The response of *Phalaenopsis amabilis* seedling (*in vitro* and greenhouse) after Salicylic acid treatment to *Dickeya dadantii* infection

UN Q Lubis¹, S Sudarsono² and D Sukma²*

¹ Plant Breeding and Biotechnology Study Program, Graduate School, IPB University
² Biotechnology Division, Department of Agronomy and Horticulture, IPB University

*Email: dewi_sukma@apps.ipb.ac.id

**Abstract.** *Phalaenopsis amabilis* is one of the popular orchid species having white flowers. However, *P. amabilis* is susceptible to soft-rot disease caused by *Dickeya dadantii* infection (a necrotrophic pathogen). This research aimed to induce *P. amabilis* resistance to *D. dadantii* using salicylic acid (SA). The SA treatment at 0 or 30 ppm was applied in the tissue culture medium of *P. amabilis* plantlets, and at 0, 15, 30, 45, 60, 75, or 90 ppm was used to four-month-old seedlings in the greenhouse. Leaves of either in vitro plantlets or seedlings were inoculated with *D. dadantii* by using the detached leaves inoculation method. Bacterial inoculation was carried out at 1, 2, or 3 days after SA treatment on *in vitro* plantlets and one day after on seedlings in the greenhouse. The results showed that SA treatment did not induce the resistance of *in vitro* plantlets. On the other hand, SA 45 ppm treatment slightly increased *P. amabilis* resistance to *D. dadantii* infection. Further studies are needed to confirm this finding and explore the SA role in *P. amabilis* resistance mechanism to necrotrophic pathogens.

1. **Introduction**

*Phalaenopsis amabilis* or known as the moon orchid has been designated as the Indonesian National Flower which is called “Puspa Pesona” based on Indonesian Presidential Decree number 4 of 1993 [1]. This orchid is one of the important native Indonesian orchids since it is widely used as the mother plant for crossing to create superior hybrid orchids [2]. It has up to 30 flowers and a long-lasting blooming phase, thus is often produced commercially as cut flowers and ornamental pot plants [3][4]. The demand for orchids increases every year in Indonesia. However, the cultivation is still relatively slow [5] and faces several problems; one of the problems is soft rot disease.

Soft-rot disease (SRD) is caused by *Dickeya dadantii* [6][7][8]. The soft-rot pathogens are transmitted through soil, water, and seeds. The disease causes crop yields and economic losses for farmers [9]. This disease spreads rapidly, and the plants can rot within 2-3 days. The symptoms are started with small wounds which have characteristic watery, moist, have a distinctive foul odor, and brownish-green spots [10]. The soft rot pathogens produce several enzymes such as pectinases, cellulases, and proteases, which can break down plant cell walls and release nutrients for bacterial growth [11].

SRD can be controlled by spraying bactericides. However, it can give the negative effects to environment and reduce plant quality due to bactericide residues in the plant surface [12]. Considering those impacts, it is essential to find alternatives that are more eco-friendly and effective in controlling SRD by inducing plant resistance. Resistance induction is a procedure to develop plant resistance by
applying an inducing agent [13]. One of the substances that have been studied to play a role in inducing plant resistance to disease is salicylic acid.

Salicylic acid (SA) is a fundamental hormone that activates plant systemic resistance. The exogenous application of SA has been found to induce plant resistance to certain pathogens [14]. Currently, many studies have identified SA as a signaling molecule in the response of plant resistance. When pathogens infect plants, the SA content increases, and signal transduction activates pathogenesis-related (PR) gene expression [15].

Lakani [13] reported that the application of 16 ppm SA increased the resistance of Dendrobium nindii orchids to Odontoglossum ringspot virus (ORSV) by 93.75%. Firgiyanto [16] also reported that the application of 5 ppm and 10 ppm SA increases the resistance of Phalaenopsis KHM 205 hybrid orchid against soft rot pathogens. Furthermore, Khotimah [17] studied the SA application for the induction of fusarium wilt resistance in shallots. However, the SA application for SRD resistance on Phalaenopsis needs to be studied in more detail. Hence, this study aimed to evaluate P. amabilis plantlets’ responses seedlings in vitro and in greenhouse to D. dadantii pathogen after resistance induction with SA treatment.

2. Materials and methods
The study consisted of two experiments, conducted from April to November 2019. The first experiment was SA treatment in P. amabilis orchid plantlet medium in vitro at the Tissue Culture Laboratory. The second one was SA treatment on P. amabilis seedling at the greenhouse. Bacterial infection to test the plant resistance to D. dadantii by using detached leaf inoculation method [17] was performed at the Plant Molecular Biology and Breeding Laboratory (PMB Lab). Green greenhouse and laboratory are located in the Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University. The peroxidase activity analysis was done at the Biorin Laboratory, Department of Food Science and Technology, Faculty of Agricultural Technology, Bogor Agricultural University.

2.1. Evaluation of plantlets response to D. dadantii infection after in vitro SA treatment
The plant material used was P. amabilis orchid plantlets, which we had evaluated for its resistance level to SRD. They were moderate susceptible (26 plantlets), susceptible (35 plantlets), and very susceptible (90 plantlets). The plantlets were cultured on a growing medium containing 2 g/L Hypone, 2 g/L activated charcoal, 50 g/L potato extract, 50 g/L banana extract, Murashige and Skoog (MS) vitamins, 30 g/L sugar and SA according to treatment. Each bottle contained a 25 ml medium with one plantlet. The plantlets were incubated in the culture room for 1, 2, and 3 days after SA treatment, and continued with a response test to D. dadantii. Bacterial inoculation was performed by the detached leaf inoculation method following the method of Tsai et al. with modifications [6][7][8].

The bacteria used in this experiment were isolated from the leaves of the Phalaenopsis orchid infected with soft rot[18]. For inoculation, a single colony subcultured from Nutrient Agar (NA) medium was cultivated in 15 mL of liquid Lactose Broth (LB) medium and shaken at 100 rotations per minute (rpm) for 24 hours. Two milliliter of bacterial suspension were taken and put into a microtube and centrifuged for 6 minutes at a speed of 8,000 rpm. The pellets obtained were dissolved in liquid LB medium, pipette as much as 2 mL and put into a microtube centrifuged again at the same speed for 15 minutes. The pellets were dissolved in 15 mL of LB medium. The bacterial suspension was then diluted in series by adding 1 mL of the suspension stock to 9 mL of distilled water; hence a 10⁻¹ dilution was obtained and continued until a 10⁻³ dilution.

The experimental design was factorial in a randomized complete block design. The factors consisted of plantlet initial resistance class against D. dadantii (moderate susceptible, susceptible, and very susceptible), SA treatment (control (0 ppm SA) and 30 ppm SA), and time length of SA treatment treatment time before D. dadantii inoculation (1, 2 and 3 days). Each treatment was repeated three times, and each replication consisted of 1 plantlet; thus, there were 54 plantlets. The biggest leaves of P. amabilis plantlet were tested to determine its resistance to pathogens causing soft-rot.

Observation of P. amabilis plantlets leaf after inoculation was conducted by measuring the diameter of soft-rot symptoms every 6 hours during the incubation period starting from 12 to 48 hours post-
inoculation (HPI). Calculation of Disease Severity (DS) and Resistance Class (RC) plantlets used the below formula in [19].

\[
DS = \frac{\sum (ni \times vi)}{Z \times N} \times 100\%
\]

Where \( ni \) the number of infected leaves in the score of \( i \), \( vi \) disease score in scale of \( i \), \( N \) the number of leaves observed per plant, \( Z \) the highest score. The symptom score (\( v \)) was determined based on the following criteria: 0 = symptom diameter of \( \leq 1 \) mm; 1 = symptom diameter of \( 1 < i \leq 2 \) mm; 3 = symptom diameter of \( 2 < i \leq 4 \) mm, 5 = symptom diameter of \( 4 < i \leq 6 \) mm, 7 = symptom diameter of \( 6 < i \leq 8 \) mm, and 9 = symptom diameter of \( >8 \) mm. The resistance class (RC) against \( D. \) dadantii was determined based on the criteria for Disease Severity (DS) as follows: resistant (R) for DS 0% - 20%, moderate resistant (MR) for DS 21% - 40%, moderate susceptible (MS) for DS 41% - 60%, susceptible (S) for DS 61% - 80%, and very susceptible (VS) for DS >80%.

The area under the disease progress curve (AUDPC) was determined based on DS at 12, 18, 24, 30 and 36 HPI following the Campbell & Madden method in Yap [20].

\[
AUDPC = \sum_{i=1}^{n-1} \frac{x_i + (x_i + 1)}{2} ((t_i + 1) - t_i)
\]

where \( x \) is the percentage of disease severity, and \( t \) is the interval of observation.

Peroxidase activity analysis was conducted according to the method of Sukma et al. [21] and Firgiyanto [16]. The samples were \( P. \) amabilis leaves at 18 HPI in medium treated with control (0 ppm SA) and 30 ppm SA which were incubated for three days. The leaf tips of orchid seedlings that were not affected by symptoms were cut from each plant and combined as a composite sample for each SA treatment. The combined sample of the leaves used for peroxidase analysis was 1 g.

The collected data were analyzed using Microsoft Excel 2016 and Statistical Analysis System (SAS) software. Data were analyzed with analysis of variance. Further analysis with Tukey’s honestly significant difference (HSD) was performed at the 5% significance level if there was significant effect from the former analysis.

2.2. Evaluation of seedlings response to \( D. \) dadantii infection after SA treatment in greenhouse

The plant materials were four months old \( P. \) amabilis seedlings after acclimatization in the greenhouse. Each seedling had 3-5 leaves on average. The seedlings were random samples from a selfing population of \( P. \) amabilis seedlings with unknown resistance response to \( D. \) dadantii. The seedlings were taken to the laboratory, sprayed with SA according to the treatment concentration until all the plant leaves were wet. The experiment used a randomized complete block design (RCBD) with treatment single factor namely the SA treatment that consisted of 7 levels (0, 15, 30, 45, 60, 75, and 90 ppm) with three replications. Inoculation of \( D. \) dadantii was conducted one day after the SA treatment. Bacterial preparation was similar to Experiment 1 and used a 10\(^{-2}\) dilution for inoculation. The inoculation and incubation methods also followed in Experiment 1. The leaves used were leaf number 3 of \( P. \) amabilis (calculated from the base stem). Soft rot symptoms, disease severity, resistance class, and AUDPC are calculated similarly to Experiment 1. The collected data were analyzed using Microsoft Excel 2016 and SAS software to variance analysis at \( \alpha \) 5%. Treatments that significantly affect the observed character were processed for further analysis with Tukey’s honestly significant difference (HSD) at the level of 5% significance.

3. Results and discussion

3.1. Plantlets response to \( D. \) dadantii infection after in vitro SA treatment

The response of \( P. \) amabilis plantlets to \( D. \) dadantii after SA treatment are presented in Table 1. \( Phalaenopsis \) amabilis plantlet with moderate susceptible and susceptible resistance level in control (0 ppm SA) and 30 ppm SA became very susceptible in all of incubation time treatment (1, 2 and 3 days). In contrast, very susceptible plantlet after the treatment of control (0 ppm SA) and 30 ppm SA in the 1
and 2 days incubation time was still categorized as very susceptible, while the three days incubation time treatment became moderate resistant and susceptible with DS of 36% and 73% respectively.

Table 1. The diameter of soft-rot symptoms, disease severity and resistance criteria of *P. amabilis* plantlets against pathogens causing soft rot disease of *D. dadantii* after in vitro SA treatment at 24 HPI.

| RC1 SA (ppm) | Incubation time (day) | Diameter of soft rot symptoms (mm) | DS (%) | RC2 |
|--------------|------------------------|------------------------------------|--------|-----|
| MS 0         | 1                      | 9.32±0.59                          | 100    | VS  |
|              | 2                      | 8.27±1.33                          | 93     | VS  |
|              | 3                      | 8.63±1.22                          | 88     | VS  |
| 30           | 1                      | 9.16±0.54                          | 100    | VS  |
|              | 2                      | 8.99±0.83                          | 98     | VS  |
|              | 3                      | 9.31±0.79                          | 100    | VS  |
| S 0          | 1                      | 9.28±1.03                          | 95     | VS  |
|              | 2                      | 9.81±0.35                          | 100    | VS  |
|              | 3                      | 7.76±1.20                          | 83     | VS  |
| 30           | 1                      | 9.78±0.33                          | 100    | VS  |
|              | 2                      | 8.79±1.07                          | 90     | VS  |
|              | 3                      | 8.13±1.27                          | 88     | VS  |
| VS 0         | 1                      | 8.30±1.24                          | 85     | VS  |
|              | 2                      | 9.96±0.10                          | 100    | VS  |
|              | 3                      | **3.57±1.51**                      | **36** | **MR** |
| 30           | 1                      | 9.54±0.66                          | 100    | VS  |
|              | 2                      | 10.00±0.00                         | 100    | VS  |
|              | **3**                  | **6.88±2.94**                      | **73** | **S** |

Note: SA = Salicylic acid; HPI = hours post-inoculation; MR = moderate resistant; S = susceptible; VS = very susceptible; DS = disease severity; RC1 = resistance class before SA treatment; RC2 = resistance class after SA treatment

The inoculation result of leaf cuttings from various plantlet resistance classes after SA treatments is presented in Figure 1. Plantlets which resistance classes were moderate susceptible, susceptible, and very susceptible had almost equal symptom area in both control (0 ppm SA) and 30 ppm SA at incubation time of 1 and 2 days.

**Figure 1.** The performance of soft rot symptom after *D. dadantii* infection. Plantlets were treated with 30 ppm of SA in the plant medium for 1, 2 or 3 days before bacterial inoculation. Control is without SA (0 ppm) treatment.
According to the variance analysis, the three treatment factors (resistance class, SA treatment, and medium incubation time) showed a significant effect on 24 and 30 HPI; meanwhile, the other observations time (12, 18 and 36 HPI) had neither significant effect. The average diameter of soft rot symptoms that showed in the 24 and 30 HPI observations are presented in Table 2. In general, 30 ppm SA treatment showed a wider diameter of soft rot symptoms than control (0 ppm SA) in 24 HPI observations. In contrast, exceptionally for the plantlet, whose resistance class is moderately susceptible that showed in one day incubation time resulted in a wider diameter of symptoms. Likewise, at the 30 HPI the control displayed a wider soft rot diameter, but the results were not significantly different from the 30 ppm SA treatment.

Table 2. Interaction of three important factors among resistance class, SA treatment, and incubation time toward the diameter of soft rot symptoms (mm) in P. amabilis plantlets.

| RC1 (ppm) | SA (ppm) | 24 HPI Incubation time (day) | 30 HPI Incubation time (day) |
|-----------|----------|-----------------------------|-----------------------------|
|           |          | 1  | 2  | 3  | 1  | 2  | 3  |          |
| VS 0      |          |    | 8.30 abc | 9.95 a  | 3.56 d | 9.93 a  | 10.00 a  | 6.84 b  |
| VS 30     |          | 9.54 ab | 10.00 a  | 6.87 c  | 10.00 a  | 10.00 a  | 8.85 a  |
| S 0       |          | 9.27 ab | 9.81 a  | 7.75 bc | 10.00 a  | 10.00 a  | 9.96 a  |
| S 30      |          | 9.77 ab | 8.78 abc | 8.13 abc | 10.00 a  | 10.00 a  | 10.00 a  |
| MS 0      |          | 9.32 a  | 8.26 abc | 8.63 abc | 10.00 a  | 9.87 a  | 10.00 a  |
| MS 30     |          | 9.15 ab | 8.98 ab | 9.31 ab | 9.97 a  | 10.00 a  | 10.00 a  |

Note: Numbers in the same column followed by the same letter were not significantly different at α 0.05 (Tukey’s HSD test). RC1 = resistance level before SA treatment; SA = salicylic acid; HPI = hours post-inoculation; VS = very susceptible; S = susceptible; MS = moderately susceptible.

The disease severity progression is the basis in determining the resistance class of each individual tested according to AUDPC formula [4]. The development of soft rot symptoms in P. amabilis plantlets at control (0 ppm SA) and 30 ppm SA for 1, 2 and 3 days incubation time are presented in Figure 2. P. amabilis plantlets’ treatment at control (0 ppm SA) and 30 ppm SA for an incubation time of 1 and 2 days showed an almost equal effect, as seen from the similar graphical form.
Figure 2. Disease severity of different initial resistance class of in vitro plantlet after bacterial infection. Initial plantlet resistance classes were very susceptible (VS), susceptible (S) or moderate susceptible (MS). Plantlets were treated by SA 30 ppm: (a) 1 day (b) 2 days and (c) 3 days before D. dadantii infection. Control (0 ppm) means without SA treatment.

3.2. Seedling response to D. dadantii infection after SA treatment in greenhouse
The SA concentration treatments results are presented in Table 3. The 45 ppm SA concentration application showed the lowest disease severity (63%) and the smallest symptom diameter (5.50 mm) at 24 HPI, but remains categorized as susceptible. In comparison, control (0 ppm SA) had the widest soft rot diameter (7.63 mm) and showed similar disease severity (85%) as 90 ppm SA treatment, which was included in the very susceptible resistance class.

Table 3 Diameter of soft rot symptoms and severity in P. amabilis seedlings in greenhouse by detached leaf inoculation method after SA treatment

| SA treatment (ppm) | Soft-rot symptom diameter (mm) ± STDEV | DS 24 HPI (%) | RC |
|-------------------|----------------------------------------|---------------|----|
|                   | 18 HPI | 24 HPI | 36 HPI |               |
| 0                 | 5.23±0.68 | 7.63±0.84 | 9.40±1.04 | 85 | VS |
| 15                | 5.03±0.40 | 6.57±1.87 | 8.63±1.40 | 70 | S |
| 30                | 5.70±1.01 | 6.83±1.47 | 8.27±1.66 | 78 | S |
| 45                | 4.87±0.81 | 5.50±0.79 | 5.67±1.89 | 63 | S |
| 60                | 4.83±1.12 | 6.53±0.97 | 7.53±2.36 | 70 | S |
| 75                | 5.23±0.32 | 6.73±1.00 | 8.30±2.61 | 70 | S |
| 90                | 4.53±0.32 | 7.57±0.84 | 10.0±0.00 | 85 | VS |

Note: SA = salicylic acid; HPI = hours post-inoculation; DS = disease severity; RC = resistance class; VS = very susceptible; S = susceptible;
the slowest progression of disease severity with the smallest symptoms occurred in 45 ppm SA treatment.

![Figure 3](image)

**Figure 3.** (a) Soft rot symptoms after *D. dadantii* infection on *P. amabilis* leaves after different SA treatment on 24 HPI. Bacterial inoculation used detached leaf inoculation method. (b) AUDPC graph from different SA concentration in *P. amabilis* seedling in greenhouse

4. Conclusion

*In vitro* treatment of SA (30 ppm) for 1, 2, or 3 days before *D. dadantii* infection did not increase *P. amabilis* plantlet resistance to soft rot disease. Evaluation of higher SA concentration *in vitro* could be considered in future experiments. SA treatment (45 ppm) on the *P. amabilis* seedling in the greenhouse slightly increased plant resistance to *D. dadantii* infection. Lower or higher SA concentration increased soft rot symptom diameter and plant susceptibility to *D. dadantii*.

4. Conclusion

In vitro treatment of SA (30 ppm) for 1, 2, or 3 days before D. dadantii infection did not increase P. amabilis plantlet resistance to soft rot disease. Evaluation of higher SA concentration in vitro could be considered in future experiments. SA treatment (45 ppm) on the P. amabilis seedling in the greenhouse slightly increased plant resistance to D. dadantii infection. Lower or higher SA concentration increased soft rot symptom diameter and plant susceptibility to D. dadantii.

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