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Full Length Article

A New Bacterial Soft Rot Disease of *Aloe vera* in Vietnam Infected by *Enterobacter cloacae*

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Abstract

Bacteria soft rot disease is an important disease of *Aloe vera* which affects both the quality as well as quantity of plant products. In this study, *Enterobacter cloacae* (*E. cloacae*) was reported as the main biological agent that causes bacterial soft rot disease outbreaks on *Aloe vera* plants in Vietnam. The typical symptoms of the disease that appear on plants include water-soaked lesions, yellow discoloration, gas formation, rotting, and collapsing at the base of the leaves. On the basic of biomedical characteristics of the studied strains, the *Aloe vera* pathogenic bacterium was identified as *E. cloacae*. The DNA sequence analysis of 16S rRNA coding gene of three isolated strains identified as *E. cloacae* shared the similarity of 97.3–98.0%. Similarly, their housekeeping genes, including the *hsp60*, *fusA* and *leuS*, indicated that they are present within the available *E. cloacae* complex. Phylogenetic analysis based on the DNA sequence of *hsp60* gene revealed that these genes belonged to the XI cluster together with the *E. cloacae* strains (*i.e.*, ATCC-13047, EN-475, and EN-287). Moreover, the pathological test revealed that the infection of healthy plants with identified bacterial isolates symptoms of soft rot disease on *Aloe vera* plants under natural field conditions. This study is the first report of *E. cloacae* which has been identified as a new pathogenic bacterium causing soft rot disease outbreaks of the *Aloe vera*. The further analysis will focus on the origin, pathogenesis and the essential control system of the disease.

Keywords: Bacteria soft rot; *Enterobacter cloacae*; *Aloe vera*; 16S rDNA; *hsp60*

Introduction

Aloe vera (*Aloe barbadensis* Miller) belongs to the Asphodelaceae (Liliaceae) family. This plant contains triangular and fleshy green leaves. The color of its flower is yellow and its fruits contain many seeds (Surjushe *et al.* 2008). The plants grow well in tropical, sub-tropical, and arid climates, including the desert, grassland and coastal areas. In particular, *Aloe vera* is cultivated mostly in the areas of sub-Saharan Africa, Saudi Arabian Peninsula and Western Indian Ocean (Reynolds and Dweck 1999). Its plants are mainly utilized for gel-production which has several uses in agriculture, food and medicine. *Aloe vera* plants have several medicinal properties like anti-cancer, anti-oxidant, anti-inflammatory and anti-bacteria effects, and is also used to treat wounds, burns and diabetes (Akinsanya *et al.* 2015).

A number of infections that lead to devastating diseases in *Aloe vera* have been identified. Some important pathogens have been identified as *Erwinia chrysanthemi (Pectobacterium chrysanthemi)* which causes the bacterial soft rot disease (Laat *et al.* 1994; Mandal and Maiti 2005) and *Fusarium* spp. that causes leaf rot/base rot diseases (Ayodele and Ilondu 2008) in *Aloe vera*. The symptoms caused by these pathogens become more serious in conditions of abundant moisture during irrigation or rainy season. The diseases caused by these pathogens lead to reduced quality and quantity of the produce.

In recent years, the bacterium, *E. cloacae* has been reported as an important pathogen that can cause diseases in

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different plant species. The infection of *E. cloacae* has been reported in several fruits, for example, in papaya fruits this bacterium causes typical symptoms around the blossom end and symptoms like soft, yellow discoloration, and offensive odor in infected fruits (Nishijima 1987); Similarly, it causes symptoms in the onions with yellow to brown discoloration, bulbs and loss of turgor (Bishop 1990; Schroeder *et al.* 2010) and in the *Odontioda* orchids symptoms like the water-soaked lesions, light to dark brown discoloration, and necrosis (Takahashi *et al*. 1997); In ginger plant, *E. cloacae* caused symptoms like yellowish-brown to brown discoloration and firm to spongy texture (Nishijima *et al*. 2004); however, in case of *Macadamia* spp. development of gray discoloration and foul odor are important symptoms caused by this bacterium (Nishijima *et al*. 2007); Likewise, in the dragon fruit, yellowish to brownish discoloration, soft rot in fruit and stem appears (Masyahit *et al*. 2009); while in mulberry (*Morus alba*), browning of vascular tissues, leaf wilt, and defoliation is caused by *E. cloacae* (Wang *et al*. 2010); Moreover, in cassava, chlorotic halo, senescence of leaves, and stem bare (Santana *et al*. 2012); in lucerne, yellowing, rot and sprout decay (Zhang and Nan 2013) and in chili pepper, brown necrosis at margins of leaf tips and defoliation (García-González *et al*. 2018). However, to the date, there has been no scientific report of *E. cloacae* infection in *Aloe vera*.

Vietnam is a tropical country that is locates on the eastern margin of the Indo-Chinese Peninsula. *Aloe vera* was the first introduced in the country in 2002. This plant has been growing mostly in Ninh Thuan province, a south-central coastal region with a typical arid climate and sandy soil conditions. Since 2011, a severe soft rot disease on the *Aloe vera* plants has frequently occurred during the wet period. This disease leads to serious economic losses due to plant deaths. The aim of this study was to isolate, characterize and identify the pathogenic bacterium and study on the disease occurrence and pathogenicity of the bacterial soft rot disease outbreak on *Aloe vera* plants in Vietnam.

Materials and Methods

Sample collection

Samples of the suspected bacterial soft rot *Aloe vera* plants were collected from the *Aloe vera* fields in Ninh Thuan province, a south-central coast region of Vietnam, during February and August 2019. The observed symptoms were water-soaked lesions, gas formation, rotting, yellow discoloration and collapsing at the base of the leaves. All the samples were stored in a cool box and immediately transported to the laboratory within 24 h for bacteria isolation.

Bacterial isolation and biochemical characteristics

Briefly, the leaf samples were surface sterilized with 70% alcohol for 3–5 min and rinsed twice with sterile distilled water (SDW) and dried for 15 min. A piece of 3 mg of the symptomatic tissues of the leaf sample was collected and suspended in 1 mL of SDW. After that, a 100 μL of each 10-times serial dilution was spread onto nutrient agar (NA) plates (Sigma-Aldrich, St. Louis, MO) in triplicate. The plates were incubated at 30°C for at least 24–48 h or until colony formation was observed. The single colonies were picked from the isolation plates for further analyses. For biochemical characterization of the isolated bacteria, 20E API kit (BioMerieux Inc., Durham, NC, USA) was used.

Pathogenicity test and re-isolation of the pathological bacteria

The single colonies were passaged on NA and incubated at 30°C for 48 h. The selected bacteria were re-suspended in SDW and adjusted to an optical density of 0.1 at A_{600} (an approximately titer of 10^8 colony forming units (cfu) per mL). Plants were grown in greenhouse for at least three months for pathological test. Briefly, 0.1 mL (about 10^7 cfu) of inoculum was injected to the base of each *Aloe vera* plant by a syringe and hypodermic needle. The SDW-injected *Aloe vera* plant was used as control. The injected plants were grown in the greenhouse and the symptoms were observed at 12–24 h. The pathological bacteria in each test were re-isolated.

Total DNA extraction and Polymerase Chain Reaction (PCR)

Bacteria DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Extracted DNA was resuspended in RNase-free water and stored at -80°C until PCR analysis was performed. The 16S rRNA gene was amplified from the bacterial genome using the 27F/1492R primer set (27F: 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R: 5'-GGTTACCTTGTTACGACTT-3') as previously described (Turner *et al.* 1999; Gloeckner *et al.* 2013). In addition, for further analysis of the *E. cloacae*, a multi-locus sequence strategy was done by using three housekeeping genes, including the *hsp60*, *fusA* and *leuS*, following the previous description (Hoffmann and Roggenkamp 2003; Miyoshi-Akiyama *et al.* 2013). The sequences of all the primers used in this study are given in Table 1. The PCR reaction was carried out at 95°C for 5 min (pre-denaturation), 35 cycles of 95°C for 1 min (for denaturation), 52 to 62°C for 30 s to 1 min (for annealing) and 72°C for 1 min (for extension), followed by 72°C for 10 min (for final extension). The size of PCR products was then studied by running it on 1.2% SeaKem LE agarose gel and the resultant bands were viewed on a BioRad Gel Doc XR image-analysis system.

Nucleotide sequencing and sequence analysis

The amplified PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA),

Table 1: List of primers used for amplification and sequencing of the *Enterobacter cloacae* isolates in this study

Gene	Primer name	Primer Sequence (5'-3')	Position	Reference
16S	27F	AGAGTTTGATCMTGGCTCAG	+	(Lane *et al.* 1991; Turner *et al.* 1999)
	1492R	GGTTACCTTGTTACGACTT	-	
Hsp60	Hsp60-F	GGTAGAAGAAGGCGTGGTTGC	+	(Hoffmann and Roggenkamp. 2003)
	Hsp60-R	ATGCATTCGGTGGTGATCATCAG	-	
fusA	fusA-f2	TCGCGTTCGTTAACAAAATGGACCGTAT	413–440, +	(Miyoshi-Akiyama *et al.* 2013)
	fusA-r2	TCGCCAGACGGCCCAGAGCCAGACCCAT	1291–1318, -	
leuS	leuS-f2	GATCARCTSCCGGTKATCCTGCCGGAAG	1342–1369, +	
	leuS-r	ATAGCCGCAATTGCGGTATTGAAGGTCT	2159–2186, -	

according to the manufacturer's instructions. For 16S rRNA gene, the amplified genes were inserted into a pCR 2.1 cloning vector and transformed into *E. coli* TOP10F (Gibco Invitrogen, Foster City, CA, USA) and the M13 reverse and T7 promoter primers were used for sequencing. For the remaining genes, including *hsp60*, *fusA* and *leuS*, the PCR primer sets were used for direct sequencing. All the amplified genes were sequenced by using a BigDye terminator cycle sequencing kit and an automatic DNA sequencer (Model 3730, Applied Biosystems, Foster City, CA, USA) at Macrogen Institute (Macrogen Co., Ltd.). The raw sequences were assembled by the SeqMan program (DNAstar package, Madison, WI). The complete sequences were aligned using BioEdit v. 7.2.5 (Yang *et al.* 2017). The resultant nucleotide sequences were aligned using the ClustalX 2.1 program (Larkin *et al.* 2007) and Lasergene software (DNASTAR; Madison, WI, USA) by using the parameters set against the corresponding *E. cloacae* sequences from the NCBI GenBank.

Phylogenetic analysis

The nucleotide sequences of the 16S rRNA, *hsp60*, *fusA*, and *leuS* genes from the isolated *E. cloacae* strain in this study were compared against representative gene sequences from the available *E. cloacae* sequences in the GenBank database. Multiple sequence alignments of the 16S rRNA gene sequence of the selected strains (NiT01/2019, NiT02/2019 and NiT03/2019) with the corresponding sequences from a broad selection of closely related strains, and calculations of the levels of sequence similarity, were made using the open DNA BLAST server (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) and CLUSTAL X 2.1 software (Larkin *et al.* 2007). Evolutionary distance matrices were generated by the neighbour-joining method described by Jukes and Cantor (1969). Phylogenetic tree was constructed by the MEGA 6.06 software package using the neighbour-joining method (Saitou and Nei 1987) and branch support in neighbour-joining tree was estimated by bootstrap resampling method with 1000 replicates (Felsenstein 1985).

Results

Infected symptoms of the *Aloe vera*

The bacteria soft rot disease symptoms of *Aloe vera* in Ninh Thuan province usually occurred after heavy rainfall and/or in the rainy season. These early conditions mainly cause the water-soaked lesions on the plant leaves followed by the infection of bacteria. Therefore, the symptoms were observed around the middle and the blossom end of the leaves. Specifically, the infection started at the water-soaked lesions and then quickly spread rounding of the leaves, leading to yellow discoloration, gas formation, rotting, and collapsing at the base of leaves (Fig. 1).

Bacterial isolation, morphology and biochemical characterization

Three strains of pathogenic agent were isolated from the typical yellow discolored lesions of the plant leaves by using NA media. The isolated strains displayed only one type of bacterial colony, including characteristics of creamy white color, opaque, mucoid, circular, and convex. The isolated strains were Gram negative, rod-shape, about 0.64 μm wide and 1.08 μm long and with peritrichous flagella (Fig. 2). The optimal growth temperature for these isolated strains is 35–37°C. The API 20E kit was used to biochemical characterization of strain NiT01/2019 and the results are shown in Table 2. Strain NiT01/2019 can produce β-galactosidase and Arginine dehydrolase enzymes and can utilize citrate. This strain used mannitol, D-Sorbitol, D-Sucrose, D-Melibiose, Amygdalin and L-Arabinose as substrate to produce acids.

Molecular identification of isolated bacteria

All the obtained nucleotide sequences of the strains under study were deposited in GenBank database under the accession numbers from MT779005 to MT779016. The partial sequence of the 16S rRNA genes of the three isolated strains, the NiT01/2019, NiT02/2019 and NiT03/2019 were amplified and the sequencing was done followed by comparison with the representative sequences which were available on Genbank database. The partial sequence of the 16S rRNA of the three isolated strains was 1500 bp in length. The nucleotide sequence of 16S rRNA gene of the three isolated strains shared 100% sequence similarity. In addition, the nucleotide sequences of the 16S rRNA of the three isolated strains shared the similarity of 97.3–98.0% to the sequence of *E. cloacae* strains available in the Genbank

Fig. 1: Symptoms produced by *E. cloacae* on *Aloe vera* in the field. (**A**) Mass of infected *Aloe vera* was culled. (**B**) The typical soft rot disease symptom of the infected *Aloe vera* with *E. cloacae*

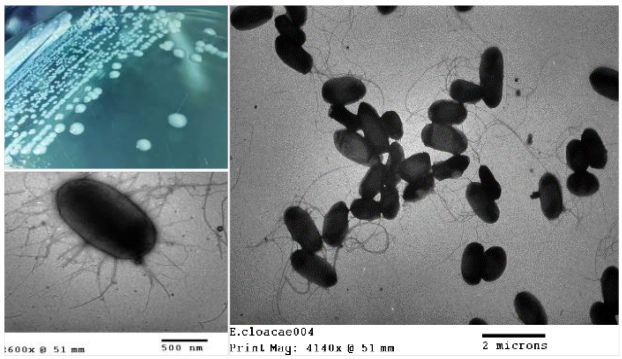

Fig. 2: Electron micrographs of the *E. cloacae* strain NiT01/2019 showing rod-shaped cells and peritrichous flagella. Black bar (nm, left panel; and μm, right panel)

database, *i.e.*, *E. cloacae* A5 strain (accession MN826713), *E. cloacae* ATCC-13047 strain (accession CP001918), *E. cloacae* R6354 strain (JQ659813), *E. cloacae* R6355 strain (accession JQ659814), and *E. cloacae* 3YN16 strain (accession GU549440). Phylogenetic analysis indicated that the isolated strains were clustered into the *E. cloacae* group (Fig. 3). Base on this nucleotide sequence, the 16S rRNA sequences of the three isolated bacteria strains in this study were identified as the members of *Enterobacteriaceae* family (Hauben *et al.* 1998).

In addition to 16S rRNA gene, three more housekeeping genes (*hsp60, fusA,* and *leuS*) were amplified and sequenced to further verify the isolated strains within the available *E. cloacae* complex. The nucleotide sequences of the *hsp60, fusA* and *leuS* genes of the three studied strains shared > 98.5%, > 99.1% and > 99.0% sequence similarity, respectively, with the available sequences of the *hsp60, fusA* and *leuS* genes of the *E. cloacae* in Genbank database. These isolates included *E. cloacae* the ATCC-13047 strain (accession CP001918), *E. cloacae* CBG15936 strain (accession CP046116), *E. cloacae* M12X01451 strain (accession CP017475), Effluent-2, -3, -4 *E. cloacae* strains (accession CP039318, CP039311, CP039303, respectively), *E. cloacae* PIMB10EC27 strain (accession CP020089), *E. cloacae* SBP-8 strain (accession CP016906), *E. cloacae*

Table 2: Physiological and biochemical characteristics of the *Enterobacter cloacae* strains

Characteristic	NiT/2019	ATCC 13047*	*E. cloacae***
Gram staining	-	-	-
Rod shaped cell morphology	+	+	+
Enzymatic activities:			
β galactosidase	+	+	+
Arginine dehydrolase	+	+	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	+	+	+
Citrate utilization	+	+	+
Hydrogen sulfide	-	-	-
Urease	-	-	-
Tryptophan deaminase	-	-	-
Indol	-	-	-
Voges-Proskauer	+	+	+
Gelatin liquefaction	-	-	-
Acid production from:			
D-Glucose	-	+	+
Mannitol	+	+	+
Inositol	-	-	-
D-Sorbitol	+	+	+
L-Rhamnose	+	+	three (+), one (-)
D-Sucrose	+	+	+
D-Melibiose	+	+	+
Amygdalin	+	+	+
L-Arabinose	+	+	+

Reference strains used: **E. cloacae* ATCC 13047 from Bergey's manual (Bergey and Holt 1994) and ***E. cloacae* isolated from cassava (Santana *et al.* 2012)

GGT036 strain (accession CP009756) and *E. cloacae* NH77 strain (accession CP040827). Phylogenetic analysis based on the DNA sequence of *hsp60* gene grouped our three isolated strains into the XI cluster within the *E. cloacae* strains (Hoffmann and Roggenkamp 2003), along with the *E. cloacae* ATCC-13047 strain (accession EU643113), *E. cloacae* EN-475 strain (accession AJ543855), and *E. cloacae* EN-287 strain (accession AJ543768) (Fig. 4).

Pathogenicity test

The NiT01/2019, NiT02/2019 and NiT03/2019 strains were found pathogenic to all healthy *Aloe vera* after 24 h post-inoculation. The symptoms of the *Aloe vera* in pathogenicity test were observed similarly to those appears on the plants observed in the field during sample collection. The symptoms start to appear at the injection sites and grew very fast leading to yellow discoloration with gas formation and the leaves became rotted and finally collapsed after 4-days post-inoculation (Fig. 5). The same bacteria strains were re-isolated and characterized. In contrast, the control group of *Aloe vera* did not show any symptoms.

Discussion

In this study, we report a new disease on *Aloe vera* in Ninh Thuan province, Vietnam caused by *E. cloacae.* Morphological, biochemical, molecular analysis and sequencing of housekeeping genes; *hsp60*, *fusA* and *leuS* demonstrated accurate identification of all the three isolated

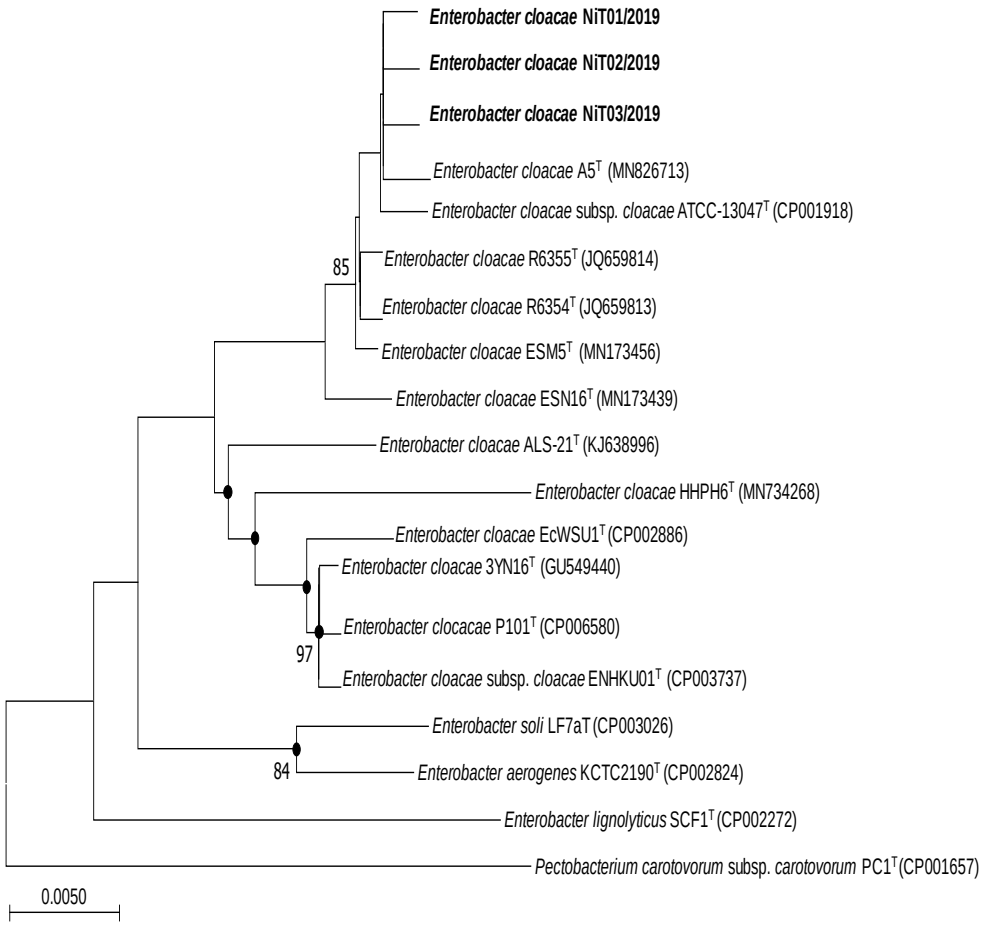

Fig. 3: Phylogenetic tree based on the 16S RNA gene sequences of the three *E. cloacae* strains (NiT01/2019, NiT02/2019, and NiT03/2019) in this study and other known *E. cloacae* strains from the GenBank database. Numbers at the nodes indicate the level of bootstrap support (%) based on neighbor-joining analysis of 1,000 re-sampled datasets. Only values greater than 70% are provided. The bar represents 0.005 substitutions per nucleotide position. The three strains in this study are marked in italic and bold

strains as *E. cloacae* (Hoffmann and Roggenkamp 2003).

E. cloacae bacteria from the genus *Enterobacter* are able to adapt, survive and proliferate in diverse environmental conditions (Sanders and Sanders 1997). In addition to infecting plant species, *E. cloacae* is also well-known as the most important pathogen of human health globally and has been found associated with up to 10% of postsurgical peritonitis cases, 5% of hospital-acquired sepsis, 5% of nosocomial pneumonias, and 4% of nosocomial urinary tract infections (Hoffmann and Roggenkamp 2003). Moreover, it has been reported as a causal agent of many kinds of plant diseases, for example, causes different diseases with diverse symptoms in papaya (Nishijima 1987), onion (Bishop 1990), orchids (Takahashi *et al*. 1997), ginger (Nishijima *et al*. 2004), macadamia (Nishijima *et al*. 2007), dragon fruit (Masyahit *et al.* 2009), mulberry (Wang *et al.* 2010), cassava (Santana *et al*. 2012), lucerne (Zhang and Nan 2013) and chili pepper (García-González *et al*. 2018). Interestingly, this study has identified *E. cloacae* for the first time as a new pathogenic bacterium causing soft rot disease outbreaks of *Aloe vera* in Vietnam.

There are the several ways through which the pathogenic bacteria may acquire plant-pathogenic potential. Deposition of animal pathogens back to environment like give these pathogens a chance to stay near or onto plants where they could easily exchange of the genetic information with other organisms in the environment (Kirzinger *et al*. 2011). For instance, in human, diarrhea disease that is primarily caused by an infection of bacteria can escape from the host into the environment (Müller 1986). This bacterial cells can move from the human wastes to natural source of irrigation water which increases the accumulation of bacterial cell communities into the above-ground portions of the plants (Cooley *et al.* 2003; Schikora *et al.* 2008). However, bacteria also infect plants through indirect routes where insects function as transports from the human host to the general environment and likely to the plants (Nadarasah and Stavrinides 2011).

In this study, *Aloe vera* plants were observed with the water-soaked lesions, dark to yellow discoloration and quick

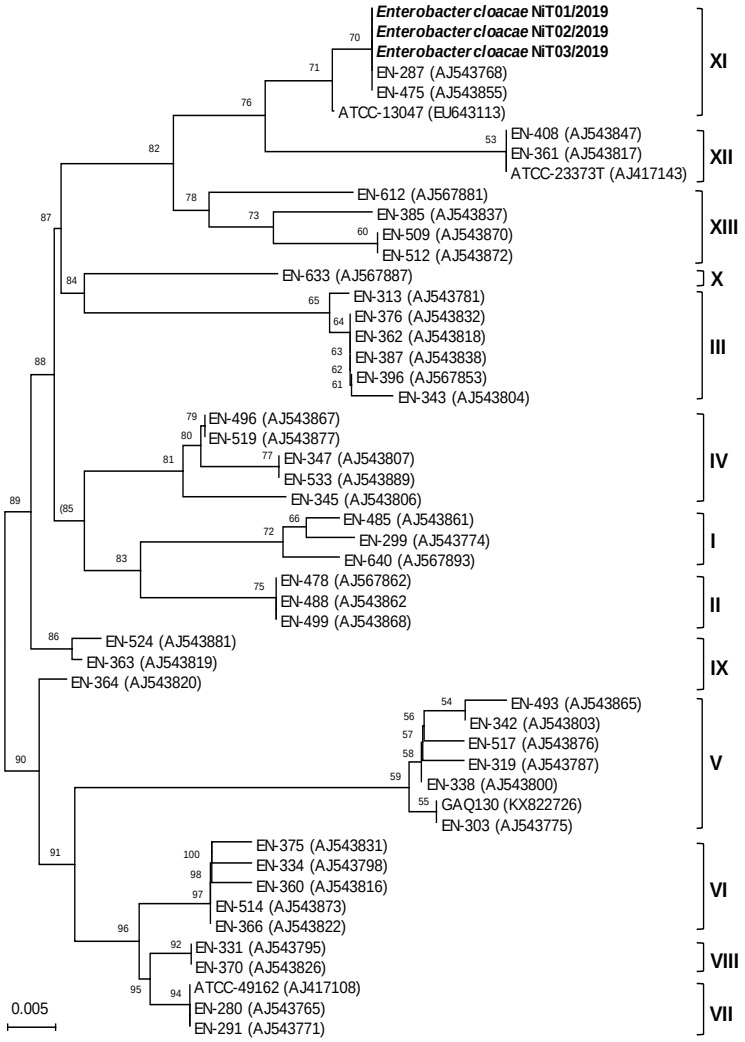

Fig. 4: Phylogenetic tree based on the *hsp60* gene sequences of the three *E. cloacae* strains (NiT01/2019, NiT02/2019, and NiT03/2019) in this study and other known *E. cloacae* strains from the GenBank database. Numbers at the nodes indicate the level of bootstrap support (%) based on neighbor-joining analysis of 1,000 re-sampled datasets. Only values greater than 70% are provided. The bar represents 0.005 substitutions per nucleotide position. The three strains in this study are marked in italic and bold

rotting with gas formation, and ultimately collapsing at the base of leaves. The disease causes the dramatic economic losses suffered by the death or culling of the infected plant. However, the pathogen has not been well identified and therefore the culling or isolating methods are used to prevent spread of the disease. Therefore, more research and experiment are needed to find out the information of epidemiology of this disease in *Aloe vera* plants which could lead to a management plan to reduce its invasion.

Conclusion

This is the first report of the *E. cloacae* that has been identified as a new pathogenic bacterium causing soft rot disease outbreaks of *Aloe vera* in Vietnam. These findings provide the important information regarding the epidemiology, physiology, pathology and genetic diversity of the bacterium and its associated strains. The further analysis will important in study of the origin, pathogenesis and establishment of essential management systems of this particular disease in *E. cloacae*.

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Author Contributions

TNN, VTT conceived and designed the proposal, and funding acquisition. TNN, TVN, TCNH, HPTN performed

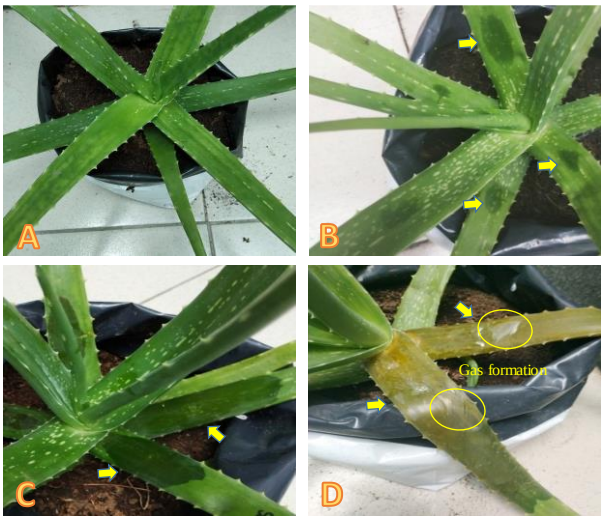

Fig. 5: Pathogenicity test in the healthy *Aloe vera* in greenhouse (**A**) The SDW-injected *Aloe vera* plant was used as control. (**B**) and (**C**) The *E. cloacae*-injected *Aloe vera* plants after one and two days of post-injection, respectively. (**D**) The *E. cloacae*-injected *Aloe vera* plant after four days of post-injection with the typical symptoms of soft rot and gas formation

the experiments. TNN, HMN, VPL, MK, VTT participated in analyzing the data. TNN, HMN, MK, VTT wrote and revised the manuscript. All authors have read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

Data presented in this study are available with the authors

Ethics Approval

There are no researches conducted on animals or humans.

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