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Research Article

The Diversity of Endophytic Bacteria from the Traditional Medicinal Plants Leaves that Have Anti-phytopathogens Activity

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ABSTRACT

Endophytic bacteria live in plant tissues which utilized in plant protection against phytopathogens. This study aims to investigate the diversity of endophytic bacteria from the leaves of traditional medicinal plants that has anti-phytopathogens properties. Isolation of endophytic bacteria was done by spread plate method. The bacteria were characterised by Gram staining and the 16S rRNA gene analysis. Further screening of anti-phytopathogen activity used disc diffusion method for *Ralstonia solanacearum*, *Xanthomonas campestris*, *Fusarium oxysporum*, and *Sclerotium rolfsii*. All togethers, sixteen isolates of endophytic bacteria from the leaves of eight medicinal plants species were obtained. Fourteen isolates had an anti-phytopathogen (with eight isolates against *R. solanacearum*, seven isolates against *X. campestris*, nine isolates against *F. oxysporum*, and five isolates against *S. rolfsii*). From the 14 isolates identified, phylum Firmicutes were dominant (64.3%), followed by Proteobacteria (28.6%), and Actinobacteria (7.1%). Phylum Firmicutes consists of *Bacillus indicus* (BJF1, TCF1, and MCF2), *Bacillus pumilus* (CAF4), *Bacillus* sp. (CAF1), *Bacillus subtilis* (AAF2, MCF1, CAF3, and MCF3); phylum Proteobacteria consists of *Pantoea agglomerans* (CAF2), *Pantoea stewartii* (AAF4), *Pseudomonas oryzihabitans* (AAF3), and *Pseudomonas psychrotolerans* (AAF1); and phylum Actinobacteria consists of *Kocuria kristinae* (CSF1).

Keywords: Diversity, endophytic bacteria, traditional medicinal plants, antimicrobial activity, anti-phytopathogens activity

Introduction

Endophytic bacteria which live in plant tissues is a very interesting subject to study. This organism significantly involves in the protection of plants, increasing growth, and overcoming environmental stresses [1, 2].

Traditional medicinal plants [such as *Annona muricata* L., *Artocarpus altilis* (Parkinson) Fosberg, *Brucea javanica* (L.) Merr., *Citrus aurantifolia* Swingle, *Cheilocostus speciosus* (J. Konig) C. Specht, *Datura metel* L., *Manilkara zapota* (L.) P. Royen, *Morinda citrifolia* L., *Syzygium cumini* (L.) Skeels., and *Tinospora crispa* (L.) Miers] have been widely reported to have antimicrobial activity against human pathogen and phytopathogen.

Endophytic bacteria isolated from traditional medicinal plants are reported to produce antibacterial, antifungal and antiseptic compounds [3, 4]. These compounds have the potential to be utilized as biopesticides in controlling pests and diseases in plants.

The use of endophytic bacteria as a biopesticide producer is very beneficial for the environ-

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ment. Biopesticide compounds are more environmentally friendly than synthetic pesticides. This is because biopesticides are easily decomposed in nature, specific work targets, have unique properties and work methods, and are not toxic to humans [5].

Ralstonia solanacearum (causes of plant wilt) [6], *Xanthomonas campestris* (causes of plant blight) [7], *Fusarium oxysporum* (cause of plant wilt) [8], and *Sclerotium rolfsii* (cause of root rot) [9] are important phytopathogens for plants. These phytopathogens are soil-borne which quite difficult to control. As phytopathogen resistance increases to existing pesticides, it is necessary to continue to explore in finding new strains of endophytic bacteria that have the potential to be developed as biopesticide producers. This study aims to investigate the diversity of endophytic bacteria from the leaves of medicinal plants that can inhibit the growth of phytopathogens.

Material and Methods

Isolation of endophytic bacteria

Leaves of plants *A. muricata* L., *Art. altilis* (Parkinson) Fosberg, *B. javanica* (L.) Merr., *C. aurantifolia* Swingle, *Che. speciosus* (J. Konig) C. Specht, *D. metel* L., *M. zapota* (L.) P Royen, *Mor. citrifolia* L., *S. cumini* (L.) Skeels., and *T. crispa* (L.) Miers were collected from the garden in Lakuk, Simpang Haru Village, Padang, West Sumatra Province, Indonesia. The collection of plant leaves was carried out according to de Melo *et al.* (2009) [10]. Leaves were sterilized by following the method developed by Araujo *et al.* (2001) [11].

Isolation was carried out using the method of de Melo *et al.* (2009) [10] with modification. Sterile plant leaf segments (1 gram) were mashed with a mortar and sterile pestle, then suspended into 9 mL physiological NaCl (Merck®) 0.85%. The suspension was homogenized and serially diluted. Then 0.1 mL of the suspension from each dilution was inoculated into a Petri dish containing medium Tryptic Soy Agar (TSA) (Merck®) using a spread plate method, then incubated at 27°C for 1-3x24 hours. Growing isolates were observed for the colony morphology.

Bacterial colonies that grow are purified on TSA plates using a quadrant streak plate method. Purified endophytic bacterial isolates were then stained through Gram staining. Gram staining is done by referring to Cappucino & Sherman (2014) [12], using the Gram (Merck®) staining kit.

Screening of endophytic anti-phytopathogens activity

All isolated endophytic bacterial isolates were screened to determine its anti-phytopathogens activity against *R. solanacearum*, *X. campestris*, *F. oxysporum*, and *S. rolfsii*. *R. solanacearum* and *X. campestris* were from culture collection of Microbiology Laboratory, Faculty of Agriculture, Andalas University, Padang, Indonesia. *F. oxysporum* and *S. rolfsii* were from culture collection of Phytopathology Laboratory, Faculty of Agriculture, Andalas University, Padang, Indonesia. Screening of anti-phytopathogen activity was carried out disk diffusion method which refers to Melliawati *et al.* (2006) [13]. Positive control used 15 ppm of chloramphenicol for bacteria, while positive control of fungus used 15 ppm of ketoconazole.

Identification of endophytic bacteria through 16S rRNA Analysis

The assay was done by planting Pure culture of endophytic bacteria was inoculated into a test tube containing 4 mL of Tryptic Soy Broth medium (Merck®) and incubated using a shaker for 24 hours. The results of the culture were taken as much as 3 mL, then centrifuged at a speed of 13,000 rpm, for 5 minutes. Pellets are taken to extract the genome DNA using the Wizard® Genomic DNA purification kit (Promega Corp.) following the manufacturer's instructions. DNA amplification was carried out by preparing the mix reagent mixture for PCR as follows (KAPA Taq ReadyMix Kit - KAPA): 20 µL dH_2O, 25 µL master mix PCR, 2 µL primer 9F (20 pmol) (5'-GAG TTT GAT CCT GGC TCA G-3'), 2 µl Primer 1541R (20 pmol) (5'-AAG GAG GTG ATC CAG CC-3'), and 1 µL DNA Template with a total volume of 50 µL. PCR amplification was carried out as many as 30 cycles with the following programs: preheat at 96°C for 5 minutes, denaturation at 96°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute, extension at 72°C for 7 minutes. PCR results were electrophoresed in 1% agarose gel using 1× TAE buffer then visualized using gel illuminator. Agarose gel is photographed as documentation. The PCR results are cleaned using the SV Gel and PCR Clean-Up System (Promega) Wizard.

The purified DNA extract was sent to First

Table 1. Cell numbers of isolated endophytic bacteria

No.	Plant species	Obtained isolate	Cell numbers (CFU/g leaf)
1.	*A. altilis* (Parkinson) Fosberg	4	4.0×10^2
2.	*Ann. muricata* L.	1	1.0×10^2
3.	*B. javanica* (L.) Merr.	1	1.0×10^2
4.	*C. aurantifolia* Swingle	4	7.0×10^2
5.	*Che. speciosus* (J. Konig) C. Specht	1	2.0×10^2
6.	*D. metel* L.		Cannot be isolated
7.	*M. zapota* (L.) P. Royen	1	1.0×10^2
8.	*Mor. citrifolia* L.	3	5.0×10^2
9.	*S. cumini* (L.) Skeels.		Cannot be isolated
10.	*T. crispa* (L.) Miers	1	1.0×10^2

Base Malaysia for sequencing of the base arrangement. Sequences are checked and edited using the BioEdit program. The similarity was analyzed using the Basic Local Alignment Tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The identified strains were then registered to obtain an accession number; then phylogenetic analysis was carried out using ClustalW2 phylogenetic tree at http://ebi.ac.uk.

Results and Discussion

Isolation and purification of endophytic bacteria

A total of Sixteen isolates of endophytic bacteria were obtained from 8 of 10 plants species in this study. These isolates were obtained from eight plant species, but the bacteria in the other two plants (*D. metel* L. and *S. cumini* (L.) Skeels.) were not isolated. The variety of isolates obtained ranged from one to four isolates for each plant (Table 1). The results of this study are not much different from the results previously reported, ranging from one to six isolates per plant [13, 14, 15]. No endophytic bacteria were found in the plants of *D. metel* L. and *S. cumini* (L.) Skeels. Presumably, when the isolation was carried out, the plant is dominated by obligate endophytic bacteria, so it cannot be cultured. Hardoim *et al.* (2008) [16] explained that many endophytic bacteria were obligate endophytes.

Based on the results of Gram staining, 12 isolates were Gram-positive and 4 isolates were Gram-negative (Table 2). It can be concluded that in this study Gram-positive bacteria were a group of dominant bacteria. Gayathri *et al.* (2010) [17] and Anjum & Chandra (2015) [18] also obtained Gram-positive bacteria as endophytic bacteria in their study.

Anti-phytopathogens activity of endophytic bacteria

In general, isolated endophytic bacteria have anti-phytopathogens activity. Eight isolates had anti-phytopathogens activity against R. solanacearum; seven isolates had anti-phytopathogens activity against *X. campestris*, nine isolates had anti-phytopathogens activity against *F. oxysporum*, and five isolates had anti-phytopathogens activity against *S. rolfsii* (Table 2). The endophytic bacteria that have been isolated have low anti-phytopathogens activity against phytopathogenic bacteria (2 mm) compared to anti-phytopathogens activity against phytopathogenic fungi (8 mm – 17 mm). Based on the criteria by Davis and Stout (1971) [19] anti-phytopathogens activity against phytopathogenic bacteria in this study was weak (inhibitory zone was < 5 mm), whereas anti-phytopathogens activity against phytopathogenic fungi was moderate (inhibitory zone was 5 – 10 mm) and strong (inhibitory zone was 10 – 20 mm).

The results obtained indicate that the endophytic bacteria obtained have the potential to be developed as a biocontrol agent or as a biopesticide producer against phytopathogenic fungi. Endophytic bacteria can produce lysis enzymes against compounds such as chitin, protein [20], cellulose, and hemicellulose [21]. These enzymes can result in direct suppression of the activity of phytopathogenic microbes [21].

The ability of endophytic bacteria to produce anti-phytopathogens compounds was very beneficial for host plants, because host plants can be used against phytopathogen [22]. According to Haas & Defago (2005) [23], this was caused by

Table 2. Characteristics of isolates and anti-phytopathogens activity against test microbes

No.	Isolate	Gram staining	Inhibitory zone (mm)			
			R. solanacearum	*X. campestris*	*F. oxysporum*	*S. rolfsii*
1.	AAF1	Negative	2	0	8	0
2.	AAF2	Positive	2	2	17	16
3.	AAF3	Negative	2	0	11	0
4.	AAF4	Negative	2	0	0	0
5.	AMF1	Positive	0	0	0	0
6.	BJF1	Positive	2	0	14	0
7.	CAF1	Positive	2	0	0	0
8.	CAF2	Negative	2	2	0	0
9.	CAF3	Positive	0	2	16	15
10.	CAF4	Positive	0	0	12	12
11.	CSF1	Positive	0	2	13	0
12.	MZF1	Positive	0	0	0	0
13.	MCF1	Positive	0	2	15	14
14.	MCF2	Positive	2	2	0	0
15.	MCF3	Positive	0	0	10	13
16.	TCF1	Positive	0	2	0	0
17.	Positive Control		21	20	26	24

active plants responding to various environmental stimuli and can also respond to various chemical compounds stimulated by microbes. Both by soil microbes, as well as microbes associated with plants.

In general, endophytic bacteria have an excellent ability to inhibit phytopathogenic fungi, when compared to phytopathogenic bacteria. According to Bloemberg & Lugtenberg (2001) [24], This ability was caused by the ability of endophytic bacteria to produce diffuse and volatile antifungal compounds. Endophytic bacteria could mediate de novo antimicrobial synthesis and new antifungal secondary metabolites, which have been accepted as potential fungicides to prevent the spread of phytopathogens [25].

To determine the anti-phytopathogens activity, the disc diffusion method was applied. This method was one of the easy-to-use methods for the selection of biocontrol bacterial agents and has proven to be the right strategy for this experimental system [26]. This method was used to determine whether the isolates used can produce compounds that can interfere with the target microbial life cycle [27]. According to Elad & Chet (1995) [28], The disadvantage of this method was that researchers sometimes inadvertently ignore endophytic bacteria that do not exhibit anti-phytopathogens activity, and this allows the discovery of antagonists in controlling phytopathogens through other mechanisms, such as inactivation of virulence factors [29].

Identification of endophytic bacterial isolates that have anti-phytopathogens activity

Based on the results of 16S rDNA sequence analysis, 14 isolates were identified into nine different species with similarity ranged from 94-99% to the nearest strain (Table 3). The isolates which have similarities > 97% can be identified as the same species, however, the similarity < 97% is identified as the same genus [30]. All identified isolates are new strains, and each is registered in the National Center for Biotechnology Information (NCBI) database with accession numbers KY806221-KY806234.

Anti-phytopathogens activity test results (Table 2) shows that strains from the isolated *Bacillus* genus have diverse anti-phytopathogens activities. *B. indicus* BJF1 has anti-phytopathogens activity against *R. solanacearum* and *F. oxysporum*; *B. indicus* TCF1 only has anti-phytopathogens activity against *X. campestris*; and *B. indicus* MCF2 has anti-phytopathogens activity against *R. solanacearum* and *X. campestris*. *B. indicus* was first discovered by Suresh *et al.* (2004) [31] in the waters of West Bengal, India, and these bacteria were resistant to arsenic. Hong *et al.* (2008) [32] report-

Table 2. Strains of endophytic bacteria based on the results of molecular identification through 16S rDNA analysis

No.	Isolate	Accession number assigned	Closest type strain (accession number)	Similarity (%)
1.	AAF1	KY806234	*Pseudomonas psychrotolerans* AP9-27B (KM891562)	99
2.	AAF2	KY806226	*Bacillus subtilis* SSCT68 (AB210968)	99
3.	AAF3	KY806233	*P. oryzihabitans* AF31 (LC015573)	99
4.	AAF4	KY806232	*Pantoea stewartii* M073	99
5.	BJF1	KY806221	*B. indicus* (KF791344)	99
6.	CAF1	KY806225	*B. cereus* RNS_01 (KT380683)	94
7.	CAF2	KY806231	*Pantoea agglomerans* ZFJ-15 (EU931554)	99
8.	CAF3	KY806228	*B. subtilis* 55C1-1 (JN366797)	99
9.	CAF4	KY806224	*B. pumilus* FI39 (KT318787)	99
10.	CSF1	KY806230	*Kocuria kristinae* LCT (KR230389)	99
11.	MCF1	KY806227	*B. subtilis* subsp. *inaquosorum* MER_77 (KT719652)	99
12.	MCF2	KY806223	*B. indicus* WJB 131 (KU877665)	99
13.	MCF3	KY806229	*B. subtilis* SR41 (KY203664)	99
14.	TCF1	KY806222	*B. indicus* WJB 131 (KU877665)	99

ed *B. indicus* to be safely used as a prebiotic. However, there have been no reports of these bacteria as endophytes and their ability to produce anti-phytopathogens compounds. This study was first reported that the bacterium as endophytic and has anti-phytopathogens activity. Further research was needed to determine the potential of these strains as biopesticide producers.

B. subtilis belongs to *Bacillus* genus which has the most strains in this study (four strains). Three strains had anti-phytopathogens activity against phytopathogenic bacteria and fungi, namely *B. subtilis* CAF3, *B. subtilis* MCF1, and *B. subtilis* AAF2, whereas one strain only had antibacterial activity, namely *B. subtilis* MCF3 (Table 2). B. subtilis was generally found in soil, water, and associated with plants [33]. Several strains of *B. subtilis* have been reported as endophytes and have the ability to inhibit soil-borne phytopathogens [34], such as *X. campestris* [35], *R. solanacearum* [36], *F. oxysporum* [37, 38] and *S. rolfsii* [39]. *B. subtilis* was reported to have the ability to produce antimicrobials, such as antimicrobial lipopeptides [40], so that these bacteria have considerable antimicrobial activity.

Strains from other *Bacillus* genera also have anti-phytopathogens activity, such as *Bacillus* sp. CAF1 against *R. solanacearum*; and *B. pumilus* CAF4 against *F. oxysporum* and *S. rolfsii*. Sturz *et al.* (2005) [41] reported that *B. pumilus* isolated from endorhiza and potato exorhiza could have anti-phytopathogens activity against *F. oxysporum*. de Melo *et al.* (2009) [10] also reported that these bacteria also had anti-phytopathogens activity against *S. rolfsii*. Therefore, it can be concluded that *Bacillus* was a genus of endophytic bacteria that has the potential to be developed as a biocontrol agent because it can inhibit the growth of bacteria and fungi. Forchetti *et al.* (2007) [42] mentions that strains of the *Bacillus* have the advantage of being developed as biopesticides compared to other bacteria. Strains from this genus are easily to be cultured and stored; can be applied as spores, or inoculants to seeds, or bioactive compounds produced; shows a protective effect on various pathogenic microbes and improve plant growth.

K. christinae CSF1 has anti-phytopathogens activity against *X. campestris* and *F. oxysporum*. This bacterium is also known as *Micrococcus kristinae*. This bacterium was first isolated by Kovacs *et al.* (1999) [43] from the roots of *Typha angustifolia*. These bacteria are found as endophytes in some plants, such as *Solanum tuberosum* [41], *Carica papaya* [44], *Panicum virgatum* [45], and *Durio* spp. [46]. *K. kristinae* can inhibit the growth of *F. oxysporum* [41], dissolve phosphate [46], and have pectinase enzyme activity [44].

Strains of the isolated *Pantoea* genus in this study only had antibacterial activity. *Pan. agglomerans* CAF2 have anti-phytopathogens activity against *R. solanacearum* and *X. campestris*, while *Pan. stewartii* AAF4 only has anti-phytopathogens activity against *R. solanacearum*. *Pantoea*

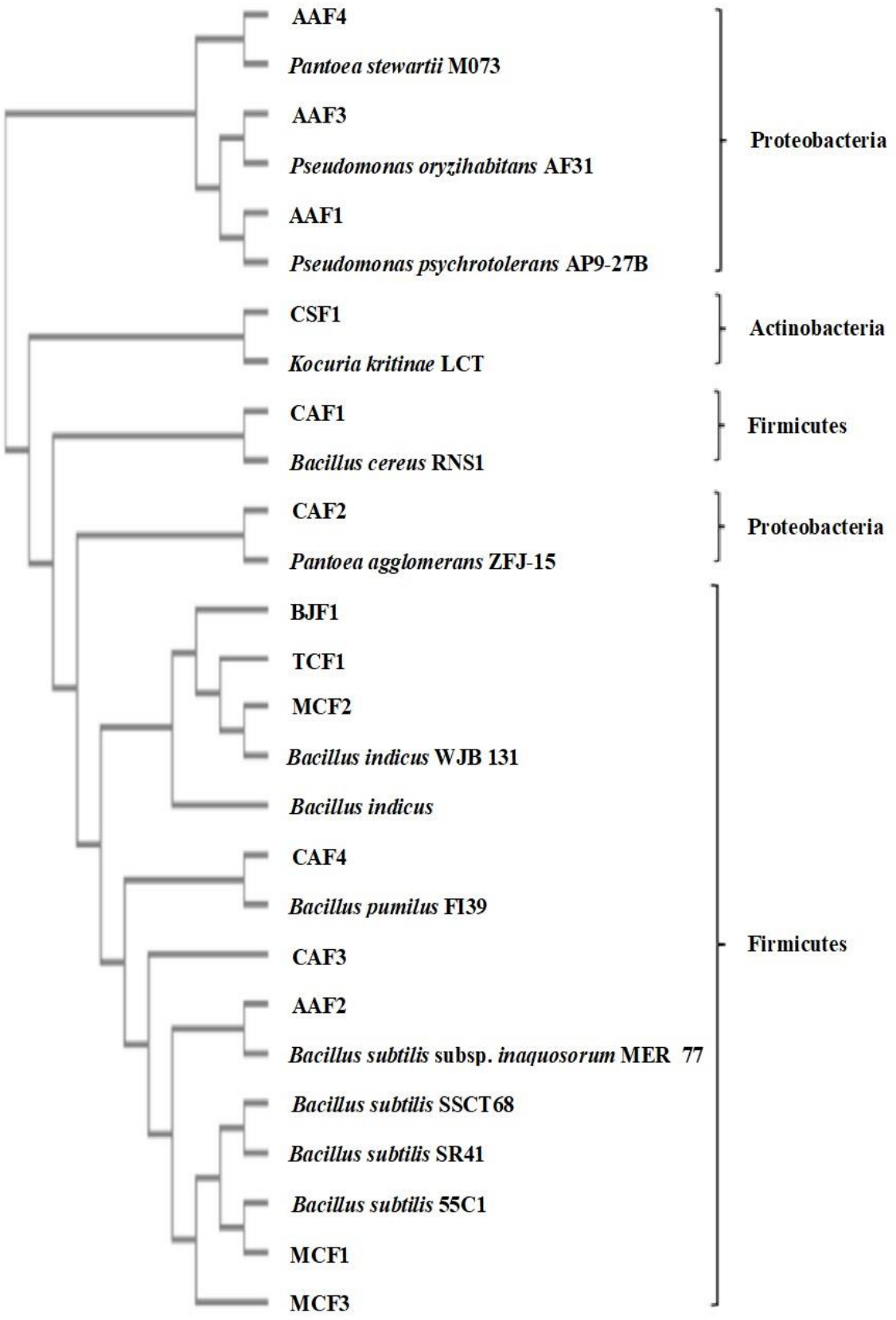

Figure 1. Phylogenetic tree strains of endophytic bacteria that have anti-phytopathogens activity

was found in *Citrus* spp. [11], *Arachis hypogea* L. [47], *Zea mays* L. [48], and in several other plants that have been studied. *Pantoea* spp. reported to produce IAA [47], siderofor [49], and have the ability to fix nitrogen [48]. This bacterium does not have antifungal activity against phytopathogenic fungi [50]. Both strains of *Pantoea* identified in this study have opportunistic properties. *Pan. agglomerans* are opportunistic to cotton plants [51], however, *Pan. stewartii* was opportunistic towards maize plants [52, 53].

P. psychrotolerans AAF1 and *P. oryzihabitans* AAF3 have anti-phytopathogens activity against *R. solanacearum* and *F. oxysporum*. Lamessa & Zeller (2007) [54] reported that Pseudomonas was a potential genus that can control R. solanacearum. Chaiharn *et al.* (2009) [55] and Munif *et al.* (2012) [56] adding this genus can also inhibit the growth of *F. oxysporum*. *Pseudomonas* was capable of producing siderophore [57], and these compounds can inhibit the growth of phytopathogenic microbes [58].

Based on the results obtained it can be concluded that endophytic bacteria that are identified are quite diverse and have various anti-phytopathogens activities. Host plants can be a limiting ability. This can be seen from the anti-phytopathogens ability of *B. subtilis* isolated from different plants having different anti-phytopathogens activity against phytopathogens. *B. subtilis* is a fairly dominant species found in this study. B. subtilis was reported by Jacobsen *et al.* (2004) [59] can produce antimicrobial peptides and contribute to leaf and root disease. Also, the bacteria also produce lipopeptide, which is an amphiphilic compound with surfactant activity [60]. *Bacillus* has secondary metabolite products that are attractive with a broad antimicrobial spectrum and very diverse structures [61].

The *Bacillus* is the most common genus found as endophytes. This is supported by the results of several studies which claim the *Bacillus* is dominant in some plants, such as *Jacarandra decurrens* [62], *Panax* spp. [63, 64], *Manihot esculenta* [10], *Solanum lycopersicum* [56], *Polygonum cuspidatum* [65], and *Musa* spp. [66]. *Bacillus* is known as a cosmopolitan genus and has high survival ability. This can be caused by the presence of endospores. Moat *et al.* (2002) [67] stated that endospores contained in cells have a role in protecting organisms against unfavorable environmental conditions. This leads to organisms that have them will be able to survive and maintain their existence.

Phylogenetic endophytic bacterial isolates that have anti-phytopathogens activity

The results of phylogenetic analysis of endophytic bacterial strains that have anti-phytopathogens activity using UPGMA (Figure 1) show strains of endophytic bacteria that have anti-phytopathogens activity grouped into three phyla. Firmicutes are dominant phylum (64.3%), followed by Proteobacteria (28.6%), and Actinobacteria (7.1%). The three phyla are commonly found as endophytes in various plants [68,69]. Some results of the study report that phylum Firmicutes is the dominant phylum [66,70,71], followed by Proteobacteria and Actinobacteria, respectively [66, 71].

Phylogenetic tree show that the strains obtained were grouped into two groups originating from different ancestors. The first group was *Pan. stewartii* AAF4, *P. oryzihabitans* AAF3, and *P. psychrotolerans* AAF1, while the second group was *K. kristinae* CSF1, *Bacillus* sp. CAF1, *Pan. agglomerans* CAF2, *B. indicus* BJF1, *B. indicus* TCF1, *B. indicus* MCF2, *B. pumilus* CAF4, *B. subtilis* CAF3, *B. subtilis* AAF2, *B. subtilis* MCF1, and *B. subtilis* MCF3. In general, the strains that were owned have a close relationship with strains that have similarities with them, only *B. subtilis* AAF2 and *B. subtilis* MCF1 strains were different. *B. subtilis* AAF2 has similarities with *B. subtilis* SSCT68, but has a kinship with *B. subtilis* subsp. inaquosorum MER_77, *B. subtilis* MCF1 has similarities with *B. subtilis* subsp. inaquosorum MER_77, but has a kinship with B. subtilis 55CI-1. Triana (2005) [87] found that different species have close molecular relationships, namely *Photorhizobium thomsonianum* BTAi1 with *Blastobacter denitrificans*.

Conclusion

Sixteen isolates of endophytic bacteria from eight species of medicinal plants were obtained. Fourteen isolates had anti-phytopathogens activity (eight isolates against *R. solanacearum*, seven isolates against *X. campestris*, nine isolates against *F. oxysporum*, and five isolates against *S. rolfsii*. Identification of the fourteen isolates showed that Firmicutes were dominant phylum (64.3%), followed by Proteobacteria (28.6%) and Actinobac-

teria (7.1%). Phylum Firmicutes consist of *B. indicus* (BJF1, TCF1, and MCF2), *B. pumilus* (CAF4), *Bacillus* sp. (CAF1), *B. subtilis* (AAF2, MCF1, CAF3, and MCF3); phylum Proteobacteria consists of *Pan. agglomerans* (CAF2), *Pan. stewartii* (AAF4), *P. oryzihabitans* (AAF3), and *P. psychrotolerans* (AAF1); and phylum Actinobacteria consists of *K. kristinae* (CSF1).

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