RELATIONSHIP OF RESISTANCE-RELATED ENZYME ACTIVITY AND SALICYLIC ACID CONTENT IN *PHALAENOPSIS* SPECIES WITH DIFFERENT LEVELS OF RESISTANCE TO *DICKEYA DADANTII*

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ABSTRACT

Orchids (*Phalaenopsis*) are ornamental plants that are cultivated commercially and in great demand in the market. Soft-rot disease (SRD) caused by the necrotrophic pathogen *Dickeya dadantii* is a cause of considerable economic loss to cultivators of many orchid species. Our previous experiment identified a limited number of species that were resistant to *D. dadantii*. This study aimed to validate the resistance level of four *Phalaenopsis* species in a detached leaf inoculation protocol to identify the resistance mechanism(s) involved. Soft-rot symptom diameter was measured from 6 to 18 hours post-inoculation (HPI) with *D. dadantii*. Disease assessment confirmed that *P. amboinensis* is a resistant species, *P. pantherina* is a susceptible species, and *P. amabilis* and *P. schilleriana* are very susceptible species. There was no difference in the lignin content between the resistant and very susceptible species. Detailed observation of resistant and very susceptible species, *P. amboinensis* vs. *P. amabilis*, revealed higher phenylalanine ammonia-lyase (PAL) and peroxidase (POD) in *P. amabilis* than in *P. amboinensis*. In contrast, there was higher salicylic acid (SA) content in *P. amboinensis* than in *P. amabilis*. These results suggest that POD and PAL activities may not be effective in defense against soft-rot disease, while SA plays an important role in the resistance of *P. amboinensis* to *D. dadantii*. Low PAL activity in *P. amboinensis* implies that the SA contents from the isochorismate pathway may be involved in the mechanism of *P. amboinensis* resistance to *D. dadantii*. Therefore, endogenous SA content may be a good indicator for screening resistant species in *Phalaenopsis*.

Key words: necrotrophic, orchid, phenylalanine ammonia-lyase, peroxidase, salicylic acid, soft-rot disease

INTRODUCTION

Plant-pathogen interaction can be diverse depending on the nature of the pathogens. Briefly, biotrophic plant pathogens stay inside living host tissues in a long-term feeding relationship. In contrast, necrotrophic pathogens are parasitic organisms that kill the living cells of their host and then feed on the dead matter. Hemibiotrophic pathogens have a biotrophic phase as the initial stage of infection and switch to the necrotrophic in the last steps of the disease or pathogen life cycle (Göhre & Robatzek 2008; Davidsson et al. 2013; Fatima & Senthil-Kumar 2015; Potrykus et al. 2018; Huang et al. 2020).

Soft-rot disease (SRD) caused by a necrotrophic pathogen is an obstacle to *Phalaenopsis* production. SRD caused up to 75% *Phalaenopsis* plant loss in Ridho Nurseries in West Java (Hanudin & Suhardi 2009). According to molecular identification, the pathogen causing SRD in *Phalaenopsis* is *Dickeya dadantii* (Sudarsono et al. 2018). Rotting, wet, and foul smells may be emitted from the SRD-infected tissue in *Phalaenopsis*. The disease spreads asymptotically on the plant surface, pots, tools, irrigation water, soil, and many surfaces around the cultivating areas and can survive for several months (Pérombelon 1992; Czajkowski et al. 2011). The SRD pathogen needs an entry point into the plants to successfully infect them.

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Natural openings such as stomata and physical wounds on the plant surface, along with plant maintenance, repotting, or distribution, may facilitate the pathogen entrance.

Most *Phalaenopsis* species, including *P. amabilis*, are susceptible to SRD, but two prospective resistant species (*P. amboinensis* and *P. pantherina*) were reported by Sukma et al. (2017). The susceptibility of *P. amabilis* was also reported by Sanjaya et al. (2020) and Lubis et al. (2020). The availability of the resistant species is essential in the resistance breeding program, and the study of resistance mechanisms is necessary for further use in a typical or molecular breeding. Our preliminary investigations did not find differences in leaf water content or leaf thickness, but we found a difference in stomata density between some *Phalaenopsis* species. However, there was no correlation between stomatal density and the SRD severity. We predicted that the leaf characteristics that we observed might not be involved in the *Phalaenopsis* resistance to SRD. We postulated that observing the initial response post-pathogen infections would give us insight into the plant defense of resistant *Phalaenopsis* species to SRD.

Constitutive features influence the plant response to biotic and abiotic stresses and modify plants' defense mechanisms. The defense mechanisms are of a physical and biochemical nature (Mithöfer & Maffei 2017). They act in a constitutive or induced manner. Most of the physical defenses such as cell walls, waxy epidermal cuticles, and bark protect the plant as barriers that keep out infection and give strength and rigidity. The biochemical defenses are commonly induced after the plant detects the pathogen infections and response (Freeman & Beattie 2009). However, there are also some induced physical defenses such as callose deposition and lignification (Bhuiyan et al. 2009; Luna et al. 2011; Wang et al. 2021) and constitutive biochemical defenses known as phytoanticipins (various antimicrobial and repellents) (Pedras & Yaya 2015).

Induced defense is activated when the host plant recognizes the presence of the pathogen, which leads to the molecular and biochemical cascades as a response. ROS, as the earliest response (including hydrogen peroxide, H_2O_2) after the perception of pathogen-associated molecular patterns (PAMPs), has been reported in many plants (Bolwell & Wojtaszek 1997; Bindschedler et al. 2006; Gill & Tuteja 2010; Wang et al. 2020).

At a low level, ROS is essential in cellular proliferation and differentiation (Mittler 2017). Under biotic or abiotic stress, plants generate many ROS involved in the regulation of pathogen defense, programmed cell death (PCD), and stomatal behavior (Gill & Tuteja 2010; Schippers et al. 2016). High ROS accumulation is often referred to as oxidative bursts (Singh et al. 2021) that are toxic to both of host and the pathogen. To deal with the high ROS level, the host plant produces enzymatic and non-enzymatic antioxidants (Hasanuzzaman et al. 2020) as the ROS scavenger that works synergistically and interactively to neutralize free radicals (Huang et al. 2019). Enzymatic antioxidants mainly include superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), while the non-enzymes antioxidant includes glutathione, ascorbic acid (AsA), and flavonoids (Gechev et al. 2006; Huang et al. 2019; Kapoor et al. 2019).

POD is one of the ROS scavengers that convert toxic hydrogen peroxide to water and oxygen (Giorgio et al. 2007). POD activities indicate signal transduction to the remote sites, resulting in necrosis and cell death (Simons & Ross 1971). PAL is an essential enzyme in the phenylpropanoid biosynthesis pathway, producing non-enzymatic antioxidants like flavonoids and salicylic acid (SA) (Zhang & Liu 2015; Lefevre et al. 2020). SA, jasmonic acid (JA), and ethylene (ET) are well-known biochemical compounds that act as signal transducers in the plant to induce defense response genes (Andersen et al. 2018). Both POD and PAL are involved in early resistance response in plants. High POD and PAL activities were found in infected resistant barley seedling, cotton, and *Hibiscus trionum* compared to control or susceptible genotypes to *Verticillium dahliae* (Walters et al. 2002; Golubenko et al. 2007; Xu et al. 2011). High PPO and POD activities were also observed in resistant potato genotype derived through somatic hybridization compared to the Polish cultivar susceptible to *Erwinia carotovora* (Łojkowska & Hołubowska 1992).

Analysis of biochemical components as POD and PAL in *Phalaenopsis* response is expected to help in understanding mechanisms of *Phalaenopsis* resistance to *D. dadantii* and, if they are related with resistance, to validate the resistance level of *Phalaenopsis* species. In this study, we intend to validate the resistance level, POD, and PAL activities of four *Phalaenopsis* species and analyze POD, PAL, and SA performance in resistant versus very susceptible species to *D. dadantii* infection.

MATERIALS AND METHODS

Plant materials

Four *Phalaenopsis* species, namely *P. amboinensis*, *P. pantherina*, *P. schilleriana*, and *P. amabilis*, were collected from nurseries in West Java. The plants of each species were adapted to experimental conditions in a plastic, shading net house under 50–80% relative humidity, at 27–30 °C, with 80% shading. Fertilizers and vitamin B1 were applied twice a week, while fungicides, acaricides, and pesticides twice a month for three months.

Plant inoculation with *D. dadantii*

D. dadantii isolate was obtained from Plant Molecular Biology Laboratory (PMB Lab), Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University. The isolate was previously confirmed as pathogenic to *Phalaenopsis* (Lubis et al. 2020; Sanjaya et al. 2020; Putri et al. 2021). Isolate preparation was made, according to Sanjaya et al. (2020). Briefly, a single colony of *D. dadantii* was cultured in 15 mL of lactose broth medium and shaken (100 rpm) overnight at room temperature. The culture was then centrifuged at 8,000 rpm for 6 minutes. The pellet was diluted with sterilized water to the concentration $OD_{600} = 0.2$. The inoculation was performed using detached leaves following Putri et al. (2021) protocol. The leaf of each plant was cut into 2 cm long parts. The abaxial sites were gently pricked using a sterilized needle. The wound was dropped with 10 µL of *D. dadantii* culture (inoculum concentration 6×10^6 CFU). The leaf cuts were then incubated in covered plastic boxes lined with wet tissue paper at the humidity of 75%.

Disease assessment

Diameters of rot lesions (SrSD) on inoculated leaves were measured every six hours until 18 hours post-inoculation (HPI) using an automatic caliper. The results were scored on the scale from 0 to 10:

- 0 (no symptoms);
- 1 (0.1–1 mm);
- 2 (1.1–2 mm);
- 3 (2.1–3 mm);
- 4 (3.1–4 mm);
- 5 (4.1–5 mm);
- 6 (5.1–6 mm);
- 7 (6.1–7 mm);
- 8 (7.1–8 mm);
- 9 (8.1–9 mm);
- 10 (> 9 mm).

Symptom scoring was used to calculate the disease severity (DS) following the formula by Sanjaya et al. (2020):

$$DS (\%) = \frac{\sum (ni \times vi)}{Z \times N} \times 100\% \quad (1),$$

ni – 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10;

vi – disease score in the score of i ;

Z – maximum score value;

N – number of samples observed.

The plant resistance level to *D. dadantii* was classified based on DS value:

DS of 0–20% – resistant (R);

DS of 21–40% – moderate resistant (MR);

DS of 41–60% – moderate susceptible (MS);

DS of 61–80% – susceptible (S);

DS of > 80% – very susceptible (VS).

Colony-forming units (CFU) from infected leaves were observed at 18 hours post-inoculation. The 50 mg leaf samples were taken from the area around the soft-rot symptom. The samples from three infected leaves from each species were mixed and ground together in 1 mL of sterile distilled water. The suspensions were diluted into seven serial dilutions. Each dilution (3 µL) was dropped into NA medium, incubated at room temperature overnight, and the growing colonies were counted and normalized as CFU per mg following the formula:

$$\text{infected leaf sample (CFU} \cdot \text{mg}^{-1}) =$$

$$\frac{\text{number of colonies}}{3 \mu\text{L}} \times \frac{1000 \mu\text{L}}{50 \text{ mg}} \times \text{dilution factor} \quad (2).$$

The area under the disease progress curve (AUDPC) was calculated by using the formula below:

$$AUDPC =$$

$$\sum \frac{\text{symptom diameter difference}}{2} \times \text{observation time interval} \quad (3).$$

POD, PAL, SA, and lignin content in resistant *P. amboinensis* and very susceptible *P. amabilis* species

To evaluate the relationship between POD and PAL activities with resistance ability, we performed quick screening for these four species (*P. amboinensis*, *P. pantherina*, *P. schilleriana*, and *P. amabilis*). Three leaf samples from three biological repeats of each species were inoculated with *D. dadantii* (inoculum concentration of 3×10^6 CFU) and taken for protein extraction at 12 and 16 HPI. Three leaves of each species were mixed into one sample for protein extraction and then used to quickly screen PAL and POD activities.

To confirm the defense mechanism, we choose two species with a distinct resistance to *D. dadantii* to perform POD and PAL activities and SA contents. This step used four biological repeats for each species inoculated by *D. dadantii* with detached leaf inoculation methods (inoculum concentration of 6×10^6 CFU) and compared them to non-inoculated leaves (mock inoculation) using sterile distilled water. Leaf samples from inoculated and non-inoculated plants were taken at 12 HPI and ground for protein extraction.

Proteins were extracted following the protocol by Sukma et al. (2012). The 0.5 g of leaf tissue was ground in cold phosphate buffer (50 mM, pH 7) at a 1 : 4 (w : v) ratio and centrifuged at 5,000 rpm and 4 °C for 10 minutes. The supernatant was then taken, and total dissolved protein was determined using the protocol outlined in Lowry et al. (1951). Buffer A (0.9 mL, 2% Na_2CO_3 in 100 mL of 1 N NaOH) was added to 1 mL of protein and homogenized by vortex, and then incubated at 50 °C for 10 minutes. The 0.1 mL of buffer B (0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 100 mL of 1% $\text{Na}_2\text{Tartrate} \cdot 2\text{H}_2\text{O}$) was added to the solution, followed by homogenization, and incubated at room temperature for 10 minutes. Then 1 mL of buffer C (50 mL of buffer A + 1 mL of buffer B) was added, followed by homogenization and incubation at room temperature for 10 minutes. As much as 1 mL of buffer D (50% Folin–Ciocalteu dissolved in H_2O) was added, homogenized, and incubated at room temperature for 10 minutes. The absorbance of the solution was measured on a spectrophotometer at a wavelength of 500 nm. The total dissolved protein was determined by using a standard curve from bovine serum albumin (BSA). Tissue protein content was determined by dividing the total dissolved protein value by the weight of the sample used, while the percentage was determined by calculating the total weight of protein (mg) per 100 mg of leaf sample.

POD activity was measured based on the Kar and Mishra (1976) protocol. A total of 100 μL of protein extract was mixed with 2.5 mL of pyrogallol 0.2 M and 250 mg of 1% H_2O_2 . The absorbance values after the reaction were measured using a spectrophotometer at a wavelength of 420 nm every 30 seconds for 150 seconds. The blank used was a mixture of the same solution without protein extract, replaced with phosphate buffer. POD activity was calculated as an increase in absorbance value per unit time per protein weight ($\Delta_{\text{A}420} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein).

$$\text{POD activity} = \frac{\text{difference in absorbance of blank and samples}}{\text{quantity of protein (mg)}} \quad (4).$$

PAL activity was measured based on Dangcham et al. (2008). Briefly, 0.1 mL protein extract was combined with 2.4 mL of L-Phe buffer (0.5 M Tris-HCL containing 6 μM L-phenylalanine) pH 8.5 and incubated at 37 °C for 1 hour. The incubation results were then combined with 0.5 mL of 5 M HCl. The absorbance value of the solution was measured at a wavelength of 290 nm. The blank used was 2.5 mL of L-Phe solution with 0.5 mL of 5 M HCl.

$$\text{PAL activity} = \frac{\text{difference in absorbance of blank and samples}}{\text{quantity of protein (mg)}} \quad (5).$$

SA contents were measured using the modified Tenhaken and Rübel protocol (1997). A total of 1 g leaf sample was ground using a pestle and mortar while adding 10 mL methanol and acetone (1 : 1, v:v) buffer. The suspension was then transferred to an Eppendorf tube and centrifuged at 5,000 rpm for 10 minutes. The supernatant was separated from the pellet. The pellet was resuspended in 3 mL methanol buffer and acetone (1 : 1, v:v) and centrifuged at 5,000 rpm for 10 minutes. The supernatant from this step was mixed with the previous supernatant and vacuum-dried in a freeze dryer. Methanol (5 mL of 30%) was added to the dry residue. The suspension was then centrifuged at 5,000 rpm for 10 minutes. The pellets were discarded, and the resulting supernatant was used for further analysis. The SA contents in sample plants were analyzed qualitatively and quantitatively using HPLC. The mobile phase used was methanol-sodium acetate buffer 50 mM (30 : 70) pH 4.5 (methanol and sodium acetate buffer as much as 500 mL prepared by homogenizing for 10 minutes on a magnetic stirrer) with a flow rate of 0.6 mL per minute. First, the sample and the solution for the mobile phase were filtered off using a 0.45 μm RC cellulose acetate filter membrane. The Chromatopac used was Shimadzu C-R7A plus. The wavelength used in this analysis was 280 nm, while the column used was a VP-DS Ultrasphere (a UV detector at a wavelength of A 280). The SA retention time is 2.549. The formula for calculating the SA contents:

$$\text{SA contents} = \frac{\text{sample area}}{\text{standard area}} \times \text{standard concentration} \times \frac{\text{dilution factor}}{\text{sample weight}} \quad (6).$$

Lignin contents in infected leaves were evaluated in three samples from three biological repeats of *P. amabilis* and *P. amboinensis* at 12 HPI. It was measured following Chesson protocol (Datta 1981). Briefly, 150 mL H₂O was added to 1 g of dry sample (a) and then refluxed at 100 °C in a water bath for 1 hour. The resulting mixture was filtered, and the residue was washed with 300 mL hot water and then dried in an oven until constant weight (b). As much as 150 mL of 1 N H₂SO₄ was added to the residue, then refluxed with a water bath for 1 hour at 100 °C. The mixture was filtered and washed to neutral pH with 300 mL of hot water and dried (c). Ten milliliters of 72% H₂SO₄ were added to the dry residue, soaked at room temperature for 4 hours. As much as 150 mL of 1 N H₂SO₄ was added and refluxed in a water bath for 1 hour at 100 °C. The solid was washed with 400 mL H₂O, strained, and heated in an oven at a temperature of 105 °C, and the result was weighed (d). Finally, the residue was heated into ash at 600 °C and weighed (e). The formula for calculating the lignin contents:

$$\text{Lignin (\%)} = \frac{(d-e)}{a} \times 100\% \quad (7),$$

a – dry sample weight;

d – dry sample post refluxed and washed weight;

e – final ash weight.

Data analysis

The experiments were performed three times. The data obtained were analyzed by analysis of variance (ANOVA) using the Statistical Tool for Agricultural Research (STAR) application. If the treatment was found to have a significant effect on the parameter, the data were further tested with the Tukey test at $p = 0.05$.

RESULTS

Disease assessment and resistance level of four *Phalaenopsis* species

The results of the disease assessment of four *Phalaenopsis* species are presented in Table 1. SRD symptoms first appeared clearly on 6 HPI. The diameters of soft-rot lesions extended with time in all species and were significantly different between the species on 6 and 12 HPI. However, on 18 HPI, there was no significant difference in soft-rot lesion diameter between *P. schilleriana* and *P. amabilis*. The most prominent lesions were found in *P. amabilis*, followed by *P. schilleriana*, *P. pantherina*, and then *P. amboinensis*, respectively. *P. amabilis* had the fastest soft-rot symptom development, from 7.9 ± 0.4 mm on 6 HPI to 20.0 ± 1.5 on 18 HPI. Conversely, lesions on *P. amboinensis* developed more slowly from 0.5 ± 0.1 on 6 HPI to 1.3 ± 0.8 on 18 HPI. From Table 1, we can see that the highest (80%) disease severity (1) was on *P. amabilis* on 6 HPI. In contrast, *P. amboinensis* showed the lowest disease severity (7%) on 6 HPI. On 12 HPI, *P. amabilis* reached 100% disease severity, while *P. amboinensis*, *P. pantherina*, and *P. schilleriana* showed 7%, 50%, and 93% disease severity, respectively. The resistance category of each species was determined based on the disease severity as described in the methods section. On 6 HPI, *P. amabilis* was classified as very susceptible, while *P. schilleriana* was moderately susceptible, and *P. pantherina* was moderately resistant. On 12 HPI, *P. schilleriana* was in the very susceptible category, while *P. pantherina* was moderately susceptible; on 18 HPI, they became susceptible and very susceptible, respectively. In contrast, *P. amboinensis* was in the resistant category from the beginning until the end of the observation. These results showed that *P. amabilis* is the most susceptible and *P. amboinensis* is the most resistant species among the four evaluated species.

Table 1. The reaction of four *Phalaenopsis* species to *D. dadantii* inoculation

Species	6 HPI			12 HPI			18 HPI		
	SrSD (mm)	DS (%)	RC	SrSD (mm)	DS (%)	RC	SrSD (mm)	DS (%)	RC
<i>P. amboinensis</i>	0.5 ± 0.1^d	7 ± 0.0	R	0.6 ± 0.5^d	7 ± 0.1	R	1.3 ± 0.8^c	20 ± 0.1	R
<i>P. pantherina</i>	2.7 ± 1.3^c	30 ± 0.1	MR	4.4 ± 3.2^c	50 ± 0.4	MS	7.9 ± 7.1^b	70 ± 0.5	S
<i>P. schilleriana</i>	4.4 ± 0.6^b	50 ± 0.0	MS	10.1 ± 2.5^b	93 ± 0.2	VS	15.4 ± 4.7^a	100 ± 0.2	VS
<i>P. amabilis</i>	7.9 ± 0.4^a	83 ± 0.1	VS	14.3 ± 1.4^a	100 ± 0.0	VS	20.0 ± 1.5^a	100 ± 0.0	VS

The leaf cuts were inoculated with *D. dadantii* suspension ($OD_{600} = 0.2$, 6×10^6 CFU) and incubated in covered plastic boxes with a base of wet tissue paper (humidity of 75%). The soft-rot symptom diameter was measured at 6 to 18 hours post-inoculation (HPI). The number within a column followed by the same letter means non-significantly different at Tukey test ($p = 0.05$). SrSD – soft-rot symptom diameter, DS – disease severity, and RC – resistant class (R – resistant, MR – moderate resistant, MS – moderate, S – susceptible, VS – very susceptible)

The soft-rot disease symptoms of the four species are shown in Figure 1A. The symptoms are characterized by necrosis, brownishness, rottenness, and wet areas around the infection site. Figure 1A also provides an average colony-forming unit (CFU) value from each inoculated leaf of the species on 18 HPI. The highest number of CFU (2×10^6) were found in *P. amabilis*, followed by *P. schilleriana* (1×10^6), *P. pantherina* (3×10^5), and *P. amboinensis* (5×10^4), respectively. This data revealed the trend of CFU among the species. The species with the heaviest soft-rot symptoms (*P. amabilis*) had a more significant number of CFU than the species with the slightest soft-rot symptoms (*P. amboinensis*), which means a higher number of bacterial cells in the infected leaves of *P. amabilis* than *P. amboinensis*. A higher CFU value seemed to correlate with broader SRD symptoms in susceptible species (Table 1), showing that the pathogen successfully broke the host cell wall as the physical barrier. Besides the CFU value, Figure 1B also shows the area under the disease progress curve (AUPDC). The highest position of AUPDC is from *P. amabilis*, and the lowest position is from *P. amboinensis*. The rapid increase of AUPDC happened on 6 to 12 HPI, as shown by *P. amabilis*, *P. pantherina*, and *P. schilleriana*. In contrast, there was no significant increase in AUPDC on *P. amboinensis*. Soft-rot disease symptoms, disease severity, CFU, and AUPDC values suggest different levels of resistance in the four species and *P. amboinensis* and *P. amabilis* as being very distinct in their response to *D. dadantii* infection.

POD, PAL, SA, and lignin content in four *Phalaenopsis* species

We performed quick screening for POD and PAL activities of these four species by using mixed samples of three biological repeats of infected leaves from each species on 12 and 16 HPI. The results showed that POD activities of *P. amboinensis*, *P. pantherina*, *P. schilleriana* were 2.0×10^{-4} , 0.77×10^{-4} , $7.25 \times 10^{-4} \text{ u} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein on 12 HPI, respectively, while those of *P. amabilis* was $8.73 \times 10^{-4} \text{ u} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein. On 16 HPI, POD activities of *P. amboinensis*, *P. pantherina*, and *P. schilleriana* were 1.14×10^{-4} , 0.57×10^{-4} , and

$4.74 \times 10^{-4} \text{ u} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein. In contrast, *P. amabilis* POD activities increased sharply to $72.49 \times 10^{-4} \text{ u} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein. PAL activities in *P. amboinensis*, *P. pantherina*, and *P. schilleriana* on 12 HPI were 1.20×10^{-4} , 0.95×10^{-4} , and $13.75 \times 10^{-4} \text{ u} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein, while in *P. amabilis*, they were $13.41 \times 10^{-4} \text{ u} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein. On 16 HPI, PAL activities in *P. amboinensis*, *P. pantherina*, and *P. schilleriana* were 1.82×10^{-4} , 1.11×10^{-4} , and $14.73 \times 10^{-4} \text{ u} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein; conversely, in *P. amabilis*, they increased to $123.51 \text{ u} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein.

Interestingly, POD and PAL activities were the highest in the very susceptible species (*P. amabilis*), increasing around nine-fold from 12 to 16 HPI. In contrast, the resistant species, *P. amboinensis*, showed much lower POD and PAL activities. The higher increase in POD and PAL activities in *P. amabilis* at 16 HPI suggested it is induced by *D. dadantii* infection. For more detailed observation of POD and PAL activities, we selected the two extreme species for their resistance to *D. dadantii* (*P. amabilis* vs. *P. amboinensis*) and observed the POD and PAL activities on 12 HPI and compared to the non-inoculated and inoculated leaves. We chose *P. amabilis*, not *P. schilleriana* because, from the quick screening, *P. amabilis* had extreme POD and PAL, even though both are included in the very susceptible class to *D. dadantii*.

As shown in Table 2, POD and PAL activities in *P. amabilis* and *P. amboinensis* had a pattern similar to the previous results from the screening of four species. POD and PAL activities in *P. amabilis* (very susceptible) were significantly higher than those in *P. amboinensis* (resistant). SA contents from *P. amabilis* and *P. amboinensis* are also presented in Table 2. The data shows that *P. amabilis*, the very susceptible species, had twice lower SA content than *P. amboinensis*, the resistant species. However, there is no significant difference in the SA contents between inoculated and uninoculated leaves in each species. Similar SA content level indicated that SA did not increase from 0 to 12 HPI. Lignin contents measured in the infected leaves of *P. amboinensis* and *P. amabilis* at 12 HPI showed no significant difference between the two species (Figure 2).

Figure 1. The development of soft-rot disease symptoms among four species: (A) SrSD on infected leaves at 0–18 hours post-inoculation (HPI), *D. dadantii* colony-forming units (CFU) in infected leaves at 18 HPI, (B) the area under disease progress curve in four *Phalaenopsis* species at various HPI

Table 2. POD, PAL, and SA content in *P. amabilis* and *P. amboinensis* 12 hours post-inoculation

Resistance level to <i>D. dadantii</i>	Treatment	POD (unit·min ⁻¹ ·mg ⁻¹ protein × 10 ⁻⁴)	PAL (unit·min ⁻¹ ·mg ⁻¹ protein × 10 ⁻⁴)	SA (ppm of fresh sample)
very susceptible	<i>P. amabilis</i> non-inoculated	15.30 ± 6.93 ^a	12.20 ± 1.60 ^a	5.26 ± 0.22 ^a
	<i>P. amabilis</i> inoculated	14.96 ± 11.78 ^a	10.78 ± 7.59 ^a	5.32 ± 0.46 ^a
resistant	<i>P. amboinensis</i> non-inoculated	2.35 ± 0.22 ^b	1.54 ± 0.16 ^b	12.56 ± 2.83 ^b
	<i>P. amboinensis</i> inoculated	1.64 ± 0.83 ^b	1.26 ± 0.44 ^b	12.88 ± 2.14 ^b

Figure 2. Percentage of lignin content in dry weight of *Phalaenopsis* species resistant (*P. amboinensis*) and very susceptible (*P. amabilis*) of *D. dadantii* at 12 HPI. There is no significant difference between the two species based on the Student's T-test

DISCUSSION

Soft-rot disease (SRD) is a very devastating disease in the production of essential crops such as potato (Łojkowska & Kelman 1994; Pérombelon 2002; Motyka et al. 2017; Rossmann et al. 2018), *Aloe vera* (Pervez et al. 2016), cucumber (Meng et al. 2017), carrot (Chandrashekar et al. 2018), pineapple (Aeny et al. 2020), Chinese cabbage (Liu et al. 2019b), chicory, *Arabidopsis*, tomato, green pepper, red pepper (Reverchon & Nasser 2013), calla lily (Luzzatto et al. 2007), *Phalaenopsis* (Sudarsono et al. 2018) and other horticulture and plants from seedling to maturity and postharvest. In *Phalaenopsis*, different species showed different resistant responses post-*D. dadantii* inoculation. The species resistant class order in this study was similar to Sukma et al. (2017). *P. amboinensis* was the most resistant species compared to *P. pantherina*, *P. schilleriana*, and *P. amabilis*.

SRD is caused by *Pectobacterium* and *Dickeya* genera, gram-negative necrotrophic phytopathogens (Agyemang et al. 2020). Necrotrophic pathogens acquire nutrients for growth and reproduction from dead cells (Mengiste 2012). Both of the genera can secrete plant cell wall degrading enzymes such as pectinase, polygalacturonase, protease, and cellulase that degrade the host cell wall, macerating host tissue, killing the host cells, and causing wetness and often an unpleasant odor from rotten host tissue that releases nutrients for bacterial growth (Toth et al. 2003; Charkowski 2018). *P. amboinensis* may inhibit the bacterial cell multiplication or control the activities of cell wall degradation enzymes resulting in minimized cell death and giving disadvantages for bacterial growth.

Necrotrophic pathogens manipulate the ROS-mediated defense response of plants to facilitate cell death. ROS accumulation is also a virulence factor for disease development in necrotrophs such as *B. cinerea* (Asai et al. 2010) and *Alternaria solani* (Kobayashi et al. 2012). The effector released by the pathogen using type II and III secretion systems induces a high level of ROS accumulation, known as an oxidative burst, leading to hypersensitive-programmed cell death (HR-PCD) of the host plant (Dey et al. 2020; Expert et al. 2018).

ROS can severely damage carbohydrates, proteins, lipids, and DNA due to the highly reactive nature of the host and the pathogen (Hasanuz-zaman et al. 2020). Interestingly, the study of necrotrophic pathogens showed that *D. dadantii* could survive in an environment with high ROS levels; the pathogen can secrete iron-binding protein that reduces the toxicity of H₂O₂ possibly by preventing the Fenton reaction (Liu et al. 2019a). We did not measure the ROS content in our study; however, we predict that *P. amboinensis* (resistant species) has a low ROS content, which is consisted with low POD activity. In contrast, *P. amabilis* (very susceptible species) may be high in ROS, which promotes POD activities; however, it cannot help the plant cell to survive *D. dadantii* infection because *D. dadantii* can control the ROS effect.

ROS accumulation could be predicted by the activities of antioxidant enzymes, POD and PAL. However, in the present results, there is no significant difference in POD and PAL activities between the non-inoculated and inoculated leaves in each species, suggesting that pathogen inoculation did not induce POD and PAL activities at 12 HPI. This result is different from the quick screening, where POD and PAL increased in *P. amabilis* at 16 HPI. The data revealed the high POD and PAL activities in a very susceptible species that are different from those previously reported; for example, POD and PAL showed higher activities and gene expression in resistant than susceptible genotypes in tobacco against *Meloidogyne incognita* (Li et al. 2018) and tomato against *Ralstonia solanacearum* (Jayanna & Umesha 2017). A similar trend in 12 HPI was also found on 16 HPI; interestingly, a high increase in POD and PAL activity from 12 to 16 HPI was found only in *P. amabilis*. Fu et al. (2012) found that POD was more dynamic in *P. amabilis* inoculated with *E. chrysanthemi* compared to the control, indicating the ROS and enzymatic antioxidant systems (as the ROS scavenger) were involved in the response of the *Phalaenopsis* to the pathogen infection. Higher POD activity in susceptible pear cultivar was also found on 1-day post-infection by necrotrophic pathogen *E. amylovora* (Honty et al. 2008).

Other research showed low PAL activities, total phenolics, total flavonoid, and total tannin in resistant *Zingiber* species compared to susceptible cultivar against necrotrophic *Pythium myriotylum*, indicating lower effectiveness of phenolics in the species, and the resistance may be controlled by single class of metabolites (Ganapathy et al. 2016). The initial stage of the necrotrophic phase was also found associated with high ROS and antioxidant activity in sesame plants against *Macrophomina phaseolina* (Chowdhury et al. 2017). Our results can suggest that the *D. dadantii* successfully targeted ROS mechanisms in susceptible *Phalaenopsis* species (characterized by high POD) to provide plant cell death and adopt the infection environment for their own survival and multiplication.

Both POD and PAL are inducible enzymes that react under stress (Fu et al. 2012; Prasannath 2017; Tsers et al. 2020). Besides the scavenging role, both POD and PAL are also involved in lignin biosynthesis (Xu et al. 2011; Liu et al. 2018). Lignin facilitates the host cell wall's physical barrier and prevents the pathogen infection by spatial restriction, which is disturbed by defects in lignin deposition, trapping pathogens and thereby terminating their growth (Lee et al. 2019). However, there was no difference in lignin contents between the two species with distinctly differing resistance levels to *D. dadantii*. All of those suggest that lignin contents may not contribute to the physical resistance mechanism to *D. dadantii* in resistant species (*P. amboinensis*).

The resistance mechanism in *P. amboinensis* may be caused by the higher SA contents in these plants. ROS, POD, and PAL are known to be synergic to SA (Herrera-Vásquez et al. 2015; Kumari & Vengadaramana 2017). However, in our present results, POD, PAL, and SA showed a contrasting pattern in *P. amboinensis*. According to Durner and Klessig (1995), SA or SA-analog can inhibit the activity of ascorbate peroxidase (APX), an enzyme scavenging H₂O₂, and induce defense-related genes, responsible for increasing the resistance to tobacco mosaic virus. It remains unclear whether SA in *P. amboinensis* also inhibits POD activities.

PAL is the first enzyme reported in SA biosynthesis (Huang et al. 2010), but lower PAL and higher SA were found in resistant *P. amboinensis*, contrasting with the very susceptible species *P. amabilis*. From these results, we predict that high SA contents in resistant species may be synthesized through the isochorismate pathway instead of the PAL pathway. Higher SA in the resistant species may be associated with the *ICS1* gene responsible for the production of ~90% defense-related SA, while the PAL pathway produced ~10% of SA in *Arabidopsis* (Garcion et al. 2008). The role of SA in the mechanism of resistance to the necrotrophic pathogen *D. dadantii* is not fully understood. Some reports suggest a positive effect of SA on *Phalaenopsis* resistance to *D. dadantii*. Exogenous SA application *in vitro* and *in vivo* slightly induced *P. amabilis* resistance to *D. dadantii* (Lubis et al. 2020; Sanjaya et al. 2020). Further research is needed to better understand the mechanism of *P. amboinensis* resistance to *D. dadantii* and the role of SA.

CONCLUSION

P. amboinensis is more resistant than *P. pantherina*, *P. schilleriana*, and *P. amabilis* to *D. dadantii*. The high activity of POD and PAL in *P. amabilis* (very susceptible species) under *D. dadantii* infection suggests that the activity of POD and PAL may not be effective in defense against soft-rot disease, while SA plays an important role in the resistance of *P. amboinensis* to *D. dadantii*. Low PAL activity with a high SA content implies that SA from the isochorismate pathway (not from the PAL-pathway) may be involved in the mechanism of *P. amboinensis* resistance to *D. dadantii*. Therefore, endogenous SA content may be a valuable marker for the detection of resistant genotypes of *Phalaenopsis* spp.

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