Antagonistic activity of volatile organic compounds of endophytic bacteria from sword brake fern (Pteris ensiformis) against soil-borne fungal pathogens

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Abstract. The use of endophytic bacteria in the control of plant diseases, especially soil pathogenic fungi such as showed good results. Endophytic bacteria have various mechanisms to control plant pathogen, but the mechanism of endophytic bacteria in producing volatile organic compounds (VOCs) has not been widely studied. The objective of this research was to evaluated the antagonistic activity of VOCs produced by endophytic bacteria from the nail sword (Pteris ensiformis) against three soil borne pathogen fungus, \(R.\ solani\), \(S.\ rolfsii\) and \(Fusarium\ sp.\). Endophytic bacterial isolates used were APE15, APE22, APE33, and APE35. The study was conducted by cupping method to determine the inhibition of endophytic bacteria VOCs against three soil borne fungal pathogens. GC-MS analysis was conducted to determine the content of volatile compounds produced by endophytic bacteria. The results showed that four endophytic bacterial isolates can inhibited \(R.\ solani\) growth (92.11%-96.05%), \(S.\ rolfsii\) (87.28%-93.10%), and \(Fusarium\ sp.\) (27.73%-57.55%). The GC-MS analysis showed that there were several antifungal compounds detected i.e. octanal, cytronellyl acetate, silane, 9-octacenamide, n-dimethylpalmitamide, and isobutyryl chloride. Octanal was presumed as the most influential compound that inhibited the three soil borne fungal pathogens because it was a dominant compound was detected.

1. Introduction

Control of plant diseases continues to lead to the development and application of biological control. The control of pathogens and plant pests is generally known to be more effective and relatively safe for the environment than synthetic chemical pesticides. Soil-borne pathogens are known as difficult pathogens to control. Several types of soil-borne pathogens found in plantations are \(Rhizoctonia\ solani\), \(Sclerotium\ rolfsii\), and \(Fusarium\ sp.\)

Various control measures that have been undertaken, such as crop rotation, use of resistant crop varieties, and synthetic chemical fungicides, are not sufficient to control these soil borne pathogens [1]. The continuous use of synthetic chemical fungicides will make the pathogen more resistant [2]
and adversely affect the environment [3]. One of the alternatives to control \textit{R. solani}, which is environmentally friendly and effective, is the use of endophytic bacteria.

Endophytic bacteria are bacteria whose life is associated with plant tissue. Endophytic bacteria can colonize intracellular and intercellular but are not pathogenic for plants. Besides, endophytic bacteria are also known to increase plant resistance to pathogenic infections and pest attacks and stimulate plant growth so that endophytic bacteria are widely used to control plant pathogens and support plant growth [4, 5].

Endophytic bacteria have various mechanisms in inhibiting pathogens and increasing the growth of host plants, such as by producing metabolites that are antagonistic to pathogens, stimulating plant growth, and induced plant resistance to pathogens. Several endophytic bacterial metabolites known to be antagonistic to pathogens include chitinase, protease, cellulase, and hydrogen cyanide. Apart from these several mechanisms, a potential pathogen inhibition mechanism has not been widely reported, namely the inhibitory mechanism due to volatile organic compounds.

Volatile organic compounds can be produced by various microbes such as bacteria and fungi [6]. Volatile metabolite compounds can inhibit the growth of pathogenic fungi [1]. The volatile compounds produced by bacteria could induce systemic resistance of Arabidopsis plants [7].

Exploring and studying endophytic bacteria that can produce volatile organic compounds is necessary to obtain biological agents to control diseases caused by soil-borne fungi. This study aims to determine the potential antagonistic activity of volatile organic compounds of endophytic bacteria isolated from ferns (\textit{Pteris ensiformis}) against three soil borne pathogens, namely \textit{R. solani}, \textit{S. rolfsii} and \textit{Fusarium} sp.

2. Methods

2.1. Preparation of endophytic bacteria and pathogenic fungi

Endophytic bacteria used are endophytic bacteria isolated from previous studies with isolate codes APE15, APE22, APE33, and APE35. The four isolates were isolated from the root of the ferns (\textit{P. ensiformis}). The available endophytic bacterial isolates were then rejuvenated on TSA media. After 24 hours, the bacterial isolates are ready to be used for testing.

Soil-borne pathogens used in this study were \textit{R. solani}, \textit{S. rolfsii}, and \textit{Fusarium} sp. \textit{R. solani} isolate was obtained from the isolation of rice stalks and midribs infected with \textit{R. solani} in the rice planting area of Situ Gede Village, West Bogor District, Bogor City. Meanwhile, \textit{S. rolfsii} and \textit{Fusarium} sp. used is a collection of the Plant Mycology Laboratory, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University. The three soil-borne pathogenic fungi isolates were rejuvenated on 100% PDA media. After 48 hours of age, the fungal isolate is ready to be used for testing.

2.2. Test of volatile organic compounds (VOC) of endophytic bacteria against pathogenic fungi

The test was carried out by the method of [8] modified on separate plates of the same size. Endophytic bacteria were streaked on 100% TSA media on the base, and \textit{R. solani} (Ø 0.5 cm) was inoculated on 100% PDA media on the lid. The cup is then closed and glued together. Observations were made on the diameter of \textit{R. solani} growth for five days. The percentage of \textit{R. solani} growth inhibition is calculated using the equation:

\[ I = \left( \frac{D1-D2}{D1} \right) \times 100\% \]  \hspace{1cm} (1)

Where: I: percentage of inhibition, D1: diameter of fungal colonies in the control treatment (cm), D2: diameter of fungal colonies on the penis of the tested endophytic bacteria (cm).
2.3. Gas Chromatography-Mass Spectrophotometry (GC-MS) analysis
Gas Chromatography-Mass Spectrophotometer analysis was carried out at the Integrated Chemical Laboratory, Center for Forest Products Research and Development, Bogor. Identification of compounds that play a role in inhibiting R. solani, S. rolfsii, and Fusarium sp. performed using GC-MS analysis. The operational conditions of the GC-MS Pyrolysis tool are:

- Type: Shimadzu GCMS-QP 2010
- Carrier Gas: Helium UHP
- Detector: MS (Mass Spectrometer)
- Column: Capiler type Phase Rtx-5MS; 60.0 M; 0.25mm
- Column Temperature: 50 °C (± 5 minutes), up to 280 °C
- Input Pressure: 101.0 kPa
- Column Water Rate: 0.85 mL / min
- Linear Velocity: 23.7 cm / sec
- Purge Flow: 3.0 mL / min
- Splitting Ratio: 1:50
- MS range temperature: 280 °C
- Ion Source Temperature: 200 °C
- Pyrolysis Temperature: 300 °C

The results obtained then identified the presence of line peaks formed in each different compound and separated based on the weight of each fragment base of the eruption.

2.4. Data analysis
The experimental design used was a completely randomized design (CRD). Data analysis used SPSS version 24 software to see each treatment's effect. A further test was carried out using Duncan's multiple range test at the 5% level.

3. Results and discussion
The results showed that the four endophytic bacterial isolates could produce volatile organic compounds. The inhibition was indicated by the colony diameter of R. solani, S. rolfsii, and Fusarium sp. in the plate that had been inoculated; each endophytic bacteria had a smaller diameter than the control colony (figure 1, 2, 3). These results showed that they could produce secondary metabolites in volatile, volatile compounds that function as anti-fungi (table 1).

| Treatments | R. solani (Inhibition rate) (%) | S. rolfsii (Inhibition rate) (%) | Fusarium sp. (Inhibition rate) (%) |
|------------|--------------------------------|---------------------------------|----------------------------------|
| APE15      | 96.05a                         | 92.25a                          | 57.55a                           |
| APE22      | 96.05a                         | 88.91a                          | 37.30b                           |
| APE33      | 96.05a                         | 93.10a                          | 43.89b                           |
| APE35      | 92.11a                         | 87.28a                          | 27.73b                           |
| Control    | 0.0b                           | 0.0b                            | 0.0c                             |

The numbers in the same column, followed by the same letters, show that they are not significantly different based on Tukey's test at the level of α = 5%.
Figure 1. The effect of volatile organic compounds from endophytic batteries from *P. ensiformis* on the growth of *R. solani*, (a) control, (b) APE15, (c) APE22, (d) APE33, and (e) APE35.

Figure 2. The effect of volatile organic compounds from endophytic batteries from *P. ensiformis* on the growth of *S. rolfsii*, (a) control, (b) APE15, (c) APE22, (d) APE33, and (e) APE35.

Figure 3. The effect of volatile organic compounds from endophytic batteries from *P. ensiformis* on the growth of *Fusarium* sp., (a) control, (b) APE15, (c) APE22, (d) APE33, and (e) APE35.

GC-MS analysis was conducted to determine the active compounds contained in endophytic bacteria isolate APE15 (figure 4). APE15 isolate was chosen for analysis because it has a relatively better ability to suppress the three types of soil-borne pathogens than the other threecendophytic bacterial isolates. The GC-MS results of the endophytic bacteria isolate APE15 are shown in figure 5. The results of the content chromatogram of endophytic bacteria isolate APE15 showed 25 detected peaks. The detected peaks were then analyzed with a mass spectrophotometer. Each peak's mass spectrum was matched with the Chemical Abstract Service (CAS) data and referred to the compounds in table 2. The GC-MS analysis results of endophytic bacteria isolate APE15 showed that 25 compounds were detected. Some of the dominant compounds detected were octanal (19.44%), cyclohexane-propanoic acid (12.34%), and silane (8.53%).
Figure 4  Colony diameter development (a) *R. solani*, (b) *S. rolfsii*, and (c) *Fusarium* sp. which grew in a dish that was exposed to the test endophyte bacteria from day 1 to day 5 after inoculation.
Figure 5. Cromatogram of VOCs profile of endophytic bacteria APE15 Isolate.

Table 2. VOCs composition of endophytic bacteria APE15 isolate.

| Compounds                                      | Retention time | Concentration (%) |
|------------------------------------------------|----------------|-------------------|
| 9-Octadecanamide                               | 17.286         | 2.10              |
| Aceton, Dipentyl Acetal                        | 17.866         | 4.05              |
| 3-Pentanol, 2-chloro-4-methyl                  | 18.049         | 2.30              |
| Cyclohexanepropanoic acid                      | 18.576         | 12.34             |
| N, N-Dimethylpamitamide                        | 19.073         | 7.87              |
| Citronellyl acetate                            | 19.542         | 5.43              |
| Silane, trichoroecicosyl-                      | 19.877         | 8.53              |
| Octanal                                        | 20.442         | 19.44             |
| Isobutyryl chloride                            | 20.809         | 5.56              |
| Heptanal                                       | 21.320         | 7.29              |
| Methanaminium                                  | 21.492         | 3.92              |
| β-Terpineol                                    | 21.642         | 2.21              |
| β-Terpineol                                    | 21.892         | 2.68              |
| 2-Hendecanone                                  | 22.242         | 0.75              |
| 2-methoxycarbonyl-3,3-dimethyl-oxirane         | 24.023         | 0.43              |
| Estran-3-one, 17-(acetyloxy)-2-methyl-          | 25.233         | 2.33              |
| 7,11-Dimethyl-dodeca-2,6,10-trien-1-ol         | 25.489         | 3.24              |
| 1,5,9-undecatriene,2,6,10-trimethyl-           | 25.742         | 1.05              |
| Estran-3-one, 17-(acetyloxy)-2-methyl-          | 27.012         | 1.96              |
| Cholesta-8,24-dien-3-ol, 4-methyl-             | 27.371         | 1.27              |
| Estran-3-one, 17-(acetyloxy)-2-methyl-2-Methyl-| 27.788         | 1.47              |
| 2-Methylnonane                                  | 28.574         | 0.37              |
| Estran-3-one, 17-(acetyloxy)-2-methyl-2-Methyl-| 28.819         | 2.52              |
| Diisopropylocarbinol                           | 30.898         | 0.29              |
| 1-Bicyclo [3.3.1]non-6-en-3-yl-ethanone        | 36.489         | 0.59              |

The inhibition of colony growth of the three types of soil-borne pathogenic fungi began to appear from day one after inoculation. It continued to increase the percentage of inhibition until day 5.
This is because the colonies of *R. solani* grown against endophytic bacteria did not show colony growth and tended to be stagnant. Meanwhile, *R. solani* in the control treatment showed very rapid colony growth.

In general, the four endophytic bacterial isolates had a high inhibitory ability to the growth of colonies of three types of soil-borne pathogens through the production mechanism of volatile organic compounds. The highest emphasis by the four endophytic bacteria occurred on the fungus *R. solani*, which ranged from 92.11% to 96.05%, the suppression against *S. rolfsii* ranged from 87.28% to 93.10%. In contrast, the lowest suppression occurred on *Fusarium* sp. namely ranging from 27.73% to 57.55%. APE isolate APE15 was the best isolate in suppressing the growth of *R. solani* and *Fusarium* sp., while the isolate with the highest suppression ability against *S. rolfsii* was the APE33 isolate.

Volatile organic compounds are secondary metabolite complexes that are volatile and produced in response to environmental conditions. According to [8], volatile compounds produced by endophytic bacteria can also cause structural changes in *Aspergillus niger*, *Fusarium* sp, and *S. sclerotium*. Volatile organic compounds are also known to have a good effect in increasing plant resistance to attack by plant pests. The volatile 2,3-butanediol released by endophytic bacteria such as *Enterobacter aerogenes* could increase maize plants' resistance to pathogenic infections and attack of *Spodoptera litura* larvae [9].

Some of the compounds detected were known to have antifungal activity, including octanal, citronellol acetate, silane, 9-octacenamide, n-dimethylpalmitamide, and isobutyryl chloride [10-13]. The compounds produced by the endophytic bacteria isolate APE15 are thought to have the ability to inhibit the growth of the third colony of soil-borne pathogens. The compound that was supposed to have the most inhibitory effect was Octanal because, in addition to being antifungal, it was the dominant compound detected (19.44%).

Octanal (C8H16O) is a colorless organic aldehyde compound. These compounds are generally used commercially as components to enhance aroma and taste in the food industry [14]. Octanal, citral, and α-terpineol could significantly inhibit *Geotrichum citri-aureanti* mycelia's growth [15]. The antifungal activity of octanal can disrupt integrity and cause leakage of cell components. Octanal in several concentrations (0, 0.25, 0.5, 1, and 2 μl/ml) was reported to inhibit the growth of *Penicillium digitatum* spores in citrus fruits [16]. Octanal can reduce the total lipid in *P. digitatum* spores, which is indicated by the destruction of the cell membrane's integrity. Octanal is also known as a compound with potential as a fungicide in post-harvest products, such as citrus fruits.

Another compound known to have antifungal activity is citronellyl acetate, also known as citronella acetate or β-citronellol. This compound shows the highest concentration after octanal, which is 12.34%. Citronellol compounds are the main compounds in *Pelargonium roseum* essential oil. These volatile compounds have antifungal and antioxidant activity, which can inhibit Candida albicans fungi' growth after 48 hours of inoculation [17].

4. Conclusion
This study provides new information that the endophytic bacteria isolated from the roots of *Pteris ensiformis* can act as biological agents by suppressing the growth of *R. solani*, *S. rolfsii*, and *Fusarium* sp. by producing volatile organic compounds. The inhibition of colony growth of the three types of soil-borne pathogenic fungi showed that volatile organic compounds such as octanal, citronellol acid were proven to have antagonistic activity against soil-borne pathogens.

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