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Identification and Characterization of Soft Rot Bacterial Pathogens on *Phalaenopsis* Orchid in Bali

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ABSTRACT

The moth orchid (*Phalaenopsis* sp.) is one of the most popular orchids due to the various colors with distinctive shapes of the flowers. Soft rot disease caused by Pectobacteriaceae (SRP) family is commonly found infected this plant. The infected orchid showed pale-colored to blackish slimy rot. This research was conducted in three locations namely Denpasar, Badung, and Karangasem, and resulted in 10 candidates for pathogenic bacteria. The pathogenicity test of the pathogen candidates was carried out by injecting bacterial suspension into the orchid leaf tissue with the result of 6 bacterial isolates showing soft rot symptoms. Moreover, two specific primers Dda1F-Dda1R and Pcc3F-Pcc3R for *Dickeya* spp. and *Pectobacterium* spp. consecutively were used for the PCR test. The electrophoresis result of the PCR product identified the bacteria isolated from infected plants as Dickeya spp. Dickeya spp. showed white to yellowish-white colony color, with convex and circular colony form on PDA medium.

Keywords: *Dickeya* spp, moth orchid, Soft Rot Pectobacteriaceae (SRP)

ABSTRAK
Identifikasi dan Karakterisasi Bakteri Patogen Busuk Lunak Pada Anggrek Phalaenopsis di Bali

Anggrek ngengat (Phalaenopsis sp.) merupakan salah satu jenis anggrek yang banyak diminati karena memiliki warna yang beragam dengan bentuk bunga yang khas. Penyakit busuk lunak yang disebabkan oleh famili Pectobacteriaceae (SRP) banyak ditemukan menginfeksi tanaman ini. Anggrek yang terinfeksi menunjukkan busuk berlendir berwarna pucat sampai kehitaman. Penelitian ini dilakukan di tiga lokasi yaitu Denpasar, Badung, dan Karangasem, dan menghasilkan 10 kandidat bakteri patogen. Uji patogenisitas kandidat patogen dilakukan dengan cara menyuntikkan suspensi bakteri ke dalam jaringan daun anggrek dengan hasil 6 isolat bakteri menunjukkan gejala busuk lunak. Selain itu, dua primer spesifik Dda1F-Dda1R dan Pcc3F-Pcc3R untuk *Dickeya* spp. dan *Pectobacterium* spp. berturut-turut digunakan untuk uji PCR. Hasil elektroforesis produk PCR mengidentifikasi bakteri yang diisolasi dari tanaman terinfeksi sebagai *Dickeya* spp. *Dickeya* spp. menunjukkan warna koloni putih sampai putih kekuningan, dengan bentuk koloni cembung dan melingkar pada media PDA.

Kata Kunci: Dickeya spp, anggrek ngengat, Soft Rot Pectobacteriaceae (SRP)

INTRODUCTION

Orchid is a popular ornamental plant favored in society because of its uniqueness and beauty. Orchids are traded around the world as cut flowers, potted plants, and are also used in the medical and food and beverage industries due to their rich content of polysaccharides, alkaloids, and other chemical components (Hinsley *et al.*, 2018; Wang *et al*., 2020). Moreover, consumer demand for orchids also continues to increase each year along with the various functions of this plant. Among thousands of types of orchids, the *Phalaenopsis* orchid, often called the moth orchid, is one of the most popular types of orchids in Indonesia. This is due to the high variation of artificial hybrid *Phalaenopsis* orchids that have been successfully developed (Joko *et al.*, 2011). The flower of *Phalaenopsis* has various colors and shapes as well as last for a long time. However, orchid cultivation has many obstacles, one of which is diseases caused by pathogens infection.

Root diseases, stem decays, leaf spots, and flower blights are common diseases found infecting the Orchidaceae family. Soft rot disease caused by bacterial pathogens is one of the most important limiting factors for cultivating *Phalaenopsis* orchids in Indonesia and all over the world (Hanudin & Suhardi, 2002; Keith *et al.*, 2005). *Dickeya dadantii* and *Pectobacterium carotovorum* subsp. *carotovorum* from the Pectobacteriaceae family has been reported to cause soft rot disease in orchids (Fu & Huang, 2011). In addition, *Pseudomonas viridiflava*, *Pseudomonas marginalis*, and *Burkholderia gladioli* can also cause soft rot disease (Gnanamanickam, 2006).

Like many fungal and bacterial pathogens, soft rot causing bacteria secrete a large arsenal of hydrolytic enzymes that exuviate the plant cell wall

and release nutrients allowing the pathogen to access (Walton 1994; Toth *et al*. 2003). The loss of the structural integrity of the host plant leads to the typical symptom of soft rot. Small water-soaked spots surrounded by yellow halos appear on the leaves of the infected plants with a distinctive rotten smell (Joko *et al*., 2014). Joko *et al.* (2011) reported the losses of orchids due to soft rot diseases in Yogyakarta and West Java were estimated at 30% or more. Research on soft rot disease infected orchids in Bali has never been reported. Therefore, this study aimed to identify the pathogenic bacteria that cause the soft rot disease of orchids in Bali.

MATERIALS AND METHODS

Sampling Site

This research involved sampling and analyzing three orchids cultivation centers to collect orchids with soft rot symptoms and measure the percentage of disease incidence in each site. The site is three of the biggest orchids cultivation center in Bali province, Indonesia: Duta Orchid Garden in Denpasar, Sanjiwani Orchid in Badung, and Wahyu Garden in Karangasem regency. The plants showing slimy rot, pale to black, and rotten smell in leaves, stem, and roots area were categorized into the host of soft rot bacterial pathogen. The percentage of disease incidence in each sampling site was counted with the following equation:

$$PS = \frac{Nh}{Nt} \times (100\%)$$

Description: PS is disease incidence
Nh is infected plants;
Nt is the total observed plants.

Plants showing symptoms of soft rot disease on the observation of disease incidence were taken for further observation regarding the cause of the disease. Five samples from each site were collected by cutting the leaves area with soft rot symptoms, wrapped with plastic, and labeled. A total of 15 leaf samples were collected by purposive sampling and transferred to the laboratory.

Isolation of bacterial pathogen candidate

Orchid leaves with soft rot disease symptoms were cut into 1 cm lengths, added with 5 ml of sterile distilled water, and then crushed using a sterile mortar and pestle. The leaf suspension was directly spread onto a PDA medium, then incubated for 24-36 hours at room temperature. Furthermore, purification was carried out using a new PDA medium and set under similar conditions to obtain single colonies. The PDA used in this research was freshly made with potato, sugar, and agar. In total, ten bacterial isolates were successfully purified from the sampling sites.

Morphological characterization

The morphological characteristics of bacterial colonies, including shape, color, edge, and elevation, were observed following Hadioetomo (1993) methods. This method is required as initial information for bacterial identification regarding the bacteria as a causal agent of soft rot disease in orchid cultivation centers in Bali. The gram assessment was conducted using 3% KOH. One full scope of bacteria isolate was spread onto the sterile object-glass, then dropped with 0.1 ml of 3% KOH. If the treated colony forms a slimy suspension, it is categorized as gram-negative bacteria. Otherwise, if there is no slimy suspension, it is typed into gram-positive bacteria (Abegaz, 2007). A motility test was examined to check bacterial movement undergrowth media. Bacteria isolate mixed with 0.3 ml of sterile distilled water then placed onto object glass, the bacteria were then observed under a microscope. If the bacteria showed some movement, they were categorized as motile bacteria. Otherwise, if the bacteria did not show any activity, they were classified as non-motile bacteria (Mustaqim *et al*., 2014).

Pathogenicity test

Healthy moth orchid leaves were injected with 0.5 ml of each bacterial isolate suspension at 108 CFU/ml (Hanudin & Rahardjo, 2011). The plants were then incubated in a sterile chamber at room temperature for seven days. After one week, the observation was conducted to examine the same disease symptoms as found at the sampling site.

DNA Isolation and Amplification

The bacterial DNA was extracted using the boiling method, 10 µl of each bacterial isolate was placed in a 1.5 ml Eppendorf tube, which contained 90 µl of TE buffer. The total volume is 100 µl with a 1:9 ratio of sample and TE buffer. The sample and buffer mixture was then incubated at 100ºC for 10 minutes. Followed by centrifugation at 4000 rpm for 3 minutes. The supernatant was then collected and placed into a new 1.5 ml Eppendorf tube and kept inside the freezer under a –20ºC temperature (Sunarno *et al*., 2014).

Previously isolated DNA template was amplified with PCR technique using a specific primer to detect Dickeya spp. and Pectobacterium spp. The primers Dda1F - Dda1R (5’-TGTTGGACGCAATA CAGRGAAAG-3’,5’-TCACTCTCCATAGGTGGCA TG-3’) for Dickeya spp. and Pcc3F - Pcc3R (5’-GGGATTCGAAAAATTACTGGCTG-3’,5’-GCTTT TCTTTCATCAACCA-3) for Pectobacterium spp. (Kabir et al., 2020) were prepared separately. The total volume of PCR reaction was 20 µl, including 10 µl GoTaq® Green Master Mix, 1 µl of 10 µM each forward and reverse primer, 7 µl DDH2O, and 1 µl DNA template. Forward and reverse primers were diluted before PCR reaction with a 9:1 ratio of DDH2O and each forward and reverse primer. The PCR reaction was set with initial denaturation under 94ºC for 5 minutes, denaturation under 94ºC for 1 minute, annealing under 57ºC for Dda1F – Dda1R,

and 50ºC for Pcc3F – Pcc3R, extension under 72ºC for 1 minute, and final extension 72ºC for 5 minutes. The PCR cycle was repeated 45 times following the reference from Kabir *et al*. (2020).

The products of PCR were then analyzed using BlueGeltm electrophoresis with 1% agarose gel (0.5xTris-Acetic acid EDTA/TAE). The electrophoresis was done under 220 volt for 28 minutes, and then the gel was incubated inside the box containing 1% ethidium bromide. The final electrophoresis results were visualized with transilluminator ultraviolet. The appeared band was captured manually using a digital camera.

RESULTS AND DISCUSSION

Symptoms and Percentage of Soft Rot Disease incidence in the Field

The observation results of soft rot infected plants at Duta Orchid, Sanjiwani Orchid, and Wahyu Garden showed the same dominant symptoms. Infected tissues showed soft rot and emitted a rotten smell. A more specific symptom was observed in the samples from Duta Orchid Garden and Sanjiwani Orchid, which had a very brittle texture and were entirely affected in the generative or active flowering phase. Meanwhile, samples from Wahyu Garden were still quite sturdy, and most of the infected plants were in the vegetative phase (Figure 1).

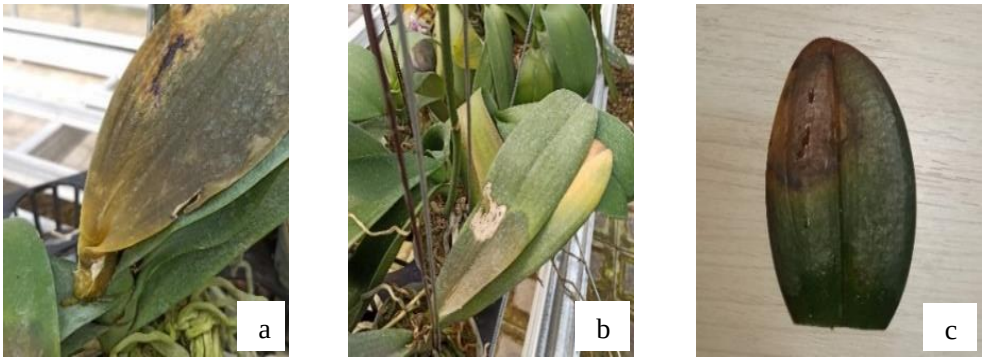

Figure 1. Symptoms of soft rot infection in orchid leaves in Duta Orchid (a), Sanjiwani Orchid (b), and Wahyu Garden (c).

The environmental conditions of all three sampling locations had temperatures ranging from 25-28ºC. The orchids were grown under shading houses, and watering was done irregularly, according to the soil moisture condition. Moreover, fungicides and bactericides were applied 2-4 times a month. The orchid plant's unhealthy or symptomatic parts were maintained by eradication at least two times a week.

The emergence of soft rot symptoms can be caused by an adequate temperature for the growth of pathogenic bacteria, which ranges from 25-30ºC with high humidity ranging from 60-80% (Elpinstone, 1987; Huang, 2008). The percentage of soft rot disease incidences on Phalaenopsis orchids in Bali were 12.1-28.7% (Table 1). Meanwhile, soft rot disease infected Phalaenopsis orchids in Lembang was reported reaching 75% (Hanudin & Suhardi, 2009). Furthermore, Hanudin & Rahardjo (2011) reported that soft rot intensity on Phalaenopsis orchid could reach 81.62% within two days after artificial inoculation. Our results of the soft rot observation and disease percentage in the field are presented in Table 1.

Table 1. The incidence of soft rot infection on *Phalaenopsis* orchids in Bali

No.	Location	Plantation	Nh	Nt	Disease incidence (%)
1.	Denpasar	Duta Orchid Garden	85	700	12,1
2.	Badung	Sanjiwani Orchid	31	108	28,7
3.	Karangasem	Wahyu Garden	103	825	12,5

Description: Nh: Number of plants with soft rot symptoms.
Nt : Total plants observed

Characteristics of the Bacteria Associated with Phalaenopsis Orchid Leaves with Soft Rot Symptoms

Several morphological characteristics of the bacterial candidates causing soft rot showed the same characteristics as Dickeya spp. reported by Muharam *et al*. (2012). Those characteristics, namely white to yellowish-white colony color, early formed colonies are circular and soft, have a convex or raised elevation, and irregular edges depending on the water content of the culture medium. Furthermore, out of ten bacterial candidates, six were shown the same

morphological characters as Dickeya spp., namely isolates B-III, D-I, D-III, K-I, K-II, and K-III. The results of the morphological identification are in Table 2, and the documentation of bacterial development can be seen in Figure 2.

Table 2. Characteristics of Isolated Bacterial Colonies

Isolates	Location	Form	Color	Margin	Elevation
B-I	Badung	Rhizoid	White	Undulate	Raised
B-II	Badung	Irregular	Transparent	Undulate	Flat
B-III	Badung	Circular	Yellowish White	Undulate	Raised
D-I	Denpasar	Circular	Yellowish White	Undulate	Raised
D-II	Denpasar	Spindle	Cloudy White	Undulate	Raised
D-III	Denpasar	Circular	White	Undulate	Raised
K-I	Karangasem	Circular	Cloudy White	Undulate	Raised
K-II	Karangasem	Circular	White	Undulate	Raised
K-III	Karangasem	Circular	White	Undulate	Raised
K-IV	Karangasem	Rhizoid	Creamy White	Undulate	Raised

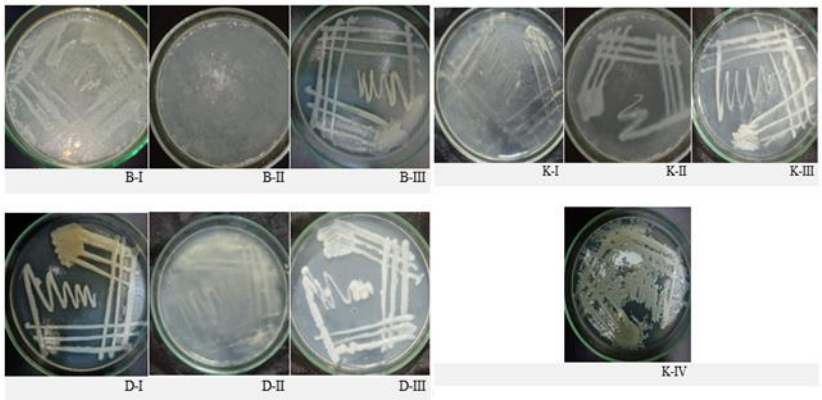

Figure 2. Three days old bacterial isolates on PDA medium.

The results of the bacterial Gram test showed that from 10 isolates, four bacterial isolates were Gram-positive bacteria and six isolates, namely B-III, D-I, D-III, K-I, K-II, and K-III were Gram-negative (Table 3). The bacteria that cause soft rot in plants are those groups of gram-negative bacteria from the Pectobacterium genera (Hanudin & Indijarto, 2012). Moreover, Kabir *et al*. (2020) reported that the disease that often occurs on orchid plants is soft rot disease caused by a group of gram-negative bacteria belonging to the soft rot Pectobacteriaceae. The motility test on pathogenic bacteria candidates showed that all bacteria were motile or had flagella to move (Table 3). Flagella help the bacteria to move and spread on or in the host plant.

Pathogenicity Test of The Bacterial Candidates

Based on the pathogenicity assays, six isolates of pathogenic bacteria candidates, namely B-III, D-I, D-III, K-I, K-II, and K-III, were able to infect plants and cause symptoms. Symptoms of the soft rot disease began to appear two days after inoculation. Initial symptoms were that the plant tissue started to become watery, yellowing, rotting, blackening, and finally falling (Figure 3). The pathogenicity test was carried out for seven days.

The key characteristic of soft rot Pectobacteriaceae (SRP) is that these bacteria have the ability to produce enzymes capable of degrading plant cell walls. This characteristic distinguishes them from new members of the Pectobacteriaceae or previous Enterobacteriaceae families. In SRP, P. carotovorum and D. dadantii are important pathogens that can cause diseases such as soft rot in potatoes or other crops in the field and storage (Kabir *et al*., 2020).

Identification of Soft Rot Bacteria Using Specific Primer

PCR test was carried out to identify pathogenic bacteria that cause soft rot disease with higher accuracy within a short time. Two specific pairs of primers were used to identify soft rot Pectobacteriacea, namely Dickeya spp. and Pectobacterium spp. Six bacterial samples with the ability to produce soft rot disease and one negative control were used in PCR and electrophoresis. The expected target of 157 bp DNA fragments was successfully obtained from PCR product with specific primers of Dickeya spp. (Figure 4A). On the other hand, no DNA fragment was observed on the electrophoresis of PCR product using Pectobacterium spp. specific primer (Figure 4B).

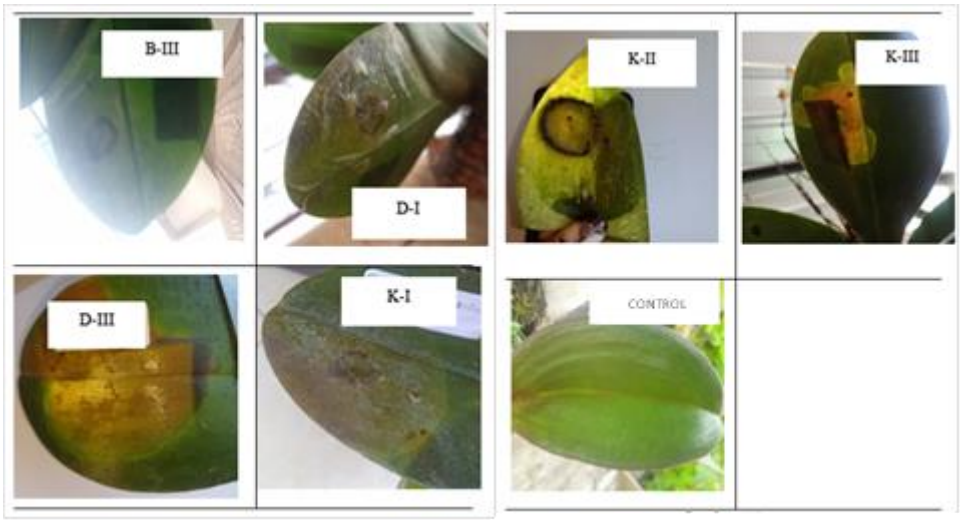

Figure 3. Results of the pathogenicity test on orchid leaves showed soft rot symptoms at day 7 after inoculation. No symptoms were observed on the control plant.

Table 3. Bacterial characteristics and the result of pathogenicity test

Isolate	Gram test	Motility	Pathogenicity
B-I	+	+	-
B-II	+	+	-
B-III	-	+	+
D-I	-	+	+
D-II	+	+	-
D-III	-	+	+
K-I	-	+	+
K-II	-	+	+
K-III	-	+	+
K-IV	+	+	-

Description: (+) : Positive
(-) : Negative

Identification of Soft Rot Bacteria Using Specific Primer

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The results of the electrophoresis visualization showed that the bacteria that caused soft rot disease in the *Phalaenopsis* orchid in Bali were from *Dickeya* spp. Kabir *et al*. (2020) reported that *Dickeya* spp. is one of the pathogenic bacteria that can cause soft rot. The biochemical and PCR identification results carried out by Joko *et al*. (2011) showed that soft rot infected orchids in West Java and D.I. Yogyakarta was caused by *Dickeya dadantii*. Elina (2016) mentions that one of the important problems in orchid cultivation is soft rot disease caused by *Dickeya dadantii*. Fu & Huang (2011) stated that the bacterium *Dickeya dadantii* is one of the bacteria that causes soft rot in orchids, including *Phalaenopsis.*

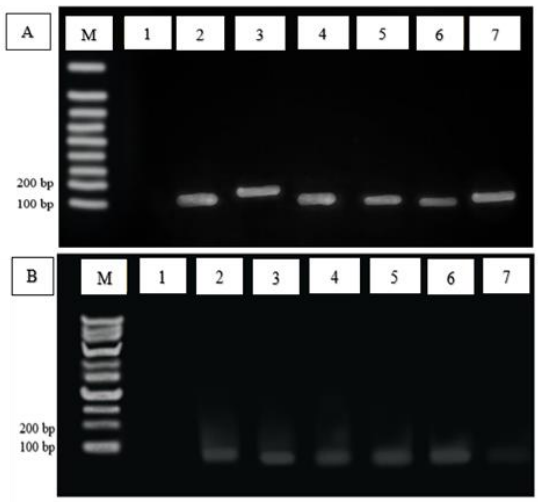

Figure 4. The results of electrophoresis using primer Dda1F-Dda1R (A) and Pcc3F-Pcc3R (B). Marker DNA (M); Negative control (1); B-III (2); D-I (3); D-III (4); K-I (5); K-II (6); K-III (7).

CONCLUSION

Based on the study results, it can be concluded that the incidence of soft rot disease in *Phalaenopsis* orchid in Bali was in the range of 12.1 – 28.7%. The bacterial pathogen causing soft rot disease in Bali was successfully identified using a specific primer as *Dickeya* spp. The results of this study are a piece of the initial information that can be used as a reference in the prevention and control of orchid diseases in Bali.

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