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Pathogen Causing *Phalaenopsis* Soft Rot Disease – 16S rDNA and Virulence Characterisation

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Abstract

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The pathogen causing *Phalaenopsis* soft rot disease and developed detached leaf inoculation methods were identified. Based on its 16S rDNA sequences, the pathogen causing soft rot disease in *Phalaenopsis* was *Erwinia chrysanthemi*/*Dickeya chrysanthemi*. Both virulent and avirulent strains were revealed. The detached leaf inoculation assay for *E. chrysanthemi*/*D. chrysanthemi* resistance evaluation included wounding and inoculating the detached leaf with 10^8 CFU/ml of bacteria. Soft rot disease symptoms in the inoculated detached leaf were measurable at 20 h after inoculation. The detached leaf assay was applicable for evaluating *Phalaenopsis* germplasm and progeny resistance in *Phalaenopsis* breeding programs.

Keywords: *Erwinia*/*Dickeya chrysanthemi*; moth orchids; detached leaf inoculation assay

Soft rot disease is a devastating disease infecting *Phalaenopsis* and other orchids grown in tropical climates (McMillan *et al*. 2007; Joko *et al*. 2012). The first reported case of soft rot disease infecting orchids was in *Dendrobium* sp. and *Phalaenopsis* sp. at Kwangyang and Sunchon, South Korea (Lee *et al*. 1999). Soft rot disease has also become the major *Phalaenopsis* disease in Florida, USA (McMillan *et al*. 2007) and Indonesia (Joko *et al*. 2012). In addition to causing a problem in orchids, soft rot disease also infects many crop species. In potato and cabbage, the soft rot disease infection may continue during post-harvest stages, making the problem more devastating (Bhat *et al*. 2012).

Soft rot disease in Indonesia exists in many orchid growing regions and the disease incidence and intensity reach 100 and 46%, respectively (Joko *et al*. 2012). Optimum environmental conditions including warm temperature and high humidity (McMillan 2007) resulted in a high soft rot disease frequency in the field. Under such optimum conditions, the pathogen associated with soft rot disease survives and quickly spreads from diseased plants to healthy ones.

Pectobacterium carotovorum (syn. *Erwinia carotovora*) is the responsible pathogen causing soft rot disease (Perombelon & Kelman 1980; Mikicinski *et al*. 2009). Therefore, soft rot disease in *Phalaenopsis* is probably because of similar bacterial pathogen infection. Tang and Chen (2007) indicated that the likely pathogen associated with soft rot disease in orchid species was either *E. carotovora* or *E. chrysanthemi* (syn. *Dickeya chrysanthemi*). Revealing the precise identity of the pathogen linked to the disease is important in the development of control strategies using resistant varieties. Therefore, further

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investigation is necessary to positively identify the pathogen associated with soft rot disease of *Phalaenopsis* in Indonesia.

The bacterial pathogen causing the soft rot disease belongs to a Gram-negative, facultative anaerobic pathogenic bacterium (Perombelon & Kelman 1980). The bacterial pathogen secretes a polypeptide having pectolytic activities which cause tissue maceration and soft rot symptom in the infected plant tissues (Joko *et al*. 2014). The soft rot symptoms in the infected *Phalaenopsis* leaf (Joko *et al*. 2012) are very similar to those caused by *Erwinia* sp. infection in potato (Rianawati 2010). One may identify more accurately the pathogen associated with plant diseases using 16S rDNA sequences. Therefore, we need to determine and analyse 16S rDNA sequences of the pathogen associated with soft rot disease in *Phalaenopsis* to reveal its true identity.

Wounding by herbivores such as insects and snails aids the spread of soft rot disease. The foraging insects may carry the bacterial pathogen from diseased orchids to healthy ones. The pathogen may also spread through other means and enter the plant tissues through the stomatal openings (Agrios 2005). In addition to causing leaf damage, a further spread of the bacterial pathogen infection in orchids may also result in stem rot (Lee *et al*. 1999).

Once the identity of the pathogen associated with soft rot disease is determined, it is necessary to investigate the effect of wounding on the soft rot symptom development after inoculation. Moreover, the pathogen may exhibit different virulence among the isolates. Therefore, evaluating strains isolated from the diseased *Phalaenopsis* is needed. In the meantime, inoculum concentration is probably another important factor in soft rot disease development (De Kievit & Iglewski 2000; Miller & Bassler 2001; Sifri 2008). Therefore, it is necessary to study the effects of appropriate inoculum levels to induce soft rot symptoms in *Phalaenopsis*. A simple and reliable method for testing the responses of *Phalaenopsis* against the pathogen causing soft rot disease is a valuable tool for finding the disease resistance donors among germplasm collections.

Indonesian Phalaenopsis Orchids Research Group (IPORG) is interested in developing better *Phalaenopsis* for the tropical region. The activities include breeding for new varieties and developing disease resistant ones (Fatimah & Sukma 2011; Elina 2015; Sukma *et al*. 2015; Sukma & Elina 2017; Handini *et al*. 2016; Haristianita *et al*. 2017).

The experiment reported here aims to isolate and identify pathogens associated with soft rot disease infecting *Phalaenopsis* in Indonesia and determine their virulence. The first activities include isolating pathogens associated with soft rot disease and assessing their identity based on the 16S rDNA sequences and the second include evaluating the effects of inoculum concentrations, strains of pathogens, and species of *Phalaenopsis* for inducing soft rot symptoms in the detached leaf inoculation assays.

MATERIAL AND METHODS

Isolation of bacterial pathogen associated with soft rot disease. We selected infected leaf samples from *Phalaenopsis amabilis* showing soft rot disease symptoms, extracted leaf sap using phosphate buffer saline (0.5 M) and centrifuged leaf extract at 5000 *g*. We spread the supernatant into Casamino Acid-Peptone-Glucose (CPG) plates and incubated the plates at 27°C in a culture incubator until bacterial colonies were visible (two days after plating). Samples of some single bacterial colonies were selected from the plates and maintained as separate isolates of the suspected pathogens. We used these isolates as inoculum in subsequent evaluations.

Validation of the isolated bacterial pathogens. To prepare bacterial inoculum, we inoculated the selected bacterial colony into 100 ml of Luria-Bertani (LB) broth and incubated it at 27°C. The cultures were shaken in a rotary shaker at 100 rpm for 15 hours. The bacterial suspension was centrifuged at 10 000 rpm for 5 minutes to harvest the bacterial cells. After discarding the liquid phase, the pellet was dissolved in 15 ml of sterile water and centrifuged at 10 000 rpm for 5 minutes. Finally, the pellets in 15 ml of water were resuspended, the concentration was adjusted to 1×10^8 CFU/ml, and it was used as inoculum for leaf infiltration and inoculation assay.

Tobacco (*Nicotiana tabacum* cv. White Burley) leaf infiltration assay using 100 µl of the resuspended bacterial isolates was used to validate the identity of the tested bacterial isolates as bacterial pathogens. Any strain capable of inducing a hypersensitive response in the tobacco leaf infiltration assay was further tested using the *Phal. amabilis* leaf infection assay. The strains causing the same symptoms as the original infected leaf were identified as the ones causing soft rot disease. We utilised the validated bacterial

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isolates capable of causing soft rot symptoms in the tested *Phal. amabilis* for subsequent evaluation.

Bacterial pathogen identification based on 16S rDNA sequences. We grew in LB broth three bacterial isolates positively identified as capable of inducing soft rot symptoms (*Dc*1, *Dc*2, and *Dc*3), and harvested the bacterial pellets by centrifugation. Total nucleic acids were isolated from bacterial pellets and used as templates for PCR amplification of 16S rDNA sequences. We amplified the 16S rDNA fragment using universal 27F forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R reverse (5'-GGTTACCTTGTTACGACTT-3') primers. The PCR reagents consisted of 2 µl genomic DNA (15–20 ng), 6.25 µl KAPA2G Fast Ready Mix +Dye (2X) (KapaBiosystems), 0.37 µl of 10 µM of each primer and 3.5 µl ddH_2O. The PCR amplification was carried out in a BIO-RAD 100™ thermocycler machine. The PCR amplification profiles included one cycle of denaturation at 95°C for 1 min; followed by 32 cycles of denaturation at 95°C for 10 s, primer annealing at 53°C for 5 s, and primer elongation at 72°C for 1 second. The final step is a final elongation at 72°C for 10 minutes. We electrophoresed the PCR amplified product in 1× TBE buffer agarose gel electrophoresis at 80 V for 45 minutes. We stained the amplified putative 16S rDNA genomic fragments using 1.2% GelRed, visualised the gel under UV transilluminator and photographed it using a digital camera. After validation, we sent the PCR amplified products to First Base Asia (http://www.base-asia.com/) for DNA sequencing.

The putative 16S rDNA genomic fragments were sequenced using BigDye® Terminator v3.1 cycle sequencing kit. After trimming of low-quality parts, the remaining sequences were aligned to all entries in the NCBI GenBank DNA database using BLAST (http://www.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments (MSA) of the 16S rDNA sequences were done using Geneious Software vR10 (http://www.geneious.com; Kearse *et al*. 2012) and a phylogenetic tree was constructed using MEGA7: Molecular Evolutionary Genetics Analysis v7.0 (Kumar *et al*. 2016). The outputs of BLAST and phylogenetic analysis were used to infer the identity of bacterial isolates.

Pathogenicity analysis of bacterial isolates. We prepared the inoculum from validated bacterial isolates (*Dc*1 and *Dc*2) using previously described procedures and adjusted the final bacterial suspension into either 1×10^6, 1×10^7 or 1×10^8 CFU/ml. Detached leaf (3×3 cm^2) inoculation assay was done to evaluate the pathogenicity of the bacterial isolates.

In the first test, we evaluated the ability of three different bacterial concentrations (1×10^6, 1×10^7, and 1×10^8 CFU/ml) to induce soft rot symptoms in detached leaves of *Phal. amabilis* either with or without wounding. In wounded leaf treatment, the wax and the epidermal layers in the centre of the detached young leaves were wounded using a needle before bacterial inoculation. We spotted as much as 10 µl of each bacterial suspension in the middle of the detached leaf using a micropipette. Three detached leaves for each treatment combination were used. All treated leaves were incubated in a plastic box, layered with a sterile dH_2O wetted sponge. The boxes were stored in an incubation chamber at 25°C and 75% relative humidity. We recorded the occurrences of tissue maceration symptoms and diameter of the symptoms for all treated detached leaves up to 48 h after inoculation.

In the second test, we evaluated the ability of two isolates (*DC*1 and *Dc*2) and three bacterial concentrations (1×10^7, 1×10^8, and 1×10^9 CFU/ml) to induce soft rot symptoms in wounded detached leaves of three *Phalaenopsis* species (*Phal. amabilis*, *Phal. cornu-cervi*, and *Phal. schilleriana*). Wounding and inoculation treatments were done using previously described procedures. The incubation conditions and the observed parameters were also the same as previously described.

RESULTS

Isolation of bacterial pathogen associated with soft rot disease. Figure 1A illustrates the diseased leaf of *Phalaenopsis* showing soft rot symptoms that was used as the source of bacterial pathogen isolation. Figures 1B and 1C show the plating of leaf sap into a plate containing CPG medium (Figure 1B), and the isolation of a single bacterial colony (Figure 1C). We used the selected isolates from the single colony plate for subsequent evaluations.

Validation of the isolated bacterial pathogens. Leaf infiltration assay into tobacco leaf using the selected bacterial isolates resulted in hypersensitive responses (data not shown), confirming their identity as the bacterial pathogen. Further tests using the selected bacterial isolates in the leaf inoculation assay of *Phal. amabilis* also resulted in soft rot symptoms (Figures 1D–E), indicating that the evaluated bacterium was the pathogen causing soft rot disease of *Phalaenopsis* in Indonesia.

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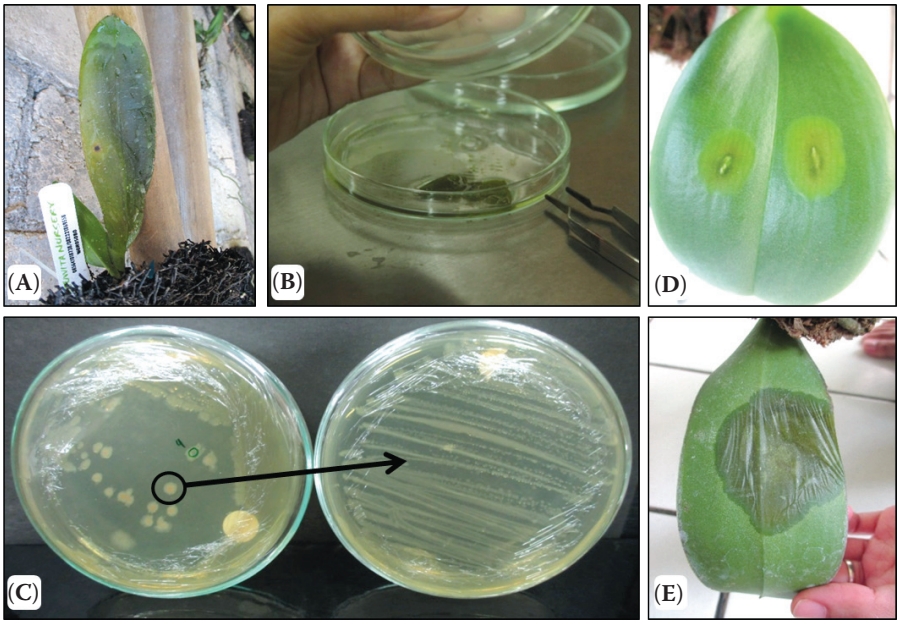

Figure 1. Steps in isolation of bacterial pathogens associated with soft rot disease of *Phalaenopsis* in Indonesia: (**A**) Diseased leaf of *Phalaenopsis* sp. showing soft rot disease symptom; (**B**) Plating of leaf sap on plate containing CPG medium; (**C**) Single colony plate; (**D**) Validation by re-inoculating selected single colony bacteria into leaf of *Phalaenopsis amabilis*; (**E**) Soft rot symptom in a *Phalaenopsis amabilis* leaf at 30 days after inoculation of the validated bacterial pathogen

To maintain the survival of the IPORG *Phalaenopsis* collections, we used the detached leaf assay instead of whole plant inoculation (Elina 2015). In a previous experiment, Elina (2015) demonstrated the effectiveness of detached leaf inoculation assay to induce soft rot symptoms in *Phalaenopsis*. Table 1 presents the effects of wounding and bacterial inoculum concentration on the occurrences of soft rot symptoms in *Phal. amabilis* detached leaf. Without wounding, detached leaf inoculation with bacterial inoculum of either 10^6, 10^7 or 10^8 CFU/ml was unable to induce soft rot symptoms (Table 1). On the other hand, bacterial inoculum at 10^8 CFU/ml was the only concentration capable of inducing soft rot symptoms in the detached leaf starting at 20 h after inoculation (Table 1). The average soft rot diameters for the *Phal. amabilis* leaf were 0.99 cm at 20 h and 1.32 cm at 30 h after inoculation (Table 1). Figure 2 presents photographs of the inoculated detached leaf samples, with or without wounding at 20 h after inoculation. We used a wounding treatment in subsequent detached leaf inoculation assays for inducing soft rot symptoms.

Table 1. Effects of wounding and bacterial inoculum concentrations on the average diameter of the soft rot symptom in a detached leaf of *Phalaenopsis amabilis*

Concentrations (CFU/ml)	Treatment	
	with wounding	without wounding
Soft rot diameter (cm) at 20 h after inoculation		
10^6	0	0
10^7	0	0
10^8	0.99	0
Soft rot diameter (cm) at 30 h after inoculation		
10^6	0	0
10^7	0	0
10^8	1.32	0

Bacterial pathogen identification based on 16S rDNA sequences. Sequencing of 16S rDNA PCR amplicons from three single colonies of the bacterial isolates (*Dc*1, *Dc*2, and *Dc*3) resulted in 1251 (*Dc*1), 1333 (*Dc*2), and 1328 (*Dc*3) nucleotide sequences, respectively. The output of BLAST analysis (accessed date Jan 1, 2017) using *Dc*1, *Dc*2, and *Dc*3 putative 16S rDNA sequences resulted in 100 positive hits including accessions of 16S rDNA from *Dickeya* sp., *Erwinia* sp., *Cedecea* sp., and an uncultured sample (data not shown).

We downloaded 22 accessions of 16S rDNA sequences representing *Dickeya* sp. and *Erwinia* sp. from the NCBI GenBank DNA Database and conducted multiple sequence alignments (MSA) for all 22 accessions. We presented the output of MSA in

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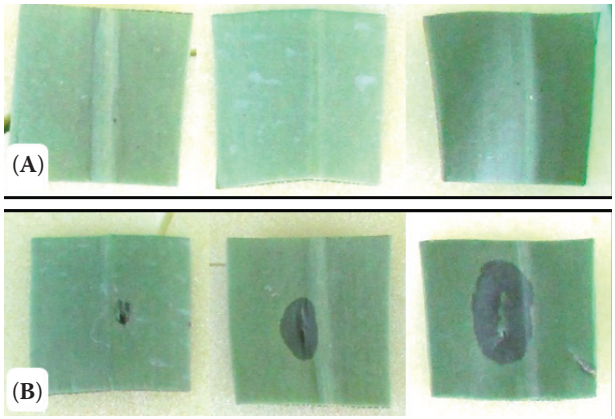

Figure 2. Effects of wounding on the occurrences of soft rot symptoms in a detached leaf of *Phalaenopsis amabilis*. (**A**) normal leaf and (**B**) wounded leaf. We wounded the detached leaves by using a needle and photograph them at 20 h after inoculation. The bacterial inoculum concentration was 1 × 10^8 CFU/ml

Supplementary Figure S1. Using the output of MSA, we constructed evolutionary relationships of the three bacterial strains (*Dc*1, *Dc*2, and *Dc*3) and 19 sequences of *Dickeya* sp. and *Erwinia* sp. available in the NCBI GenBank DNA Database. Figure 3 shows the output of the constructed evolutionary relationships based on their 16S rDNA sequences, which indicates that the three bacterial strains isolated from soft rot disease infected *Phalaenopsis* from Indonesia were closely related with those of the species *E. chrysanthemi* or *D. chrysanthemi*, respectively. Moreover, the *Dc*1 and *Dc*3 isolates were more closely related than that of *Dc*2 based on their 16S rDNA sequences.

Pathogenicity of bacterial isolates. Previously we have tested the 10^6–10^8 CFU/ml bacterial concentrations and found that the bacteria induced soft rot symptoms only at 10^8 CFU/ml (Table 1). We further tested the virulence of isolates *Dc*1 and *Dc*2 to *Phalaenopsis* sp. at higher bacterial concentrations (10^7–10^9 CFU/ml). The results indicated the *Dc*1 isolate was unable to induce soft rot symptoms in all bacterial levels at 20 h after inoculation (Table 2). On the other hand, the *Dc*2 isolate caused soft rot symptoms at 20 h after inoculation of the detached *Phalaenopsis* leaf for all bacterial concentrations. The soft rot symptom diameters increased with the increase in bacterial inoculum concentrations from 10^7 to 10^9 CFU/ml (Table 2).

Table 2. Effects of two bacterial strains and inoculum concentrations on average diameters of the soft rot symptom in a detached leaf of *Phalaenopsis* at 18 h after inoculation

Concentrations (CFU/ml)	Bacterial strains	
	*Dc*1	*Dc*2
10^7	–	4.1[c]
10^8	–	8.8[b]
10^9	–	10.5[a]

– no symptom in leaf; values in a column with the same letter indicated mean values were not significantly different at α = 5% using LSD test; average values were the averages over three samples and three *Phalaenopsis* species

We also tested the ability of the two isolates (*Dc*1 and *Dc*2) to induce soft rot symptoms in the detached leaf of three *Phalaenopsis* species (*Phal. cornu-cervi*, *Phal. schilleriana*, and *Phal. amabilis*). Table 3 shows the results of the evaluation, which further confirmed the inability of the *Dc*1 isolate to induce soft rot symptoms. Figure 4 presents typical soft rot

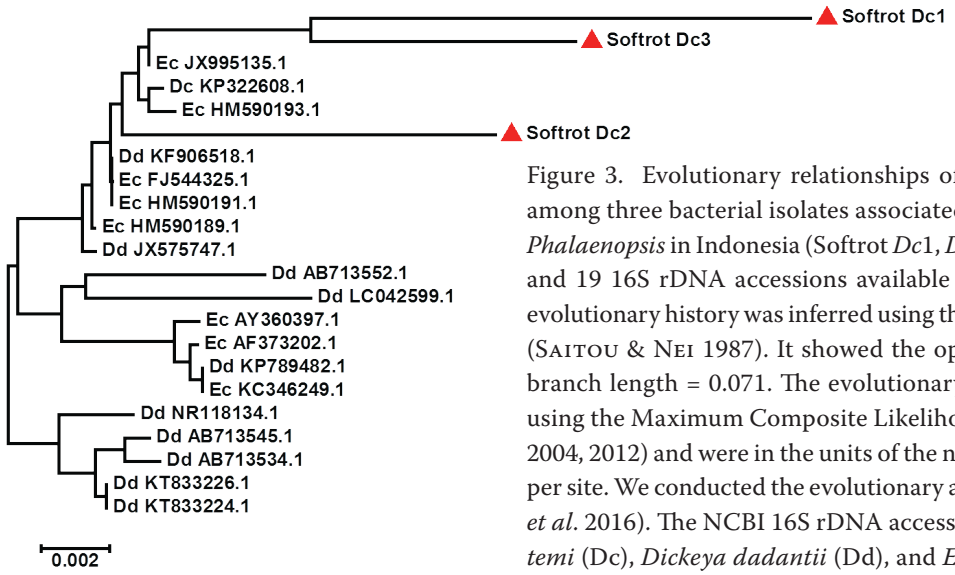

Figure 3. Evolutionary relationships of the 16S rDNA sequences among three bacterial isolates associated with soft rot disease from *Phalaenopsis* in Indonesia (Softrot *Dc*1, *Dc*2, and *Dc*3 – red triangles) and 19 16S rDNA accessions available in the DNA database. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). It showed the optimal tree with the sum of branch length = 0.071. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004, 2012) and were in the units of the number of base substitutions per site. We conducted the evolutionary analyses in MEGA7 (Kumar *et al.* 2016). The NCBI 16S rDNA accessions were *Dickeya chrysantemi* (Dc), *Dickeya dadantii* (Dd), and *Erwinia chrysantemi* (Ec)

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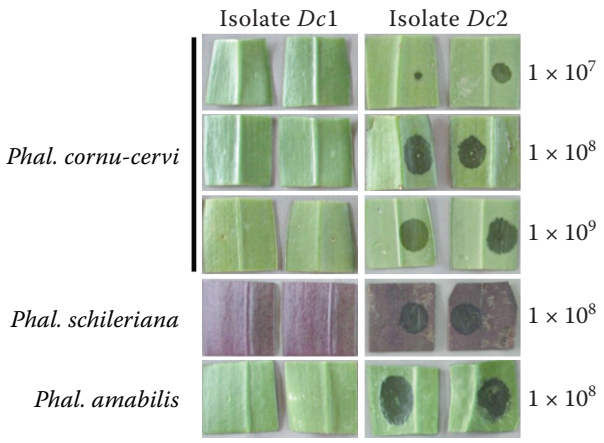

Figure 4. Effects of two bacterial isolates and inoculum concentrations on occurrences of the soft rot symptom in detached leaves of three *Phalaenopsis* species. We photographed the symptom at 20 h after inoculation

symptoms in the detached leaf of *Phalaenopsis* at 20 h after inoculation with *Dc*1 and *Dc*2.

DISCUSSION

We had isolated a single colony of bacterial isolates associated with soft rot disease in the infected leaf of *Phalaenopsis* from Indonesia and confirmed that the isolated bacterial strains were pathogenic bacteria based on the tobacco leaf infiltration assay. The infiltration assay induced a hypersensitive response in tobacco leaves, indicating they were pathogens to plants (Morel & Dangi 1997; Lam *et al*. 2001). Validation of the selected strains by inoculating the strains into the leaf of *Phalaenopsis* resulted in similar soft rot symptoms like the original ones (positive result for Koch postulate; Raza *et al*. 2014). Therefore, *Phalaenopsis* leaf inoculation assay using the selected bacterial strains confirmed their identity as pathogens associated with soft rot disease in *Phalaenopsis*.

Results of the 16S rDNA sequence analysis indicated that the three bacterial strains (*Dc*1, *Dc*2, and *Dc*3) associated with soft rot disease in *Phalaenopsis* from Indonesia were closely related to known isolates of either *Erwinia chrysanthemi* or *Dickeya chrysanthemi*. Therefore, the *Dc*1, *Dc*2, and *Dc*3 strains were most probably the isolates of either *E. chrysanthemi* or *D. chrysanthemi*.

Inoculation of the selected bacterial pathogen onto wounded detached leaves induced soft rot symptoms, as in the *de novo* leaf inoculation (Joko *et al*. 2012).

Table 3. Effects of two bacterial isolates and *Phalaenopsis* species on the average diameters of the soft rot symptoms in leaf at 20 h after inoculation

Phalaenopsis species	Bacterial isolate	
	Dc1	*Dc2*
Phal. cornu-cervi	–	8.06[c]
Phal. schilleriana	–	9.83[b]
Phal. amabilis	–	12.56[a]

– no symptom in leaf; values in a column with the same letters indicated the mean values were not significantly different at $\alpha = 5\%$ using LSD test; average values were the averages over three samples and three bacterial concentrations

Such results also indicated that the detached leaf might be used to evaluate responses of *Phalaenopsis* against *E. chrysanthemi*. The use of detached leaves was advantageous since it would not kill the evaluated mother plants. In sensitive *Phalaenopsis* species, the soft rot disease infection in the leaf may spread systemically, infect the stem, and result in mortality. Moreover, one might modify and provide controlled environment conditions suitable for soft rot symptom development in the detached leaf inoculation assay which would give more reliable evaluation results. Researchers have used the detached leaf inoculation assay effectively for evaluating disease responses in plants (Olmstead & Lang 2000; Yusnita & Sudarsono 2004; Ocktavia *et al*. 2016).

Wounding treatment to the detached leaf was necessary for inducing soft rot symptoms. The inoculated detached leaf without wounding showed no soft rot symptoms. The *E. chrysanthemi* might not have the ability to remove physical barriers in the surface of the detached leaf. Wounding probably removes physical barriers, such as the wax and the epidermal layer and results in the infiltration of bacterial inoculum into the leaf tissues. Such results were similar to the observed disease spread in the glasshouse in Indonesia. Our glasshouse observations indicated there was more rapid disease spread when herbivores were foraging *Phalaenopsis* plants, causing physical injury. Injured plant tissues were also an entry for the spread of the bacterial disease in another plant (Hadiwiyono *et al*. 2007).

We used various bacterial inoculum concentrations and evaluated the diameter of the macerated tissues in the *Phalaenopsis* species. The bacterial concentration of 10^6 CFU/ml was not high enough to induce the soft rot symptoms even in the most

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susceptible *Phal. amabilis*. Moreover, there was inconsistency in the diameter of soft rot symptoms among 10^6 CFU/ml inoculated detached leaves. The bacterial concentration of 10^8 CFU/ml gave the best results in terms of consistency of soft rot symptom diameters and increased rate of diameters among the treated detached leaves. The detached leaf inoculation procedures developed in this study using the bacterial concentration of 10^8 CFU/ml and wounding treatment were the best for inducing soft rot symptoms. In practice, the bacterial concentration of 10^8 CFU/ml was approximately equal to the optical density (OD) value of 0.181. For evaluating responses of *Phalaenopsis* species against soft rot disease, one should observe the soft rot symptoms as early as 20 h after inoculation.

Not all of the identified strains of the bacteria associated with soft rot disease were virulent strains. The *Dc*1 was an avirulent strain of the bacteria associated with soft rot disease while *Dc*2 is the virulent one. The virulence of *Dc*3 strain was not tested in this experiment, however, since the *Dc*1 and *Dc*3 are closely related based on their 16s rDNA sequence, they both were probably the avirulent isolates. However, further virulence evaluation for *Dc*3 and other bacterial strains associated with soft rot disease is necessary. We have also used the suggested detached leaf inoculation procedures for evaluating responses of three *Phalaenopsis* species against soft rot disease. The tested *Phalaenopsis* species were all susceptible to the soft rot disease infection.

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