plants (Hidayati et al. 2014). The ability of endophytic bacteria as plant growth-promoting bacteria can support the growth of sweet sorghum as well as a solution to reduce the use of chemical fertilizers and pesticides.

4. Conclusions

In this study, the endophytic isolates have been proven for their great potential to promote plant growth based on several factors, including fixation of nitrogen, dissolution of phosphate, production of IAA and ACC deaminase, and antagonistic activity. Additionally, three endophytic bacterial isolates of *Paenibacillus* sp. BB7, *Bacillus* sp. PIB1B, and *Bacillus* sp. PLB1B demonstrates the ability in almost all tests and shows positive results for the growth of sweet sorghum plants. The study on growth-promoting microbial inoculants for growing sweet sorghum should be further studied. The particular strain may give great potential and commercial interest to produce inoculants for sweet sorghum cultivars.

Authors' contributions

CED, DW, YAP, TRN wrote the manuscript. CED, DW, YAP, TRN designed the study. CED was responsible for the overall stage of research. CED, DW, YAP, and TRN analyzed the data. All authors read and approved the final version of the manuscript.

Competing interests

The author declare that they have no competing interest.

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