Isolation and screening of rhizobacteria as biocontrol agents against *Fusarium oxysporum f.sp vanillae*

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**Abstract.** One of the major diseases that can reduce vanilla growth and production is stem rot caused by *Fusarium oxysporum f.sp. vanillae*. The objective of this study is to evaluate the rhizobacteria as biocontrol agents against *Fusarium oxysporum f.sp vanillae*. This research consists of isolation of rhizobacteria, antagonism assays, and identification of the rhizobacteria. A total of 263 isolates were evaluated in antagonism assays. There were 250 isolates isolated from vanilla, and 13 isolates were the collection of Plant Protection Laboratory, Indonesian Spice and Medicinal Crops Research Institute. The results showed that seven rhizobacteria isolates, i.e., L34, PS4, V116, KB7, KB10, M117a, and V112, could inhibit the mycelium growth by more than 60%. Rhizobacteria isolate L35, PS4, and V112 showed the highest inhibition, i.e., 64.92%, 72.72%, 68.72%, respectively. Based on the 16S rRNA sequence, isolate L35, PS4, and V112 were identified as *Burkholderia vietnamiensis*, *B. ambifaria*, and *B. lata*, respectively. Further studies are needed to investigate the interaction between rhizobacteria and the host plants and formulate products to evaluate their effectiveness in the fields.

**Keywords:** Antagonism, Biocontrol, *B. ambifaria*, *B. lata*, *B. vietnamiensis*

1. **Introduction**

Indonesia is the second-largest producer and contributes 43% of vanilla supply worldwide [1]. Vanilla is cultivated to produce vanillin, the flavoring agent used in food additives and the various pharmaceutical formulation [2]. The yield production of vanilla reached 3.341 tons in 2009 but decreased to 2.600 tons in the following year [3]. Various plant pathogens can reduce vanilla production, including *Fusarium sp.*, *Rhizoctonia sp.*, *Colletotrichum sp.*, *Phytophthora sp.*, *Sclerotium sp.*, *Uromyces sp.*, *Cucumovirus*, *Potexvirus*, *Tobamovirus*, and *Potyvirus* [4]. However, the primary pathogen that responsible for significant losses in Indonesia is *Fusarium oxysporum f.sp. vanillae* (*Fov*) [5].

In Indonesia, *Fov* was first reported in 1960 in Central Java [6]. This pathogen causes the rotting of the stem and root of the plants and yields losses of up to 80% [4, 7]. Using resistant varieties of vanilla is the best option for limiting the disease’s development, but it does not exist yet. Synthetic pesticides have been used widely to control pests and diseases. However, excessive use of synthetic pesticides can leave residue in the plants and environment that affect human health [8]. A reliable alternative method to control the disease and to improve the yields is the application of microbial [9]. The use of microbial agents is extensively researched and applied to various crops [10]. One of the microbial that has been used as biocontrol agents is rhizobacteria. Rhizobacteria has been studied as biocontrol agents against fungal pathogens [11, 12], nematodes [13], and bacteria [14]. Other advantages of rhizobacteria
inoculants are environmentally friendly, non-toxic, sustainable, long-term use, and they have a broad range of mechanisms to control pathogen [15].

Previous studies reported that *Streptomyces, Arthrobacter, Pseudomonas,* and *Staphylococcus* isolated from Lauraceae inhibited the *Fov* growth in *in-vitro* assays [16]. Moreover, Rhizobacteria can reduce the risk of severe symptoms of infection of *Fov* in vanilla plants [17]. *Bacillus amyloliquefaciens* FLN13 secretes a secondary metabolite that reduces Fusarium head blight in wheat [10]. Although numerous studies have described the use of rhizobacteria to control *Fov,* it is still limited in Indonesia. The objective of the study was to determine the rhizobacteria as biocontrol agents against *Fov* *in-vitro.*

2. Materials and methods

2.1. Isolation of Rhizobacteria

Samples of rhizosphere soil were obtained from the vanilla plantation in Experimental Garden Sukamulya, West Java (6°56′34.6″S, 106°46′3.2″E). The isolation of rhizobacteria was carried out by dissolving 10 grams of soil in 90 ml of sterile water, then diluted to 10^{-3} and 10^{-4} dilutions [12]. As much as 50 µl solution in the last dilution was spread in *Peptone Sucrose Agar* (SPA) (Sucrose 20 g; peptone 5 g; K_{2}HPO_{4} 0.5 g; MgSO_{4} 0.25 g; agar 17 g; and distilled water 1 l) in a petri dish, then incubated at room temperature. A single colony of bacteria with different morphological characteristics was subcultured to a new petri dish containing SPA media and incubated for 3 days to be used in antagonism assays.

2.2. Fungal pathogen preparations

The fungal pathogen is a collection from Plant Protection Laboratory, Indonesian Spice and Medicinal Crops Research Institute (ISMCRI). The isolate was reculture on *Potatoes Dextrose Agar* (PDA [Sigma Aldrich]) and incubated at room temperature. Pathogenicitassay was carried out by putting a mycelial plug (Φ= 5 mm) of *Fov* on the surface-sterilized leave and stem of vanilla. The isolate that causes necrosis on the leaves and stem was used for the antagonism assays (data not shown).

2.3. Antagonism assays of Rhizobacteria against Fusarium oxysporum f.sp. vanillae

Antagonism assays were performed using the double culture method to determine rhizobacteria’s inhibitory ability against of *Fov.* A mycelial plug (Φ= 5 mm) of 5-day-old purified *Fov* was put on a petri dish containing PDA media at a distance of 3 mm from the edge. The rhizobacteria were streaked at a distance of 3 cm from the *Fov* and incubated at room temperature. Each radial growth of the fungal colony was measured for 7 days.

The antagonism assays used rhizobacteria isolated in this study and collected from previous studies. Equation (1) was used to calculate the inhibitory ability by measuring the radius of mycelial growth of *Fov* toward the edge of the petri dish (R1) and the radius of mycelial growth of *Fov* toward the rhizobacteria (R2).

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\text{Inhibitory ability} = \frac{R1 - R2}{R1} \times 100\%
\]  

2.4. Molecular identification of Rhizobacteria

Three (3) isolates of rhizobacteria with the highest inhibitory effect were identified based on 16S rRNA gene sequences. The DNA were extracted from bacterial using CTAB buffer (CTAB 2% [Sigma H-5882]; 1.4 M NaCl; 100 mM Tris-HCl pH 8.0; 20 mM EDTA pH 8.0; PVP-40 1%) [18]. The 16S rRNA region was amplified by PCR using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1392R (5′-ACGGGCGGTGTTGTCAG-3′) [19], in 50 µl reactions containing DNA template 2 µl, PCR Mix 25 µl, Nuclease Free Water 21 µl, Primer Forward 1 µl, and Primer Reverse 1 µl. SensQuest labcycler is used for the reaction with the conditions as follows: initial denaturation at 95 °C for 4 min; 30 cycles of denaturation at 95 °C for 45 s; annealing at 53 °C for 45 s; and extension at 72 °C for 2 min; and a final extension at 72 °C for 5 min. The amplification results were electrophoresed using 1.2% agarose gel with RedSafe™ Nucleic Acid Staining Solution (EtBr Alternative) at 100 volts for 25
minutes. Purified DNA amplicons were then sent to 1st Base Singapore. The sequence results were compared to Gen Bank’s data using the Blast program (http://www.ncbi.nlm.nih.gov).

2.5. Experiment design and data analysis
The antagonism assay was carried out through a randomized completely design with three replicates. Sequence alignment and a Neighbor-Joining tree were constructed in MEGA X.

3. Results
3.1. Isolation of Rhizobacteria
Isolation of rhizobacteria from the rhizosphere of vanilla obtained a total of 250 isolates (Table 1). In addition, 13 isolates were taken from the collection from the previous studies that have been shown to have antagonism to fungal pathogens.

| Host     | Number of |
|----------|-----------|
| Vanilla  | 250       |
| Pepper   | 6         |
| Mimosa   | 3         |
| Maize    | 2         |
| Others   | 2         |
| **Total**| **263**   |

3.2. Antagonism assays
The mycelial growth of *Fov* was inhibited by 19 isolates of rhizobacteria, with inhibition ranging from 8-70% (Figure 1.). Isolate P2 showed the lowest inhibition of 8.9%, while isolate L35 showed the highest inhibition of 72.72%. Seven isolates were antagonists to *Fov* with >50% inhibitory, i.e., V112, V116, KB7, KB10, PS4, L35, and M117a. The three isolates with the highest inhibitory were L35, V112, and PS4 with inhibition of 64.92%, 68,72, and 72.72%, respectively.

![Figure 1. Mycelial growth inhibition of *Fov* by the rhizobacteria isolates.](image-url)

Seventeen rhizobacteria isolates showed inhibition zone ranging from 1.67-18.67 mm (Figure 1.). Isolate KB4 showed the lowest inhibition zone of 1 mm, while isolate PS4 showed the highest inhibition zone of 18.67 mm. The three isolates with the highest inhibition zone were V112, L35, and PS4, with
an inhibition zone of 16.67 mm, 18.33 mm, and 18.67 mm, respectively (Figure 2.). Isolates L35, PS4, and V112 were isolated from the rhizosphere of pepper, maize, and vanilla, respectively.

Figure 2. Antagonism assays of rhizobacteria isolates PS4 (a); PS9 (b); V112 (c); V116 (d); L35 (d); and control (e).

3.3. Identification of Rhizobacteria
Based on the 16S rDNA sequences, the three isolates belong to the bacterial genera *Burkholderia* (Table 2.; Figure 3). Isolate L35, PS4, and V112 were identified as *B. vietnamiensis*, *B. ambifaria*, and *B. lata*, respectively

Table 2. Identification of three rhizobacteria isolates

| Isolates | Closest match          | Query Cover (%) | Similarity (%) | Accession number |
|----------|------------------------|-----------------|----------------|-----------------|
| V112     | *B. lata*              | 100             | 100.00         | MH537749        |
| PS4      | *B. ambifaria*         | 100             | 100.00         | KF733685        |
| L35      | *B. vietnamiensis*     | 99              | 99.93          | FJ436055        |

Figure 3. Phylogenetic relationships of L35, PS4, and V112 with other species based on 16S rDNA sequence (Neighbor-joining Method).
4. Discussion
The *Burkholderia cepacia* complex consists of several closely related species that have high 16S rRNA gene sequence similarity (>97.5%) [20, 21]. The rhizobacteria used in this study: *B. vietnamiensis* L35, *B. ambifaria* PS4, and *B. lata* V112, belong to *Burkholderia cepacia* complex [22]. They effectively suppressed the mycelial growth of *Fov* caused by the production of antimicrobial metabolites. *Burkholderia* spp. produce many antifungal compounds such as xylocandins, cepacidines, burkholdines, and occidiofungins [23]. Other studies reported that *Burkholderia* spp. had been used in plants disease control. Inoculation using *B. vietnamiensis* can enhance the resistance of *Eucalyptus grandis* by decreased volatiles compounds [24]. *B. latata* can inhibit the anthracnose disease caused by *Colletotrichum acutatum* on pepper [25]. *B. ambifaria* produced occidiofungins that are responsible for their antifungal activity against *Fusarium* spp. [26]. In addition, inoculation of *B. vietnamiensis* on sugarcane is an economical approach because it can save the cost using N-fertilizer [27]. Other benefits of *B. cepacia* inoculation were phosphates solubilization, nitrogen fixation, potassium utilization, siderophore production, and indole acetic acid production [28, 29, 30].

5. Conclusion
Rhizobacteria *B. vietnamiensis* L35, *B. ambifaria* PS4, and *B. latata* are effective antagonists against *Fov* and have potential to be developed as control for *Fov* in vanilla. However, further studies are required to investigate the interaction between rhizobacteria and the host plants and formulate products to evaluate their effectiveness in the fields.

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Author’s Contribution
S Hardiyanti, Sukamto, R Noveriza, and M Mariana are the main contributors.

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