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Endophytic Bacteria of *Pemphis acidula* on Karst Ecosystem of Gorontalo, Indonesia

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Wild plant *Pemphis acidula* in coastal habitats is often associated with microbes on their root, stem, or leaf. The objective of the study was to reveal the endophytic bacteria associated with *Pemphis acidula* in the coastal area of Gorontalo, the study antibacterial activity of endophytic bacteria against pathogenic bacteria, and identify endophytic bacteria based on molecular characteristics. The *Pemphis acidula* sampling was conducted on three coastal areas of Gorontalo, including Biluhu beach, Dulanga beach, and Olele beach. The sample consists of the root, stem, and leaf of *Pemphis acidula*. The result showed that the endophytic bacteria were just found on the leaf. There were four isolates with similar morphological features. The antibacterial activity on a broad spectrum of two endophytic bacteria against both *Escherichia coli* and *Staphylococcus aureus*, whereas two isolates others on a narrow spectrum against *Staphylococcus aureus*. The BDPa-01 endophytic bacterial identify as *Bacillus tequilensis* based on 16S rRNA gene sequence and this isolate is closely related to *Bacillus tequilensis* strain 20Q9-B-4-13 OK653944.1.37-1458 on a similarity index of 100%.

Key words: Endophytic bacteria, Karst ecosystem, *Pemphis acidula*

Tumbuhan *Pemphis acidula* di wilayah pesisir sering ditemukan berasosiasi dengan mikroba pada bagian akar, batang, dan daun. Penelitian ini bertujuan untuk mengungkap bakteri endofitik yang berasoasiasi dengan *Pemphis acidula* di pesisir pantai Gorontalo, mempelajari aktivitas antimikroba bakteri endofit melawan bakteri pathogen, dan identifikasi bakteri endofitik berdasar karakteristik molekular. Sampling *Pemphis acidula* dilakukan di tiga wilayah pesisir Gorontalo, yaitu pantai Biluhu, pantai Dulanga, dan pantai Olele. Sampel terdiri atas akar, batang, dan daun *Pemphis acidula*. Hasil penelitian menunjukkan bahwa bakteri endofitik hanya ditemukan pada bagian daun *Pemphis acidula*. Terdapat empat isolat bakteri endofit yang berhasil ditemukan dengan karakter morfologi yang seragam. Bakteri endofit menunjukkan aktivitas antibakteria dengan mode *broad spectrum* melawan *Escherichia coli* dan *Staphylococcus aureus*, dan dua solate lainnya menunjukkan mode *narrow spectrum* hanya melawan *Staphylococcus aureus*. Bakteri endofitik BDPa-01 diidentifikasi sebagai *Bacillus tequilensis* berdasar karakter sekuen gen 16S rRNA dengan indeks similaritas 100% terhadap type strain *Bacillus tequilensis* strain 20Q9-B-4-13 OK653944.1.37-1458.

Kata kunci: bakteri endofitik, ekosistem karst*, Pemphis acidula*

Pemphis acidula is a wild plant in coastal and mangrove ecosystems. This plant is in unique wood texture, also shown in herbal medicines traditional, ceremonies, and building materials functions. As a medicinal herbal, this plant showed the antibacterial activity against pathogenic bacteria (Samidurai and Saravanakumar 2009; Hamdillah *et al.* 2019), antioxidant (Lalitha *et al.* 2019), and ovicidal activity against *Culex quinquefasciatus Say* (Tennyson *et al.* 2011), also as biocontrol of *Aedes aegypti* (Silverio *et al.* 2020).

Pemphis acidula on Olele beach, Dulanga beach, and Biluhu beach of Gorontalo that found growing on coral with a root system penetrating of coral structure. Rindyastuti *et al.* (2018) reported that *Pemphis acidula* has a high resistance to extreme environments, thin soil layers, poor nutrients, and drought. The ability to grow in this extreme environment is supported by endophytic microbes. Guerrero *et al.* (2018) reported that fungi *Rhizopus microsporus* Tiegh., *Nigrospora oryzae* H.J. Huds, *Nigrospora sphaerica* Mason, and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. were associated with *Pemphis acidula* on the leaf, whereas, Vangronsveld *et al.* 2002; Ferrando and Scavino 2013; Shahzad *et al.*, 2017 reported that Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes are types of endophytic bacteria that support plant growth in extreme environments.

Endophyte microbes were associated with *Pemphis acidula* in the roots, especially on rhizosphere area, stems, and leaves. Endophytic microbes have a role to increase plant growth and development, as well as tolerance to environmental stresses, synthesize plant

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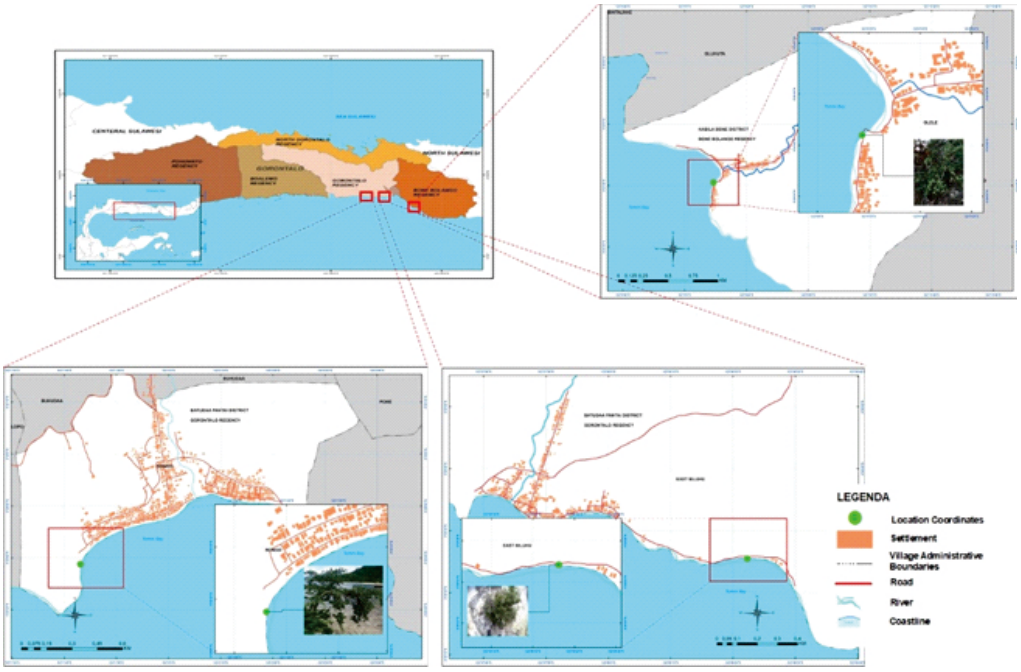

Fig 1 Location of Olele Beach, Dulanga Beach, and Biluhu Beach of Gorontalo Province indicating the sampling sites of *Pemphis acidula*: point 1 (123° 9' 9,360" E; 0° 24' 40,980" N), point 2 (123 1' 43,076" E; 0° 29' 38,752" N), and point 3 (123° 58' 41,640" E; 0° 29' 12,600" N).

hormones such as indole-3-acetic acid, solubilize phosphates, secrete siderophores, release antimicrobial compounds to compete for space and nutrients, and modulate plant resistance responses (Vangronsveld *et al.* 2002; Ferrando and Scavino 2013; Bibi *et al.* 2017; Shahzad *et al.* 2017). The ability of endophytic bacteria to produce antimicrobial compounds has the potential to be developed commercially. Extreme environmental conditions allow endophytic microbes to produce specific antimicrobial compounds. Exploration of antimicrobial compounds, especially antibacterials, needs to be carried out continuously considering the many types of microbes that are resistant to various types of antibiotics. Studies on endophytic microbes in *Pemphis acidula* on the coastal area of Gorontalo and analysis their potency as antibacterial compound-producing have never been reported. This study was focused to reveal the endophytic bacteria associated with *Pemphis acidula*, studying their antibacterial activity, and determining their identity on the species level based on molecular characters.

MATERIALS AND METHODS

Study area

Study area and sampling of *Pemphis acidula.* Sampling of leaf, stem, and root of mature *Pemphis acidula* was conducted at karst ecosystem on Olele Beach, Dulanga Beach, and Biluhu Beach of Gorontalo Province on ordinate 123°9' 9,360" E; 0° 24' 40,980" N of point 1; 123 1' 43,076" E; 0° 29' 38,752" N for point 2, and 123° 58' 41,640" E; 0° 29' 12,600" N for point 3, respectively (Fig 1). The sample of *Pemphis acidula* on three replicates. The physicochemical characteristics of site sampling include soil acidity, temperature, humidity, and light intensity.

Procedures

Isolation and purification of Endophytic bacteria. Leaf samples were surface-sterilized by the method described by Yan *et al.* (2018) with minor modifications. The samples were sterilized in the following order: a 1-min soaking in 70% ethanol, followed by a 6-min soaking in 3.25% NaOCl, a 1-min soaking in 70% ethanol, and a 1-min rinse with sterile water five times. The sterilized tissues were imprinted onto nutrient agar (NA, Difico), then incubated at 30°C for 1 week. After surface-sterilization and drying under aseptic conditions, 5-g surface-sterilized samples from three samplings were cut up in a sterile mortar and grinding to homogenate, followed by dilution to 10^{-1}, 10^{-2}, and 10^{-3} with sterile water. An aliquot of 200-µL dilutions was spread over the surface of solid media and incubated at 37°C for 48 hours. The Nutrient Agar (NA) (Merck) medium was used as isolation media. The NA medium was sterilized at 121°C for 15 min. After incubation, the bacterial colonies were picked and repeatedly re-streaked onto an agar plate until their purity.

Screening of endophytic isolates for potential antibiotics production. The screening of endophytic for potential antibiotics production was based on the Kirby Bauer method using Escherichia coli, and Staphylococcus aureus as a microbial test. Each microbial test was grown overnight in Nutrient Broth onto OD 0,7 of cell mass turbidity. The antibiotic production by endophytic isolates was based on the fermentation method on Nutrient Broth Medium for 72 hours. The crude extract of antibiotic was collected using a centrifuge at 45000 g. The paper dick 5 mm in diameter was soaked in the crude extract for about 5 minutes. Afterward, 300 µL of the microbe test was surface inoculated in the NA medium, then the soaked paper disk was placed on the surface of the NA medium. The plate was incubated at 37C for 24-48 hours. The antibacterial activity was determined based on the diameter of clear zone formation.

Identification of endophytic bacteria producing antimicrobial of Pemphis acidula

Extraction of genomic DNA. The DNA genomic of endophytic bacteria was carried out by following the protocol of the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). The bacterial cells as much as 50-100 mg (wet weight) were resuspended in 200 µL of PBS isotonic buffer to a ZR BashingBead™ Lysis Tube (0.1 mm & 0.5 mm). Add 750 µl Bashing Bead™ Buffer to the tube. Secure in a bead beater fitted with a 2.0 mL tube holder assembly and process at maximum speed for 5minutes. Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at ≥10,000 x *g* for 1 minute. Transfer up to 400 µL supernatant to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuge at 8,000 x *g* for 1 minute. Add 1,200 µL of Genomic Lysis Buffer to the filtrate in the Collection Tube from Step 4. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IICR Column^3 in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through from the Collection Tube and repeat Step 6. Add 200 µL DNA Pre-Wash Buffer to the Zymo-Spin™ IICR Column in a new Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Add 500 µl gDNA Wash Buffer to the Zymo-Spin™ IICR Column and centrifuge at 10,000 x *g* for 1 minute. Transfer the Zymo-Spin™ IICR Column to a clean 1.5 ml microcentrifuge tube and add 100 µL (35 µL minimum) DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x *g* for 30 seconds to elute the DNA. Characterization of DNA extraction product for quality (purity) and quantity (concentration and extraction efficiency) was determined by using a spectrophotometer (Dangre-Mudey and Tankhiwale 2016).

Amplification of 16S rRNA gene. Amplification PCR by using 2X MyTaq HS Red Mix (BIO-25048). PCR Master Mix 1x25µL consist of 9.5µL ddH2O; 12.5 µL MyTaq HS Red Mix, 2x; 1 µL 10 µmol/µL 27F Primer (5' –AGAGTTTGATCMTGGCTCAG– 3'); 1 µL 10 µmol/ µL 1492R Primer (5' –GGTTACCTTGTTACGACTT– 3'), and 1 µL DNA Template (Okolie *et al.* 2013). PCR Condition (35 cycles) followed an initial denaturation of 95°C for 3 min; denaturation at 95°C for 15sec; annealing on 52°C for 30 sec; extension on 72°C for 45 sec; and final extension on 72°C for 3 min. The process was held at 4°C for more than 48 hours. The PCR product was detected on agarose gel electrophoresis using a 1 Kb DNA ladder as a marker.

16S rRNA gene sequencing and phylogenetic analysis. The PCR products of endophytic bacteria were purified by using ZymocleanÔ Gel DNA Recovery Kit (Zymo Research) and sequenced based on bi-directional sequencing method. All the sequences obtained from the sequencing phase were analyzed and edited by using BioEdit software (Retnowati *et al.* 2017). Initially, all the 16S rRNA gene sequences were compared to sequences in GenBank by using the online service of Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the approximate phylogenetic position. Sequences were aligned using ClustalW with representative bacteria 16S rRNA sequences, and a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software VII. An unrooted neighbor-joining tree was constructed using the sequence of the 16S rRNA gene *Pseudomonas fluorescens* strain KU-7 AB266613.1 as an outgroup, obtained from GenBank as outgroup species (Retnowati *et al.* 2017).

Data analysis

The population of endophytic bacteria was analyzed based on descriptive quantitative analysis. The antibacterial activities were analyzed based on clear zone diameter. Molecular characterization data were compared with the NCBI GenBank.

RESULTS

Physicochemical characters the habitat of *Pemphis acidula*. Gorontalo Province is geographically bordered by Tomini Bay. Along the

Table 1 Physicochemical characters of *Pemphis acidula* habitat at the coast of Tomini Bay

Location	Physicochemical characters			
	Light intensity	Soil acidity	Humidity	Temperature (°C)
Biluhu	0.4515	7.885	57.625	26.375
Dulanga	0.5225	8.185	69.5	24.5
Olele	0.5195	7.72	60.5	25.5

Table 2 Morphology character of endophytic bacteria of *Pemphis acidula* leaf

No	Location	Isolate	Isolate description
1	Biluhu Beach	BDPa-01 and BDPa-02	12BDPa-01 isolate shows a circular colony, white color, and entire of the colony edge; while BDPa-02 isolate shows a circular the colony, brown in color and entire the colony edge
2	Dulanga beach	DDPa-01	DDPa-01 isolate shows a circular colony, white color, and entire of the colony edge
3	Olele beach	ODPa-01	ODPa-01 isolate shows a circular colony, white color, and entire of the colony edge

Table 3 Antibacterial activity of endophytic bacteria of *Pemphis acidula* leaf against Gram-negative and Gram-positive bacteria

No	Isolate	Diameter zone (mm)		Spectrum activity
		Escherichia coli	*Staphylococcus aureus*	
1	DDPa-01	7	8	Broad-spectrum
2	BDPa-01	10	10	Broad-spectrum
3	BDPa-02		8	Narrow spectrum
4	ODPa-01		8	Narrow spectrum

southern coast of Gorontalo is a coastal area with varied geographical conditions. Biluhu Beach shows a dominant sandy soil structure, some parts of Olele beach show a sandy-coral soil structure and coral hills, while Dulanga Beach is characterized by a rocky structure and steep cliffs. The physicochemical conditions of the *Pemphis acidula* growing habitat in the three locations tend to be uniform with the air humidity at Dulanga Beach tending to be higher, whereas the soil acidity on normal levels tends to be alkaline (Table 1).

Endophyte bacteria of *Pemphis acidula.* Endophytic bacteria were isolated successfully from the leaf of *Pemphis acidula*. There were two isolates of endophytic bacteria in *Pemphis acidula* leaf from Biluhu beach, while leaf samples from Olele and Dulanga beaches found one bacterial isolate respectively. The morphological characters of bacterial isolates tended to be similar at all three locations (Table 2).

Endophytic bacteria show the antibacterial activity to *Escherichia coli* and *Staphylococcus aureus* (Table 3). DDPa-01 and BDPa-01 isolates were shows a Broad-spectrum mode than the Narrow spectrum of BDPa-02 and ODPa-01 isolates.

Identification of endophytic bacteria. BDPa-01 isolate showed the highest antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. For cultivation reasons, it's important to know the character and identity of the isolate, so phylogenetic analysis required DNA extraction, amplification, and sequencing of the 16S rRNA gene. Genomic DNA of BDPa-01 isolates extraction using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005) obtained a level of purity of genomic DNA at 1.90. Amplification using universal primers 27F and 1429R succeeded in amplifying the 16S rRNA gene sequence with a size of 1500 bp. Sequencing the 16S rRNA gene based on the bi-directional method obtained a sequence with a size of 1422 bp. The results of analysis of the similarity of the 16S rRNA gene sequence isolate BDPa-01 using the BLAST algorithm obtained a 100% similarity index to several strains belonging to the genus Bacillus. Reconstruction of

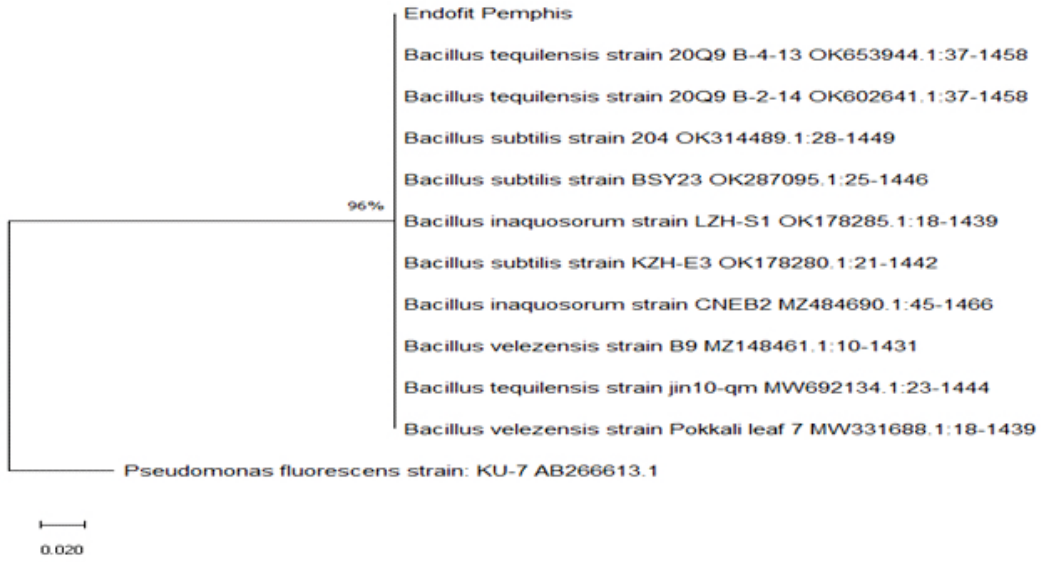

Fig 2 Neighbor-joining phylogenetic tree inferred from 16S rRNA gene sequences of BDPa-01 isolate. The phylogenetic tree shows the phylogenetic relationship of isolates with related genera. Bootstrap values are expressed as percentages of 1000 replications. Bootstrap values, >50% are shown at branch points. The score bar represents 1 nucleotide substitution per 100 nucleotides

phylogenetic trees based on neighbor joining algorithm showed that the BDPa-01 isolate form a sister clade with 10 strains from NCBI gene bank data and show kinship with *Bacillus tequilensis* strain 20Q9-B-4-13 OK653944.1.37-1458 (Figure 2).

DISCUSSION

Pemphis acidula is a type of plant that is commonly found in rocky coastal environments or on rocky cliffs, rocky coastal areas (Lewis and Rao 1971; Ellison *et al.* 2010), sandy beaches, or at the edges of mangrove forests. The ability to live in these extreme environments is supported by the ability to associate with microorganisms in plant tissues. The results showed that *Pemphis acidula* on the rocky coast of Biluhu, Dulanga, and Olele was associated with endophytic bacteria on the leaves. The presence of endophytic bacteria in the karst ecosystem is one of the adaptation efforts of plants in extreme environments. Endophytic bacteria play an important function in the adaptation and evolution of plants (Martin *et al.* 2013; Thomas *et al.* 2014; Afzal *et al.* 2019). The presence of endophytic bacteria benefits plants and can increase plant growth under normal and extreme conditions. They can benefit host plants directly by increasing plant nutrient uptake and by modulating growth and stress-related phytohormones. Indirectly, endophytic bacteria can improve plant health by targeting pests and pathogens with antibiotics, hydrolytic enzymes, nutrient restriction, and helping plants to tolerate various biotic and abiotic stresses that can inhibit plant growth.

Endophytic bacteria of *Pemphis acidula* on the coast of Biluhu, Dulanga, and Olele of Gorontalo were just found associated with the leaves. The competence of endophytes bacteria to colonize plants is strongly influenced by the host plant and environmental factors. Host plant age, genotype, geographical location, and even the tissue can determine the type of endophytic bacteria (Afzal *et al.* 2019). Host plant growth stages can also determine the endophytic diversity of a plant, where plant stages enriched in nutrient availability tend to have increased bacterial diversity (Shi *et al.*, 2014). Not only that, the climatic conditions can also influence the endophytic colonizers of a plant species. Penuelas *et al.* (2012) observed that changes in a climate significantly altered the abundance and composition of endophytic bacteria within the leaf tissues.

Endophytic bacteria on the leaves of *Pemphis acidula* plants at three locations showed similar morphological characters. The diversity of endophytic colonization depends on several factors specific to bacteria, plants, and the environment (Afzal *et al.* 2019). Fei Li *et al.* (2019) reported that the community structure of endophytic bacteria is different in each type of plant that grows in the karst ecosystem. The diversity of endophytic bacteria is also influenced by the plant's

intrinsic factor in producing root exudate which is one of the stimuli for the interaction of plants and endophytic bacteria. It is assumed that the endophytic bacteria in leaf tissue found in *Pemphis acidula* originates from the root system, so it is suspected that there are similar types of root exudates produced by *Pemphis acidula* from the three locations (Canarini *et al.* 2019).

Endophytic bacteria on *Pemphis acidula* leaves showed antimicrobial activity, especially antibacterial against gram-positive and gram-negative bacteria. As reported by Afzal *et al.* (2019) that the presence of endophytic bacteria in host plant tissues has a beneficial effect by improving plant health through the production of antibiotics that target pests and plant pathogens. Endophytic bacterial isolates in *Pemphis acidula* leaves are thought to play an important role in plant metabolism in relation to metabolism for adaptation in response to the physicochemical habitat conditions. The presence of endophytic bacteria *Bacillus sp*. on host plants can produce phytohormones such as indoleacetic acid, indolebutyric acid, gibberellins, cytokinins, octadecanoids, and compounds that mimic the action of jasmonates, and assist in survival of the plant. Control of opening and closing of stomata, osmotic adjustment, modification of root morphology, photosynthetic efficiency, increased uptake and modification of mineral buildup, supply of essential vitamins, and nitrogen metabolism are examples of benefits related to the association between endophytes and plants (Santoz *et al.* 2018). Kiran *et al.* (2018) reported that *Bacillus tequilensis* associated with sponges in marine environments has the ability to produce pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro compounds which exhibit antimicrobial activity against *Staphhylococcus aureus*. Meanwhile, Guan *et al.* (2018) reported that the endophytic *Bacillus tequilensis* in *Angelica dahurica* has the potential as a biological control agent against *Magnaporthe oryzae*.

Endophytic bacteria (BDPa-01 isolate) on *Pemphis acidula* leaves were identified as *Bacillus tequilensis*. The blast results showed that the isolate was closely related to *Bacillus tequilensis* strain 20Q9-B-4-13 OK653944.1.37-1458 with a similarity index of about 100%. *Bacillus tequilensis* is a type of bacteria that are mostly found living solitary in extreme environments such as deserts (Zhao *et al.* 2019), fermented intestines (Hidayati *et al.* 2021), as well as in association with plants (Guan *et al.* 2018; Bhattacharya *et al.* 2019). Dong *et al.* (2021) reported that plant-associated *Bacillus tequilensis* showed biocontrol activity against soft root disease in *Colocasia esculenta* (L.) Schott.

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