An ability of endophytic bacterial isolated from chilli to reduce seedling-off caused by *Fusarium oxysporum*

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Abstract. Isolation and assay of endophytic bacteria from red chili plant to reduce chilli seedling-off caused by *Fusarium oxysporum* has been conducted. Endophytic bacteria were isolated from stem, root, and leaves of healthy red chili plant, while the fungi was isolated from infected root. Antagonistic assay were conducted by dual culture method. In vivo assay of reducing seedling-off was conducted by dipping red chilli seeds in endophytic bacterial solution. Two (SDW1 and SDW2) out of five endophytic bacterial isolates showed more in inhibiting growth of *F. oxysporum*. The isolates showed to reduce chilli seedling-off. Furthermore, these two isolates increase seedling height and leaf number compared to treatment without bacterial application.

1. Introduction
Red chili (*Capsicum annum* L.) is one important cultural and economical spicy crop in Indonesia. Chili price becoming expensive especially during religion holidays. It gives more benefit to farmers. However, chili production may fluctuate due to plant pest and disease. Of those, one soil-borne fungal pathogen *Fusarium oxysporum* is one of the most responsible fungal agents for chili seedling-off diseases [23]. Infection of healthy plant can occured if chili seed grows in soil contaminated with the fungus. Fungal mycelium invades through the root developing and spreading in root, enter plant vessel, inhibiting water and nutrient transport causing chili seedling to wilt.

To control fungal disease of plant, synthetic fungicide are used as primary method of control. However, this fungicide harm to environment, human, and increase pathogen resistance. One alternative to control plant diseases is to used biological control using bacterial isolates. Many have been reported the success of using bacterial isolates as biological control agent in reducing seedling-off disease [7, 28, 23, 25]. *Aeromonas hydrophila*, *A. caviae*, *Pseudomonas maltophilia*, *Bacillus licheniformis*, *B. circulans*, *Vibrio furnissi*, *Xanthomonas* spp., and *Serratia marcescens* have been evaluated to as biological control agents of plant pathogenic fungi [8]. Fusarium wilt particularly can be suppressed through the activity of *Bacillus subtilis*, *Pseudomonas fluorescens* [28], *Pseudomonas putida* [5], and *Streptomyces* sp. [7].

Bacterial isolates as biological control agents of fungal disease have been isolated from soil and rhizosphere. However, potential bacteria have also been found in many plant species and living asymptomatically within plant tissues. Isolation of endophytes have been carried out from root, stem, leaf, and seed [11, 17]. A plant may harbor more than one endophytes of fungi and bacteria [6, 17].
even more than one species of endophyte is found in one plant tissue [23]. Endophyte increases plant performance and protects plant against plant disease [6, 17, 18] by producing plant growth hormone, chitinase and β-1,3-glucanase, peroxidase, phenylalanin ammonia-lyase, polyphenol oxidase, phenolat, jasmonic acid, and salicylic acid [4, 6, 9, 22]. Using endophytic bacteria rather than rhizobacteria was more promising since much less microbial competition because of less diversity and lower populations of indigenous microorganisms inside plant tissues [10, 12]. Furthermore, the internal tissues of plants protect endophyte from environmental stress [11, 17]. Several studies have been conducted to evaluate endophytic bacteria as biological control agent of fungal disease in organic farming and sustainable agriculture [10]. In this study, isolation of endophytic bacteria from healthy chilli plant and assay of the endophytics to reduce seedling-off cause by F. oxysporum has been conducted. One possible approach in biological control of soilborne plant diseases is to apply potential isolates to seeds [17, 25].

2. Materials and Methods

2.1. Bacterial and Fungal Isolation
To isolate endophytic bacteria, pieces of healthy chilli stem, root, and leaf were surface-sterilised using 75% ethanol, 5.3% NaOCl, and washed with sterilized water [21]. The pieces were cut longitudinally and put on nutrient agar with cut part facing to the culture medium. Similar work was done for pathogenic fungal isolation using potato dextrose agar (PDA) as a growing medium. Infected plant with obvious fungal symptom disease was used. Culture was incubated for 1 day in ambient temperature. Single isolate was obtained by sub-culturing growing colony of the culture. Macroscopic and microscopic features of cell and colony of bacterial isolates as well as several biochemical characterization was conducted. Characterization and identification of fungi was done using book of [20].

2.2. In vitro Assay of Endophytic Bacterial Isolate Against Fusarium oxysporum
To assay antifungal activity of endophytic bacterial isolate, dual culture methods was utilized. The isolate was sub-cultured for 2 days in ambient temperature. Fungal isolate was grown in center of PDA + 3% yeast extract and incubated for 2 days. Blank disc (Oxoid) was dipped in bacterial suspension (OD$_{600}$=0.5) and put on both sides of growing fungi with a distance of 2 cm. Culture was incubated for 3 days in ambient temperature. Inhibition activity was measured as radius of normal growing hyphae subtracted with radius of inhibited hyphae.

2.3. In vivo Assay of Endophytic Bacterial Isolate to Reduce Chili Seedling-off
Fungal isolates was sub-cultured in PDA fro 7 days in ambient temperature. Mycelia was removed to glucose yeast broth and incubated for 10 days. Fungal culture was mixed with planting media of 1 kg of sterilized soil : compost (3:1) in plastic tray of 30 cm x 20 cm x 10 cm. Twenty chili seeds was grown in the tray. For endophytic bacterial treatment, the seeds was immersed in bacterial culture (OD$_{600}$=0.5) for 30 minutes prior planting. (-) control was growing seed without any bacterial and fungal investment, while (+) control was seed growing in the media with fungal infestation. To evaluate possibility of endophytic to harm to seedling, the seeds was grown in planting media without fungal infestation. Re-isolation of pathogenic fungi causing seeding-off was conducted at the end of study. Observation of seedling-off, seedling height, dry-weight, and leaf number was conducted after 30 days [25].
3. Results and Discussion

3.1. Bacterial and Fungal Isolation and Reisolation

Five different endophytic bacteria (SDW1, SDW2, SDW3, SDW4, and SDW5) was isolated from red chili healthy plant. Characterization of the isolates is showed in Table 1.

Table 1. Characterization of morphology and biochemistry of bacterial isolates showing to inhibit other microbial colony of soil isolation

| Bacterial isolates | Colony characterization | Gram | Cell shape | TSIA | Glucose | Sucrose | Lactose | Sediment | Splinter | Catalase | Motility | Citrate | Starch hydrolysis | Gelatinase |
|--------------------|------------------------|------|-----------|------|---------|---------|---------|----------|----------|----------|----------|---------|----------|---------------|-----------|
| SDW 1              | Irregular, flat, browny white | -(ve) | Coccus    | +    | +       | +       | -       | -        | +        | +        | +        | +       | +       | -             | -         |
| SDW 2              | Irregular, flat, white   | +(ve) | Rod       | +    | +       | +       | -       | -        | +        | +        | +        | +       | +       | +             | +         |
| SDW 3              | Irregular, flat, browny white | -(ve) | Coccus    | +    | +       | +       | -       | -        | +        | +        | +        | +       | +       | -             | -         |
| SDW 4              | Irregular, flat, browny white | -(ve) | Rod       | +    | +       | +       | -       | -        | +        | +        | +        | +       | +       | -             | -         |
| SDW 5              | Irregular, flat, white   | +(ve) | Rod       | +    | +       | +       | -       | -        | +        | +        | +        | +       | +       | -             | -         |

Note: + = able to, - = unable to

Characterization of fungal isolate showed that fungal was white cottony colony, macroconidia 2 septate, hyphae septate, purple at the base of medium, purple and smooth at the surface of medium. On PDA, mycelium that initially was white, but became pale yellow brown with age. Fungal isolation from fungal infected plant showed that the isolate was Fusarium oxysporum. Reisolation of infected seedling showed the same disease agent F. oxysporum (Figure 1). This clearly confirmed that F. oxysporum used was pathogen to chili.

![Figure 1. (a). Colony of F. oxysporum from infected chilli (b). hyphae and conidia, (c). infected seedling, and (d). colony of F. oxysporum reisolation](image-url)
Disease manifestation were observed as abnormal, seedling wilted with yellowing leaf followed by seedling slunted and stem desiccated, collars appeared slunted and discolored with yellowish gray and then leaf turning to brown and brittle. The pathogen infects young root, growing, developing and spreading in root and stem vessel, inhibiting water and nutrient transport.

3.2. In vitro Assay Of Endophytic Bacterial Isolate Against Fusarium oxysporum

From previous studies, it has been confirmed that several soil bacteria like Bacillus spp., Pseudomonas spp. and Streptomyces sp. have antagonistic and biologically control potential against F. oxysporum [5, 7, 28]. Antagonistic assay of endophytic bacterial isolates to F. oxysporum demonstrated that all isolates inhibited fungal growth shown as inhibition zone (Figure 2).

![Figure 2. Inhibited F. oxysporum hyphae depicted as clear zone (arrow) as a result of antagonistic assay against (a). SDW2, (b). SDW1, (c). SDW5, (d). SDW3, and (e). SDW4.](image)

The antagonism work through antibiosis, competition, predation, or parasitism [1, 15, 19]. It seemed that competition was not mechanism of bacterial inhibition to fungi, since PDA+3% is rich in nutrient [13]. Many antagonistic microbe produces antimicrobial compound that released to the environment and inhibit other microbes of the same ecological niche [17].

The ability of bacterial isolates to inhibit fungal growth varied to some extent (Figure 3.). SDW2 showed more to inhibit followed by SDW1. An interaction between bacterial ability to produce antifungal metabolites, bacterial culture age, growth medium composition and incubation time might affect the ability of the isolates to inhibit fungal growth in vitro. Antifungal metabolites interfere fungal cell wall manifestated as broken, curled, twisted, and petite fungal hyphae [16, 26].
3.3. In vivo Assay of Endophytic Bacterial Isolate to Reduce Chili Seedling-off

Two isolates SDW1 and SDW2 were used for in vivo study based on their ability to inhibit fungal growth. To know the effect of endophytic bacterial isolate infestation to chili seedling, seedling-off number, seedling height, dry-weight, and leaf number were observed. Chili seeds were treated by soaking them into endophytic bacterial solution prior planting in soil inoculated with F. oxysporum. [16] and [17] demonstrated that seed bacterization prior planting was to significantly increase seed germination, shoot length, shoot fresh and dry weight, root length, root fresh and dry weight and leaf area.

Direct observation showed that seedling without bacterial application (control (+)) were more susceptible to seedling-off disease than those of seedling with bacterial application (Figure 4.).

Figure 3. Inhibition zone of F. oxysporum hyphae caused by endophytic bacterial isolates after 7 days of incubation

Figure 4. Growth of (a). non infected chilly seedling ((-) control), (b). seedling without bacterial application ((+) control); seedling with bacterial application of (c). SDW2, (d). SDW1, and seedling with fungal infestation treated with endophytic bacterial isolates of (e). SDW 2 and (f). SDW1
Seedlings-off caused by *Fusarium* was observed after planting the seed in fungal inoculated soil. Chili seed treated with endophytic bacteria showed to reduce seedling-off to some extent (Figure 5). No infected plant were observed in seed planted in soil without fungal infestation. Bacterial treatment of SDW2 with and without fungal infestation was more to reduce seedling-off. Many reported the ability of bacterial endophyte to reduce fungal disease on individual [18, 28] and bacterial combination treatment [17, 24]. Although bacterial application of SDW1 also reduce seedling-off caused by the fungi, it seemed that it might slightly harm to the seed.

![Figure 5](image)

**Figure 5.** Seedling-off reduction of chilli treated with endophytic bacterial isolates SDW1 and SDW2

Manifestation of the disease was clearly observed in (+) control a week after planting, seedling-off occurred and increased hereafter. Morphological and physiological alteration of seedling may occur as manifested in alteration of seedling height and dry-weight. All treatment were likely to contribute in increasing seedling height compared to that (-) control (Figure 6). [11] and [16] indicated that the growth promoting substances and plant growth hormones such as IAA, auxins, cytokinins and gibberellic acid etc., produced by endophytes were responsible for the increased plant growth.
Figure 6. Seedling height of chilli treated with endophytic bacterial isolates SDW1 and SDW2

Seedling dry-weight treated with endophytic bacterial isolates varied (Figure 7.). Instead of increasing seedling height, all bacterial treatments did not contribute to increase dry-weight. This indicated that water content was high in seedling of bacterial and fungal infestation. It seemed that fungal dry-weight contributed to seedling dry-weight shown by higher seedling dry weight in bacterial and fungal infestation than that of bacterial infestation only.

Figure 7. Seedling dry-weight of chilli treated with endophytic bacterial isolates SDW1 and SDW2

Leaf number was observed to leaf left on seedling. All treatment were to show higher leaf number compared to that of (+) control or even to (-) control (Figure 8.). Beside to give lower seedling-off,
higher seedling height and dry weight, SDW2 also contributed to higher leaf number. In the absence of pathogens, endophytes promote plant growth directly, or indirectly by protecting the plant against soilborne diseases, most of which are caused by fungi [14]. The role of these bacteria to contribute higher seedling performance by producing plant growth promoting hormone should be investigated.

Figure 8. Seedling leaf number of chili treated with endophytic bacterial isolates SDW1 and SDW2

4. Conclusions
Five different endophytic bacteria namely SDW1, SDW2, SDW3, SDW4 and SDW5 have been isolated from chili healthy plant. Fungal isolation of chili infected plant showed F. oxysporum a plant pathogenic fungi was causative agent of chili seedling-off. SDW1 and SDW2 inhibit fungal growth the most in vitro. The two isolates reduced chili seedling-off, as well as chili seedling height and leaf number compared to those of treatments without any bacterial application (+) control. This indicated that despite of protecting plant from disease, endophytic bacterial isolates contribute to increase seedling performance.

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