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Induction of Maize Resistance to Downy Mildew Disease *Peronosclerospora Spp.* Using an Endophytic Consortium

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ABSTRACT

Maize resistance to downy mildew is connected with the activation of multiple defense responses that slow or stop infection at specific stages of the host-pathogen interaction. The interaction between the pathogen and the host plant induces several changes in cell metabolism, especially phenolic content, activity of peroxidase enzyme (POD), and salicylic acid. In this study, an analysis was carried out on the effect of a consortium of endophytic microbes from the fungal group (isolates of AC-1, AC-2, AC-3, and DC-5) and bacterial group (isolates of II-D1, IV-B2, I-A1, III-A2, and I-D3) to control downy mildew in maize through seed treatment and watering of the isolate suspension around plant roots at 14 days after planting under screen house conditions. The results showed that DC-5+II-D1endophytic consortium significantly suppressed downy mildew disease up to 71 %, with the total phenolic content after inoculation 10.3 ppm. In comparison, the DC-5+I-A1 endophytic consortium treatment recorded the highest increase in salicylic acid concentration of 330 %. This treatment did not significantly affect the peroxidase enzyme activity. This study contributes to the understanding of potential mechanisms involved in the defense of maize against downy mildew, highlighting the role of POD and salicylic acid in plant susceptibility to pathogens.

Keywords: Disease suppression; Enzyme activity; Resistance induction; *Peronosclerospora philipinensis*

1. INTRODUCTION

Maize production tends to decrease every year. The decline of maize production is caused by several factors, one of which is pests and disease infections. Important diseases of maize include downy mildew, stem rot, leaf blight, and leaf rust. Downy mildew can be controlled by planting resistant varieties, environmental sanitation, crop rotation, uniform timing of sowing, and seed treatment with synthetic fungicides such as metalaxy[1]. The yield potential of maize production will not be achieved if the crop is infected by downy mildew[2,3].

Plants generate defense systems to fight biotic stressors, such as pathogens (disease-causing microorganisms), viruses, tissue-destroying herbivores, nematodes, and plant parasites, as well as allele-chemicals produced by other plants. However, suppose the pathogens can generate chemicals that are not recognised by plant defense mechanisms. In that case, the pathogens will be able to infect and exploit plants, causing disease symptoms. There are two types of resistance mechanisms that occur in plants to fight pathogenic infections that SAR (Systemic Acquired Resistance) and ISR (Induced Systemic Resistance)[4].

Plant immunity is a complex process and a basic defense system that protects host plants from competing with pathogens. The induced response can be local or systemic depending on the physical barrier and several signaling pathways, which are involved in pathogen recognition through signal transduction and respond defensively through the expression of several genes and their products[5,6] According to Pieterse,[7] *et al*., activation of plant immune signal transduction networks consists of Salicylic Acid (SA), Jasmonic Acid (JA), and/or Ethylene (E).

Beneficial microbes can be used to increase maize plant resistance to pathogens by inducing resistance. Endophytic microbes are an alternative biological control agent, such as the application of endophytic fungi[8] and endophytic bacteria[9]. A prior study examined the development of resistance to downy mildew in maize[10]. Application of the rhizosphere bacteria of *B. paramycoides* Ga3 successfully causes resistance in maize to downy mildew, with an accumulation of salicylic acid levels of 9.6 ppm[11].

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Endophytic microbes have potency for field application because of their ability to colonize root plants, and several studies have demonstrated specific and intrinsic communication between bacteria and host plants of different species and genotypes[12].

Endophytic microbes are living microorganisms that interact with plants, can be used in biological control, and induce resistance by removing antimicrobial metabolites, forming a specific chemical or physicochemical barrier that suppresses or reduces the stimulus of the plant's immune response and acquiring symptom-free habitation within the host plant[13,14.]

Based on this, research to study the best effect of the endophytic microbial consortium to trigger maize plant defense enzymes as biochemical markers for the efficacy of the endophytic microbial consortium against the downy mildew disease *Peronosclerospora philipinensis* was carried out.

2. MATERIAL AND METHODS

The study was conducted out from February to August 2021 at the Disease Laboratory and screen house of the Indonesian Cereals Research Institute, Agriculture Department Republic of Indonesia.

2.1 Isolation of Endophytic Microbes

2.1.1 Isolation of Endophytic Fungi

The source of the endophytic fungus was isolated from maizeroot and leaves collected from several maize production centers in the South Sulawesi region. Isolation of endophytic fungi was carried out following a modified surface sterilisation method[15].

2.1.2 Isolation of Endophytic Bacteria

The isolation of bacterial endophytes from leaves and root was carried out using the surface sterilization method according to Desriani,[16] *et al.*

2.2 Effectiveness of Endophytic Consortia in Inducing Resistance of Maize

The microbial consortium applications were carried out twice on beds per treatment plot according to the experimental design. Twice applications were conducted as seed treatment and sprinkling around the stems and roots of plants at 2 Weeks After Planting (WAP) with a dose of 50 mL per plant. Inoculation was carried out by spraying the suspension of *P. philipinensis* conidia (10^6 spores/ml) onto the leaves at 10 Days After Planting (DAP).

The study conducted in a screen house was arranged in a randomized block design. Seven endophytic consortium as treatments and one control were performed. Each treatment was repeated three times. Therefore, there were a total of 24 experimental plot units. Treatment details as below:

1. AC-1 fungus + II-D1 bacteria
2. AC-2 fungus + IV-B2 bacteria
3. AC-3 fungus + I-A1 bacteria
4. AC-3 fungus + III-A2 bacteria
5. DC-5 fungus + I-A1 bacteria
6. DC-5 fungus + I-D3 bacteria
7. DC-5 fungus + II-D1 bacteria
8. Control/sterile water (without endophytic consortium)

2.3. Effect of Endophytic Microbes on Total Phenolic Content, Salicylic Acid, and Peroxidase

2.3.1 Analysis of Total Phenolic Content

Total phenolic content was measured using leaf samples at 7 and 14 DAP using a spectrophotometer. As many as 0.1 mL of maize leaf samples in 80 % ethanol (1:10 w/v) and 1.2 mL of aquadest were homogenized, and 0.1 mL of Folin-Ciocalteu's reagent was added. After being left for 5 minutes, 0.4 mL of 20 % Na_2CO_3 was added and incubated for 30 minutes. The absorbance values of the samples were read at 765 nm[17].

2.3.2 Analysis of Salicylic Acid Concentration

Analysis of salicylic acid content was carried out based on the method of[18]. Absorbance values were measured at a wavelength of 278 nm on a spectrophotometer. A stock solution of the salicylic acid standard was prepared at a concentration of 0.1 mg/mL. The stock was diluted to 0.001 %. A 0.50 g of maize plant leaves were extracted with chloroform. The extract was mixed with distilled water to 100 mL. The increase of salicylic acid (%) was calculated from the observations at 7 and 14 DAP with a formula:

$$\textbf{Percentage of increase} = \frac{\mathbf{a}}{\mathbf{b}} \times \mathbf{100}\ \%$$

Information:
a: content of salicylic acid 14 DAP
b: content of salicylic acid 7 DAP

2.3.3 Peroxidase Enzyme Analysis

Peroxidase enzyme activity was measured using a spectrophotometer based on a modified method by[19]. The leaves were weighed for1 g and crushed with a mortar in 0.01 M phosphate buffer, pH 6, with a ratio of 1:4 (w/v). The leaf extract was centrifuged at 5000 rpm for 30 minutes at 4 °C and then filtered using Whatman filter paper. The supernatant was used as an enzyme source.

Enzyme activity was measured by pipetting 0.2 mL of enzyme source, diluted 1:3 (v/v) with 0.01 M phosphate buffer pH 6, into a test tube containing 5 mL of 0.5 M pyrogallol solution and 1 mL of 1 % H2O2. The mixed solution was homogenized for 5-10 seconds, and absorbance measurements were taken at 420 nm at 30-second intervals over 150 seconds. The average absorbance values (AOD = b) were calculated using a regression equation Y=(a + b*x*) Enzyme Activity Units (EAU) are calculated by the formula:

$$\textbf{EAU} = \textbf{A OD} \times \frac{\textbf{enzyme source (mL)}}{\textbf{control wet weight (g)}}$$

2.4 Observation Parameters

Parameters observed in this study were *P. philipinensis* downy mildew Disease Incidence (DI), Disease Suppression Index (DSI), and cumulative disease transmission based on the Area Under the Disease Development Curve (AUDPC)[20], with their respective equations using the formula as follows:

$$\mathbf{DI} = \frac{Total\ number\ of\ infected\ plants}{Total\ plants\ observed} \times \mathbf{100}\,\%$$

Each treatment was classified based on the category of plant resistance to downy mildew as follows: disease incidence 0–5 % Highly Resistant (HR);>5 –20 % Resistant (R);>20–40 % Moderately Resistant (MR);>40 – 60 % Susceptible (S); and >60 % Highly Susceptible (HS).

The formula for calculating disease suppression and the area under the disease development curve was as follows:

$$DSI = \frac{I_0 - I_i}{I_0} \times 100\%$$

Here I_0 is the control disease suppression index, and I_i is the disease suppression index in the treatment

$$\mathbf{AUDPC} = [(\mathbf{Y_i}/\mathbf{100} + \mathbf{Y_i} + \mathbf{1}/\mathbf{100})/\mathbf{2}][\mathbf{t_i} + \mathbf{1} - \mathbf{t_i}]$$

where Y_i = disease severity in the i-th observation, and T_i = the i-th observation time.

2.5 Statistical Analysis

All statistical analyses in this study were conducted using STAR 2.0.1 v13[21]. The data were analysed using one-way ANOVA, and the means were separated using the Least Significant Difference test. P-value of 0.05 was used to evaluate significance.

3. RESULTS

3.1 Isolation of Bacteria and Endophytic Fungi

Based on the isolation results of endophytic fungi from parts of maize including roots, stems and leaves from 3 locations in South Sulawesi and 1 location in Central Sulawesi, 4 fungal isolates were obtained (Table 1). Based on a previous study, the results of the isolation of endophytic fungal from maize at 4 locations showed that 4 isolates of endophytic fungi were superior in suppressing downy mildew infections in screen house. One isolate each came from Maros, Palu, Bone, and Gowa. The endophytic fungi obtained mostly emerged from the roots of 3 isolates and the remaining 1 isolate was isolated from the leaves of the plant.

The results of the isolation of endophytic bacteria from maize at two locations showed that 5 isolates of endophytic bacteria were superior in suppressing downy mildew infections in screenhouse (Table 2), based on a previous study. Two of the isolates were isolated from roots, 2 isolates were isolated from leaves, and 1 other isolate was isolated from maize plant stems. Maros obtained 2 superior isolates, while the other 3 isolates came from Palu.

Table 1. Isolation of biological control endophyte fungi from maize of south sulawesi and central sulawesi

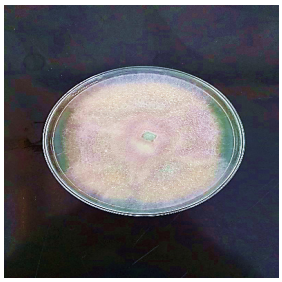
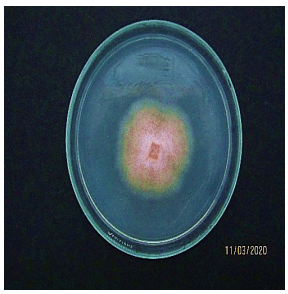
Code	Isolated plant parts	Origin of isolate	Colony morphology on PDA, Genus Assumption
AC-1	Root	Maros, South Sulawesi	 *Fusarium* spp.
AC-2	Root	Palu, Central Sulawesi	 *Aspergillus* sp.
AC-3	Root	Bone, South Sulawesi	 *Trichoderma* sp.
DC-5	Leave	Gowa, South Sulawesi	 *Fusarium* spp.

Table 2. Isolation of biological control endophyte bacteria from maize of south sulawesi and central sulawesi

Code	Isolated Plant Parts	Origin of Isolate
I-A1	Root	Maros, South Sulawesi
I-D3	Leave	
II-D1	Leave	Palu, Central Sulawesi
III-A2	Root	
IV-B2	Stem	

3.2 Effectiveness of Endophytic Consortia in Inducing Resistance of Maize

The difference in the growth of corn plants that were treated with consortium endophytes and those that were not can be seen in Fig. 1. Plants that were treated with consortium endophytes, on average, grew well in the screen house even though they were inoculated with downy mildew pathogens, green plant leaves, and normal growth (a). While in infected plants, the downy mildew is characterised by chlorosis on the leaves with a yellow line of bleaching following the leaf bone direction, infecting from the base leaf and spreading to the ends of leaves (c), leaves becoming stiff and growing vertically, as well as plants becoming dwarfs (b).

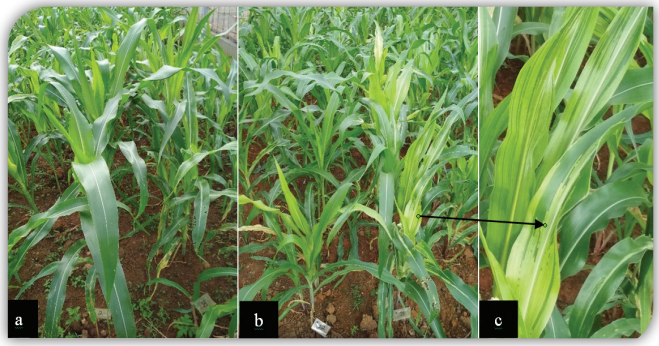


Figure 1. The appearance of maize inoculated by downy mildew.
a. endophyte consortium treatment;
b. control/without endophyte consortium;
c. symptoms of chlorosis on leaves infected by downy mildew.

The endophytic consortium treatment under screen house conditions to reduce the incidence of downy mildew in maize showed that not all treatments significantly reduced disease infection (Table 3). The incidence of downy mildew in the endophytic consortium treatment showed a variation of infection between 3.8 and 17.6 % at 42 DAP. The DC-5+II-D1 treatment reduced downy mildew infection as shown in low disease incidence (3.8 %) which was significantly lower than the AC-2+IV-B2 treatment and the control.

Table 3. Disease incidence of downy mildew and response criteria of Bima3 maize variety treated by the endophytic Consortium and Downy Mildew Inoculated

Endophytic consortium treatment	Disease incidence (%) at 42 DAP	Plant response
AC-1 + II-D1	8.7 ± 4.21	Resistant
AC-2 + IV-B2	17.6 ± 7.50	Resistant
AC-3 + I-A1	8.6 ± 7.56	Resistant
AC-3 + III-A2	12.5 ± 5.95	Resistant
DC-5 + I-A1	13.7 ± 6.00	Resistant
DC-5 + I-D3	12.0 ± 12.53	Resistant
DC-5 + II-D1	3.8 ± 3.36*	Highly Resistant
Control	17.9 ± 7.10	Resistant

Note: *p< 0.05 Least Significant Difference test.

Observations of the AUDPC value showed that the smaller the AUDPC value in the treatment, the greater the ability of the endophytic consortium to suppress downy mildew (Table 4). AUDPC values were between 0.28 and 0.87.The DC-5+II-D1 treatment showed the smallest AUDPC value. In addition, the disease suppression in the DC-5+II-D1 consortium (71 %) was significantly higher than that of the control, while the percentages of disease suppression in other endophytic treatments were between 18 and 60 %.

Endophytic consortium treatment	AUDPC	Disease suppression (%)
AC-1 + II-D1	0.41 ± 1.39	51.6 ± 12.07
AC-2 + IV-B2	0.67 ± 0.29	18.6 ± 32.19
AC-3 + I-A1	0.30 ± 0.08*	60.9 ± 34.82*
AC-3 + III-A2	0.70 ± 0.21	27.3 ± 37.06
DC-5 + I-A1	0.68 ± 0.09	26.6 ± 34.36
DC-5 + I-D3	0.50 ± 1.12	33.3 ± 57.74
DC-5 + II-D1	0.28 ± 0.40*	71.0 ± 32.27*

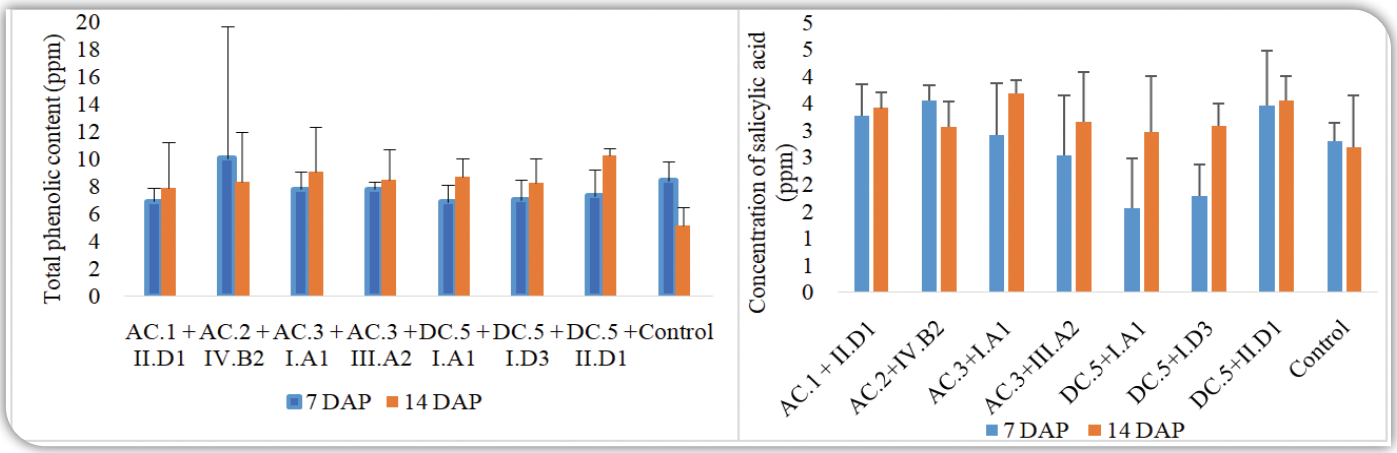
Note: *p < 0.05 Least Significant Difference test
± standard deviation

3.3 Effect of Endophytic Microbes on Total Phenolic Content, Salicylic Acid, and Peroxidase

Total phenolic compounds and salicylic acid concentrations were observed before downy mildew inoculation at 7 DAP and the endophytic consortium treatment had no significant effect on both parameters (Fig. 2).Total phenolic contents before pathogen infection ranged from 6.9–10 ppm. After being inoculated with pathogens, the increase of total phenolic contents was observed on the endophytic consortium, except for the AC.II+IV-B2 treatment. The DC-5+II-D1 treatment had a significant effect on total phenolic content after being inoculated with downy mildew of 10.3 ppm.

The endophytic consortium treatments had no significant effect on the salicylic acid concentration produced by maize. The concentrations of salicylic acid ranged from 1.5–3.5 ppm at 7 DAP. The AC-2+IV-B2 endophytic consortium (3.56 ppm) was significantly different from the DC-5+I-D3 and DC treatments; however, not significantly different from those of other treatments. Treatments of the endophytic consortium were not significantly different in producing salicylic acid at 14 DAP.

The endophytic consortium treatments were able to increase total phenolic and salicylic acid contents, except for the AC-2+IV-B2 treatment (Table 5). The increases in total phenolic content were 108–140 %, with the DC endophytic microbe as the consortium component. The treatment of DC-5+II-D1had the highest increase (140 %). The highest increase in salicylic acid concentration was observed in the DC-5+I-A1 treatment. The increase in salicylic acid ranged from 106-330 %, except for the AC-2+IV-B2 treatment and control.



Bars represent the standard deviation

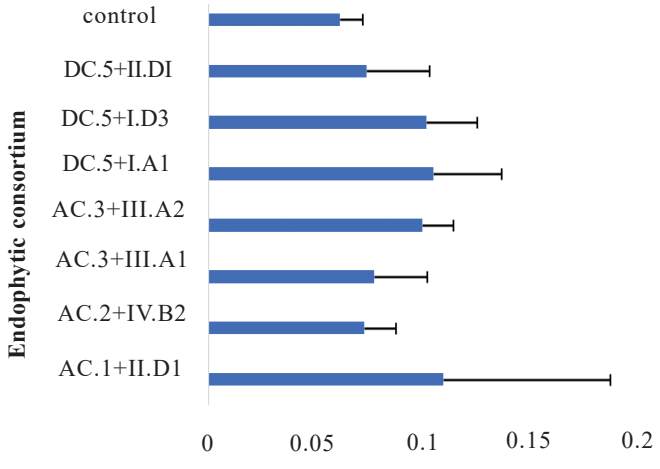
Figure 2. Content of total phenolic compounds and concentrations of salicylic acid in the bima3 maize variety before and after inoculation with downy mildew pathogens.

Table 5. Increase of total phenolic content and salicylic acid concentration in the bima3 maize variety treated by endophyte consortium and downy mildew inoculated

Endophytic consortium treatment	Increase in total phenolic content (%)	Increase in salicylic acid concentration (%)
AC-1 + II-D1	114.89 ± 47.15	106.77± 23.43
AC-2 + IV-B2	-83.05 ± 167.91	-87.01 ± 18.56
AC-3 + I-A1	116.81 ± 17.15	135.22 ± 43.90
AC-3 + III-A2	108.58 ± 10.12	162.28 ± 132.89
DC-5 + I-A1	126.59 ±32.46	330.32 ± 350.83*
DC-5 + I-D3	116.96 ± 37.79	195.77 ± 104.93
DC-5 + II-D1	140.08 ± 33.20	106.17 ± 19.38
Control	-60.85 ± 27.09	- 99.88 ± 47.72

Note: *p < 0.05 Least Significant Difference test ± Standard deviation

The endophytic consortium treatment also had no significant effect on peroxidase enzyme activity (Figure 3). The peroxidase enzyme activity ranged from 0.059 to 0.106 units/mg.



Bars represent the standard deviation

Figure 3. Peroxidase enzyme activity in the bima3 maize variety treated by endophyte consortium and downy mildew inoculated.

4. DISCUSSION

The endophytic microbial isolates used in this study consisted of 4 fungal isolates and 5 bacterial isolates. All of these isolates are superior isolates based on the results of previous study against downy mildew in maize. The fungal isolates, in terms of colony morphology,are classified into 3 large genera consisting of *Fusarium*, *Aspergillus*, and *Trichoderma*. According to Kupper[22], *et al.* the effect of *Trichoderma* application on downy mildew, besides the direct effect as an antifungal, also occurs through the indirect effect as an inducer of resistance. The bacterial isolates used have the potential to become biological control endophyte agents in plants. The application of a consortium of antagonistic bacteria is a potential prospect for controlling downy mildew in maize[23].

Infectious symptoms of the downy mildew begin with a change in the color of leaves and plants into dwarfs, and some individuals in the population dry up and die, mainly in susceptible varieties[24] the production of maize continues to increase along with the escalation of population growth and animal feed requirements in the last few years. The potential to increase the national production of maize is still feasible because of the yield gap between the potential yields of new superior varieties and the level of yields obtained by farmers. The yield gap caused by biotic stress in maize is mainly

caused by pathogens such as downy mildew due to Peronosclerospora spp. Downy mildew distribution is sporadic that can infect a wide area.

In Indonesia, it spreads widely and significantly reduces yields in the areas of maize production centres in East Java, Central Java, South Sulawesi, North Sulawesi, Gorontalo, Lampung, and Sumatera. These obstacles can be overcome by integrated pest and disease control technology. One strategy is to discover downy mildew-resistant varieties that can be combined with other control treatments. The phenomenon of resistance to downy mildew infection of several hybrid maize strains began to be detected in the vegetative growth phase, with symptoms beginning at 14 days after planting (DAP. The resistance of the maize variety Bima3 was classified as tolerant– to downy mildew, but the resistance could be induced to highly resistant" in the DC-5+II-D1 consortium treatment as shown by the lowest disease incidence and the highest disease suppression. This could be caused by interaction between the microbes and the maize variety, thereby showing an increased level of resistance to downy mildew pathogens. This finding was consistent with the results of Prihatiningsih,[25] *et al*. research, which discovered that the application of *Bacillus* sp. was able to raise the resilience of tomato plants from susceptible to resistant.

The high phenolic content in the DC-5+II-D1 treatment correlated negatively with the ability of downy mildew pathogens to develop in plant tissues, causing significant disease suppression. Phenolic compounds increase the mechanical strength of host cell walls and also inhibit infecting pathogenic organisms. As described by [26]the defense response of the cucumber to downy mildew disease *P. cubensis* is stimulated by the presence of total phenol synthesis. Peroxidases and polyphenol oxidases are both involved in the lignification of plant host cells and are considered key enzymes associated with defense reactions against pathogen infection[27,28]. Similar results were also reported by[29] in which the application of rhizobacteria *P. fluorescens*, *B. subtilis*, and *Azotobacter* sp. as a single application and as a consortium were able to significantly increase total phenolic content and salicylic acid concentration. The increase in total phenolic and salicylic acid contents affected the reduction of root-knot disease intensity in kenaf plants.

The AC-2+IV-B2 treatment showed the lowest disease suppression or the highest disease incidence. This treatment showeda high concentration of salicylic acid before downy mildew pathogen inoculation. It may be assumed that this endophytic consortium was not able to degrade the high content of salicylic acid in plants, so it could not increase plant resistance to downy mildew disease. A study conducted by Ibrahim & Leiwakabessy,[30,31] proved that salicylic acid plays an important role in increasing the induction of rice plant resistance to bacterial pathogens.

5. CONCLUSIONS

The application of the DC-5 + II-D1 endophytic consortium was the most effective treatment in suppressing downy mildew disease (> 70 %), with the highest total phenolic content (10.3 ppm) after being inoculated with the pathogen. The DC-5+I-A1 endophytic consortium treatment showed the highest increase in salicylic acid concentration (330 %). However, the application of the endophytic consortium did not significantly affect the activity of the peroxidase enzyme.

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