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Pathogenicity and Molecular Characterization of *Burkholderia cepacia* Causing Sour Skin Disease of Onion (*Allium cepa*) Bulbs

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ABSTRACT

In this study, *Burkholderia cepacia* was identified as the causal agent of sour skin disease of onion in the Philippines. The bacterium's identity was validated by analyzing the DNA sequence of the 16S ribosomal RNA gene region. The Philippine isolate had 99.51% identity to *B. cepacia* (GenBank accession number: KF681774). *B. cepacia* caused sour skin symptoms in inoculated red and yellow onions. Repeated assays also revealed that scallions are susceptible to *B. cepacia* infection. Since *B. cepacia* was previously reported to infect bulb onions in the field and this study showed severe rotting once the disease developed during storage, efficient detection methods and integrated pest management strategies would be needed to reduce the source of inoculum in the field and mitigate disease development when onion bulbs are in storage.

Keywords: *Allium* disease, bulb rotting, onion disease, *Pseudomonas cepacian*, scallion rot

INTRODUCTION

Onion (*Allium cepa* L.) is the most cultivated species in the genus *Allium*, ranking worldwide as the second vegetable crop with the highest average production quantity (99,968,016 tonnes) next to tomato (Food and Agriculture Organization, 2021). Onion is a rich source of phosphorus, calcium, and carbohydrates, essential elements in the human diet. Onion bulbs, commonly Red Creole and Yellow Granex as the prevalent varieties, are extensively cultivated in 22 provinces of the Philippines, with Region III as the top-producing region with an average annual production volume of 241,030 metric tons in 2022 (Philippine Statistics Authority, 2023). It is commonly planted after rice towards the dry season, utilizing rice straw as mulch material (Lopez & Anit, 1994). The onion industry is one of the most significant contributors to domestic vegetable earnings (Alberto et al., 2018), with the biggest increment in production among all the country's major crops, with a 32.9% increase (Philippine Statistics Authority, 2021). However, onions are more susceptible and predisposed to plant-pathogenic fungal and bacterial diseases.

Burkholderia cepacia (formerly *Pseudomonas cepacia*; Palleroni & Holmes 1981 ex Burkholder 1950) is an important bacterial pathogen that infects onion in field and develops at storage, causing "sour skin disease." Since the mid-90s, it has been known that *B. cepacia* is a complex species, including more than 20 different species sharing similar phenotypes but distinguishable phylogenetically. The use of DNA-based techniques, in addition to morpho-cultural characterization, has made identification more accurate (Jin et al., 2020). This pathogen generally causes rot in onions, which gains entry to the bulbs through various ways such as wounding, insect feeding, use of contaminated farm equipment during harvest, and removal of foliage, rendering the bulb infected at the field before storage. The disease gradually spreads to a few inner scales of mature onion bulbs, causing pale yellow to brown decay. In developed symptoms, rotted outer scales slip off during handling, leaving the healthy inner part (Burkholder, 1950; Sotokawa & Takikawa, 2004). In the Philippines, in an unpublished thesis study, *B. cepacia* was only reported 47 years ago, causing soft rot in onion bulbs. However, soft rotting lacking typical sour skin disease symptoms may indicate that the causative agent could be a different *Pseudomonas* species or bacterium.

This study aimed to identify the causal agent of an onion disease in the Philippines and confirm, using a molecular assay, that *B. cepacia* is causing the observed sour skin diseases. In addition, this study further examines the pathogenicity of *B. cepacia* to locally available selected *Allium* species. The information from this study will help assess postharvest losses and disease management in bulb onions.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation

Several red onion bulbs showing advanced rotting symptoms were observed in a market stand at Los Baños, Laguna, Philippines. Randomly selected infected bulbs were brought to the Plant Pathology Laboratory, Institute of Plant Breeding, University of the Philippines Los Baños, for processing and isolation. The diseased bulbs were washed with running tap water and then subjected to surface sterilization with 10% sodium hypochlorite solution (NaOCl) (v/v, Chlorox, GreenCross, Philippines) for 5 minutes and then washed thrice with sterilized distilled water for 2 minutes for each rinse. Inside the fume hood, small portions of the inner portion (below the onion surface) of the washed rotted tissues of the bulbs were transferred in a 1.5-mL sterilized Eppendorf tube containing sterile distilled water. Tissues were then macerated aseptically using a sterilized micro-pestle. The homogenized mixtures were left to stand for 15 minutes and then streaked into Petri plates containing nutrient agar (NA; Himedia Laboratories Pvt. Ltd., India) using the streak plate technique as described by Kim et al. (2002). Plates were incubated at room temperature (28°C) for 48 hours and then examined for the development of bacterial growth. The single colony technique was performed to obtain pure cultures from the resulting bacterial colonies. The bacterial isolates were preserved according to the method of Akar et al. (2019) with modifications; single colonies of the pathogenic isolate were preserved on Eppendorf tubes containing sterilized distilled water as used in succeeding studies.

Colony Morphology

A bacterial isolate PHOBI-001 purified from single colonies was streaked on NA (Himedia Laboratories Pvt. Ltd., India) and incubated for 48 hours at 28°C. Colony morphology was assessed based on form, elevation, margin, odor, texture, opacity, and color. Fifteen pure representative colonies' average size was measured using the software "ImageJ" (Version 1.51s, Wayne Rasband, National Institutes of Health).

Bacterial DNA Isolation

Bacterial DNA was extracted following the method of Chen and Kuo (1993). The quality of bacterial DNA was checked by gel electrophoresis in 1.5% agarose gel (Vivantis) in 0.5X Tris-acetate (TAE) buffer amended with 1.2 µL of GelRed (Biotium).

Polymerase Chain Reaction Assay, 16S Sequencing, and Phylogenetic Analysis

The 16S ribosomal RNA gene was amplified through a polymerase chain reaction (PCR) performed in the MyCycler™ Thermal Cycler (Bio-RAD, USA) in a 25-µL reaction volume comprised of 0.2 µM each of the universal primer pairs 27F and 1492R (Heuer et al., 1997), 1x PCR buffer (Invitrogen), 2.0 mM $MgCl_2$ (Invitrogen), 0.2mM deoxynucleotide triphosphates (Invitrogen), 1 U *Taq* DNA Polymerase (Invitrogen), 10 ng of bacterial DNA, and diethyl pyrocarbonate water (Sigma) to volume up reaction mix. The thermal cycling condition was as follows: initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes, and a final extension at 72°C for 7 minutes. The PCR product was resolved by gel electrophoresis in a 1.2% agarose gel (Vivantis) in 0.5X TAE buffer, added with 1.0 µL of GelRed (Biotium), and ran on the Mupid-One Electrophoresis System (Advance). The amplified PCR product was visualized using GelDoc™ XR+ with the Image Lab Software (Bio-Rad Laboratories).

For DNA sequencing, the amplified PCR product of primer pairs 27F and 1492R (Heuer et al., 1997) was sent to Apical Scientific Sdn. Bhd. (Malaysia). The consensus sequence was then obtained from the forward and reverse sequences using the Geneious R9 Software (Biomatters, New Zealand) and compared with the 16S rDNA sequences of *B. cepacia* and other *Burkholderia* species (Table 1). A distance tree (maximum-likelihood tree method) was constructed using the MEGA7 software (Kumar et al., 2016). The analysis was performed with 1,000 bootstrap replications using the Tamura and Nei (1993) evolutionary model with gamma rate distribution with invariant sites (G+I).

Pathogenicity Testing on Red Onion Bulbs

The pathogenicity of the bacterium obtained from the diseased onion bulbs was determined by inoculating healthy red onion bulbs. Each isolate was inoculated on three onion bulbs using the methods done by Kim et al. (2002) and Schroeder et al. (2010) with modifications. Instead of boring a hole up to the inner middle of the bulb, only a 5-mm wound was made. Bacterial suspensions from 48-hr-old culture were obtained into sterile distilled water with an optical density (OD_{600}) of 0.3 (at 1×10^7 CFU/mL). Using a sterile needle, the bacterial isolate was inoculated longitudinally on the upper shoulder, running transversely from the outer to the inner part of each surface-sterilized red

onion bulb, replicated at a depth of 5 mm using 100 µL. Inoculated onion bulbs were

Table 1. Type Strains of *Burkholderia cepacia* and Other Related *Burkholderia* Species Were Compared With Bacterial Isolate PHOBI-001 and the Corresponding 16S rRNA GenBank Accession Number of the Sequences Generated

Species	Strain	GenBank Accession Number	Reference
Burkholderia cepacia	SE-1	KF681774	Zhu et al., 2016
Burkholderia cepacia	ATCC 25416	U96927	Viallard et al., 1998
Burkholderia cepacia	PHOBI-001	OP090404	This study
Burkholderia gladioli	CIP 105410	EU024168	Tayeb et al., 2008
Burkholderia thailandensis	E264	U91838	Brett et al., 1998
Burkholderia glumae	LMG 2196	U96931	Viallard et al., 1998
Burkholderia lata	ATCC 17760	MT940984	Feria, 2020
Burkholderia aenigmatica	LMG 13014	NR_174230	Depoorter et al., 2020
Burkholderia pseudomallei	ATCC 23342	DQ108392	Glass et al., 2006
Burkholderia caledonica	RA57	AY949197	Salles et al., 2006
Burkholderia xenovorans	LB400	U86373	Lau and Bergeron, 1997
Burkholderia sabiae	Br3407	AY773186	Chen et al., 2005
Burkholderia sacchari	PAS44	AF263278	Bramer et al., 2001
Burkholderia mallei	ATCC 23344	AF110188	DeShazer and Woods, 1998
Burkholderia megapolitana	LMG 23650	AM489502	Vandamme et al., 2007
Burkholderia dolosa	LMG 18943	NR_118058	Lemaire et al., 2012
Burkholderia stagnalis	LMG 28156	NR_136495	De Smet et al., 2015
Burkholderia multivorans	LMG 13010	Y18703	Bauernfeind et al., 1999
Burkholderia ubonensis	CIP 107078	EU024179	Tayeb et al., 2008
Pectobacterium carotovorum[a]	CFBP2046	NR_118226	Nabhan et al., 2012

Note. [a]Served as an outgroup.

incubated in moistened, clean, sterile plastic containers (12 x 8 x 5 inches, Sunnyware, Philippines) at 28°C for 72 hours. Infection was examined by cutting the bulbs longitudinally. The same method was used for the control treatments; inoculated with 100 µL of sterile distilled water was instead used. To confirm and fulfill Koch's postulates, bulbs showing infection were used to reisolate the bacteria using the above-described method. Pathogenicity trials were done twice.

Cross Infection in Yellow Onion Bulbs and Scallions

Pathogenic isolate PHOBI-001 was inoculated to the healthy yellow onion and scallions. Bacterial inoculum from 48-hr-old culture was prepared using the same procedure as above. The same method and incubation measures were conducted for the yellow onion bulbs using three surface-sterilized yellow onion bulbs and evaluated for infection by cutting the bulbs longitudinally, exposing the inner scales. The same methods were used for the control treatment, using sterile distilled water. In the detached scallions, healthy scallions with intact roots were washed thoroughly with running tap water to remove adhering soil and debris. Scallions were then subjected to surface sterilization. Scallions were soaked and washed in 10% NaOCl for 5 minutes and then subjected to three series of rinsing with sterile distilled water for 2 minutes in each wash. The bacterial inoculum was injected at approximately 1 cm above the basal stem of each surface-sterilized scallion replicate. Inoculated scallions were kept and incubated inside moistened, clean, and sterile plastic containers at 28°C for 72 hours. Scallions inoculated with sterile distilled water served as the control checks. The cross-infection trial was conducted twice.

RESULTS AND DISCUSSION

This study confirmed the presence and the identity of *B. cepacia* causing sour skin disease of onion bulbs in the Philippines from four randomly representative onion bulbs. In addition, this study provided evidence of the pathogenicity of *B. cepacia* to locally available onion varieties (red and yellow) and the damage of the disease to the bulbs. This is the first demonstration of the pathogenicity of *B. cepacia* to scallions in the Philippines.

Bacterium Identity

Bacterial isolate PHOBI-001, grown on NA medium, showed circular colonies in form with entire margin, semi-umbonate elevation, opaque, with viscid texture, and has a yellow pigmentation (Figure 1) and a distinct sulfuric odor. The measured average colony diameter of the isolate on NA medium was 3.21 mm (±0.54; range from 2.54 mm to 4.27 mm) 2 days after incubation.

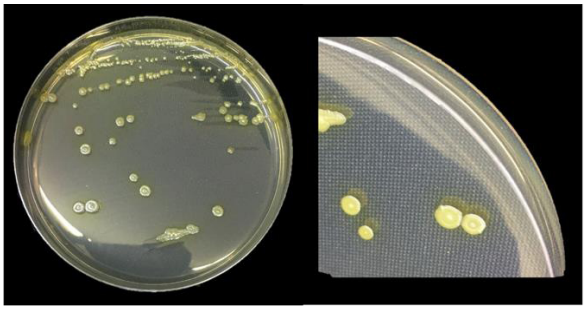

Figure 1. Growth of two-day-old *Burkholderia cepacia* PHOBI-001 in a nutrient agar medium.

A 1,419 base pair product was amplified by PCR assay using the universal primers 27F/1492R. The sequence of the Philippine isolate (GenBank accession number: OP090404) showed 99.51% homology with *B. cepacia* (GenBank accession number: KF681774;

Zhu et al., 2016), confirming the identity of the bacterium. In the constructed phylogenetic tree, using the sequences of *B. cepacia* and other *Burkholderia* species, the isolate PHOBI-001 clustered with *B. cepacia* (Figure 2).

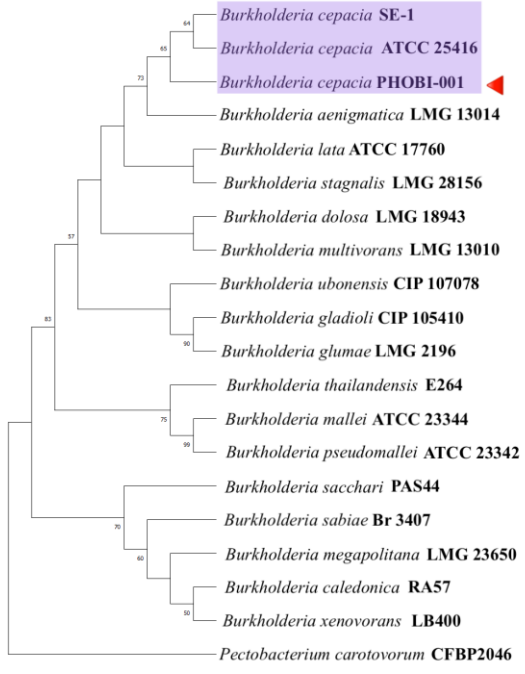

Figure 2. Maximum-likelihood phylogenetic tree reconstruction based on 16s rRNA sequence of *Burkholderia cepacia* PHOBI-001 (GenBank accession number: OP090404) isolate with sequences of *B. cepacia* and other related *Burkholderia* species conducted with MEGA7, with 1,000 bootstrap replicates using Kimura-2 parameter with gamma rate distribution with invariant sites (G+I). Bootstrap support values are indicated at the nodes (values lower than 50 are not shown), with the tree rooted with *Pectobacterium carotovorum* strain CFBP2046 serving as an outgroup.

While *B. cepacia* was previously reported (as *Pseudomonas cepacia*) and was associated with soft onion rot (Tangonan, 1999), this study validated the identity of *B. cepacia* by analyzing the DNA sequence of the 16S rRNA gene region. Nucleotide BLAST searches and phylogenetic reconstruction strongly supported the identity of the bacterium. Nevertheless, recent taxonomic studies revealed that *Burkholderia cepacia* belongs to the *B. cepacia* complex (Bcc), a group of at least 20 genetically related but phenotypically diverse organisms. The Bcc has been reported to have significant biotechnological potential. Some species produce hydrolytic enzymes, and bioactive substances beneficial for plant health and growth have bioremediation properties. In addition, they can produce compounds with biocontrol activity against various phytopathogens. However, despite the agricultural importance of Bcc, there are specific safety issues. These organisms are also known as opportunistic human pathogens associated with cystic fibrosis in patients (Coenye & Vandamme, 2003; Eberl & Vandamme, 2016; Jacobs et al., 2008).

Pathogenicity in Red Onion Bulbs

Inoculated red onion bulbs showed initial rotting symptoms three days postinoculation (dpi). At five dpi, bulbs were cut transversely, showing the extent of infection, with a depth of 8 mm, characterized by slimy, yellow to brown rot on consecutive infected scales (Figure 3B). Pungent, sour, and sulfur-like odors from the infected tissues were also present. Symptoms progressed to neighboring scales, eventually leading to the complete rotting of the onion bulbs at prolonged incubation/storage. Reisolation from the infected tissues showed the same colony and molecular characteristics as with bacterial isolate PHOBI-001 and thus

satisfying Koch's postulates, confirming the pathogenicity of the isolate. No other bacteria or fungi grew from the samples taken from the infected tissues. No infection was observed in the negative control.

Figure 3. Symptom of *B. cepacia* isolates PHOBI-001 showing severe rotting on onion scales. (A) Negative control and (B) experimentally infected red onion bulbs.

Infection in Yellow Onion Bulbs and Scallions

In the inoculated yellow onions, initial symptoms were evident also at three dpi. On the fifth day, deteriorated tissues were evident on the outer scale. Bulbs showed similar rotting symptoms (Figure 4) as observed in the red onion bulbs, with infected tissues having 4.5-mm depth. Reisolation resulted in the same bacterium colony morphologically, and DNA sequences and no other bacteria or fungi grew from the samples taken from the infected tissues. No infection was observed in the negative control.

Figure 4. Symptom of *B. cepacia* isolates PHOBI-001 showing severe rotting on onion scales. (A) Negative control and (B) infected yellow onion bulbs.

The inoculated scallions resulted in initial symptoms (three dpi) of rotting and an irregular growth on the inner portion of the spring onion stem. Curled or semi-twisted portions of the scallions (pointed with a red arrow) were observed (Figure 5). The curling is accompanied by rotting inside and the surrounding stems of the inoculated part. At more prolonged incubation, up to 7 days, rotted tissues progressed, showing slimy, pungent, and deteriorated tissues. The same bacterium was reisolated based on colony morphology, confirming the pathogenicity of *B. cepacia* to scallions.

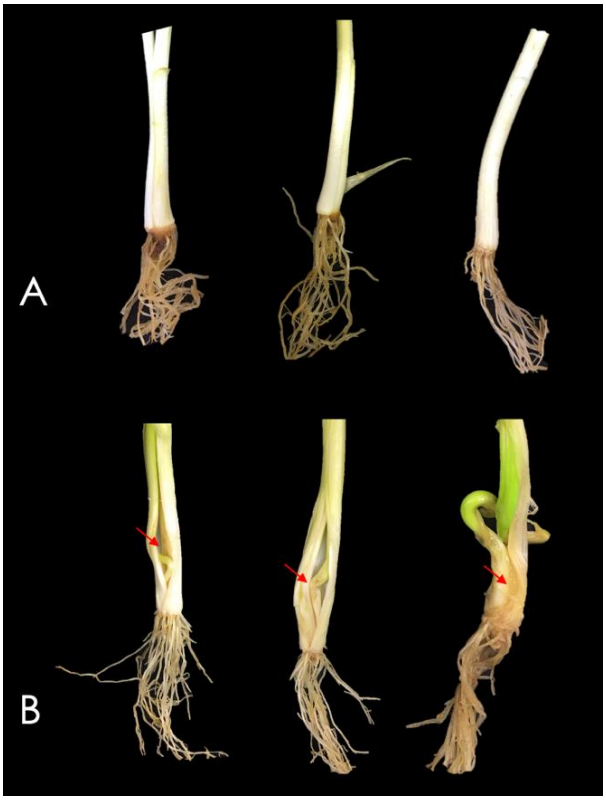

Figure 5. Symptom of *B. cepacia* isolates PHOBI-001 showing rot infection on the basal stem of scallions. (A) Negative control and (B) infected basal stems.

In this study, *B. cepacia* PHOBI-001 could infect and produce rotting symptoms on scallions, a crop belonging to the same genus as onion. The result suggests the pathogenicity of *B. cepacia* to other *Allium* species. Therefore, the contamination of this bacterium during marketing and storage may negatively affect other *Allium* species. The bacterium has also been reported to infect bananas, causing finger-tip rot (Lee et al., 2003) and causing bacterial fruit rot on apricot, plum, nectarine, and kiwifruit (Fang et al., 2009). Thus, future studies on the host range of *B. cepacia* should be conducted to determine susceptible plants and possible alternative hosts. Moreover, plant-pathogenic *B. cepacia* and other Bcc species have been previously reported to predominate in soil, water, certain crops' rhizosphere, and field soil (Jacobs et al., 2008; Tabacchioni et al., 2002). Thus, contamination in the field and postharvest storage poses a problem in onion productivity and could threaten profitability.

Burkholderia cepacia PHOBI-001 infected both red and yellow onions. In the Philippines, bulb rots remain one of the storage problems encountered (Bureau of Postharvest Research and Extension, 2000). As postharvest practices with bulb onions are reported manually by farmers and other stakeholders, culling infected bulbs may not be possible as the early stages of sour skin and internal rots are often overlooked. Further, externally visible symptoms are not also evident during sorting and grading. With this, infected onion bulbs are stored with healthy bulbs, eventually showing latent infection and spreading inoculum during storage, resulting in losses in the entire storage unit (Watson-Selph, 2016). Hence, it is important to develop effective integrated management strategies to detect and reduce inoculum levels and disease incidence in the field and before storage to prevent pre- and postharvest quantity and quality losses.

CONCLUSION

In conclusion, the repeated pathogenicity and cross-infection trials confirmed that *B. cepacia* causes sour skin disease in red and yellow onion varieties. This study also demonstrated that scallions, relative to onion bulbs, are susceptible to *B. cepacia* infection. Since preinfected bulbs at an early stage of the disease are often overlooked before storage, early detection of plant pathogenic *B. cepacia* in the field and effective disease control measures that would suppress inoculum and infection spread should be

developed to prevent infection during storage. Furthermore, research on the environmental conditions during bulb storage should also be considered to determine the optimum storage conditions suitable for suppressing disease development. Additional studies on the possible host range of this bacterial pathogen in the Philippines are also needed. Previous studies elsewhere reported that *B. cepacia* is not only limited to bulb onions.

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