Biological Control of Strawberry Crown Rot Disease (*Pestalotiopsis* sp.) using *Trichoderma harzianum* and Endophytic Bacteria

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**Abstract.** Strawberry crown rot is one of the diseases caused by several soil-borne pathogens. Control of the disease is quite difficult, especially on unhealthy soil condition. The study was conducted to examine the potential of *Trichoderma harzianum* and consortium endophytic bacteria application to control strawberry crown rot disease and resistance of strawberry varieties to crown rot disease based on control treatment. The research stages were isolation and identification of the pathogen, antagonist test of *T. harzianum* against the pathogen, potency test of both biological control agents to control crown rot disease on three strawberry varieties (Dorit, Rosalinda and the hybrid of Early Bright and Tokii varieties). This study used a Randomized Block Design. The results indicated that *Pestalotiopsis* sp. was one of the pathogens causing strawberry crown rot. The pathogen was inhibited by *T. harzianum* using antagonistic mechanism. Although, based on the percentage inhibition of radial growth, only 32% of inhibition of *T. harzianum* against *Pestalotiopsis* sp. when both isolates laid out on PDA in same time. Application of both biological controls lead to the increasing resistance of three strawberry varieties against crown rot disease. The hybrid of Early Bright and Tokii variety was the most resistant varieties compared than 2 others varieties after biological control application.

1. Introduction
Strawberry (*Fragariae ananassa* Duch.) is one of the most economically important crops worldwide. Strawberries are appreciated worldwide for their unique flavour, importance as a source of macronutrients and beneficial dietary compounds [1] with benefits on neurodegenerative and cardiovascular [2]. Strawberries are high in the nutrients including vitamin C, folate, manganese and they are also rich in phenolic compounds, including anthocyanins, hydrolysable tannins, and phenolic acids [3]. In Indonesia, strawberry is mainly grown in highland, where the elevation is over 1,000 meter above the sea level.

Strawberry plants reported being infected by several soil borne pathogens causing root rot and crown rot. Crown rot is a complex disease caused by one or more of fungal pathogens, including *Colletotrichum gloeosporioides* and *Colletotrichum fragariae* [4], *Macrophomina phaseolina* and *Fusarium solani* [5], *Pestalotiopsis* sp. [6,7] and other pathogens. *Pestalotiopsis* is an important fungal plant pathogen causing leaf spot, fruit rot and crown rot symptoms. *Pestalotiopsis clavispora*, recently named as *Neopestalotiopsis clavispora* [8] is causing crown rot in strawberry in Pakistan, Spain, Argentina, and Italy [7, 9-11].
The typical symptoms of the infected plant were the drying of edge leaves, the black lesion appears first on the leaves and spread down the crown causing orange-brown colored tissue of the crown. Stem and root are black, then the plant wilting, stunting, collapse and death of the entire plant [7, 12].

Screening for strawberry disease resistance before the introduction and use of strawberry cultivars in commercial strawberry production is important to develop. This is because susceptible plants will affect the spread of diseases. Furthermore, the planting of disease-resistant strawberry cultivars will reduce the number of chemical treatments. Strawberry cultivars have different resistance to crown rot disease. That it might be related with time for the pathogen to infect the crown and to cause the typical discoloration and wilting [12].

The method that usually applied to control pathogenic fungi is spraying by fungicides. Giving fungicides is a common way for farmers to suppress the growth of plant diseases, but pesticides can cause various problems and disturb the environmental balance [13]. Pesticide residues can kill non-target organisms, increase the resistance of target organisms, absorb and accumulate in fruit, seep in the soil, carried by wind and water that can kill aquatic organisms, and are harmful to farmers. Therefore, there is a need for other alternatives in controlling these pathogens using environmentally friendly way. Applying of antagonistic microbes that live in plants (endophytes) and roots such as *Trichoderma* sp., *Bacillus* spp., *Pseudomonas* sp. and *Gliocladium* sp. are an environmentally friendly method to control soil borne pathogens. Antagonistic microbes can directly or indirectly control the development of soil borne pathogens.

Systemic acquired resistance (SAR) is the activation of plants defense mechanism leading to the induction of systemic resistance to subsequent pathogens infection. The resistance of plant to pathogens can be enhanced by the application of various biotic and abiotic agent, called induce systemic resistance in plants [14,15]. Microorganisms acting through antibiosis, generally have a wide action spectrum. Pathogen inhibition by producing toxic substances is more effective than any other mechanism of action [16]. Their protective effect involves different mechanisms of action that directly antagonize pathogen growth. *B. subtilis* group is known to produce a variety of bioactive metabolites leading to antibiosis [17,18] and able to compete for space and nutrients. The effectiveness of *B. subtilis* in inhibiting the reproduction of pathogenic fungi to control disease in various plants has shown significant results [18-21]. *Trichoderma* spp. uses many antagonistic mechanisms against pathogens of crops for their colonization, such as lytic enzymes, mycoparasitism, competition for nutrients and space and so on [22]. The effectiveness of *Trichoderma* spp. against soil borne pathogens and biotic stress has shown significant results [23, 24].

The study was aimed to identify the causal agents of strawberry crown rot and to know the potency of *T. harzianum* and consortium endophytic bacteria as biological control agents to manage the disease on three strawberry varieties.

2. Methodology

2.1. Isolation and identification of the pathogen

The study was conducted at Phytopathology Laboratory, Indonesian Citrus and Subtropical Fruits Research Institute, East Java, Indonesia. Isolation of infected-strawberry crown rot was growth on agar plate contain PDA (Potato Dextrose Agar) medium and incubation one week. Identification of fungal by a light microscope on 400 X magnification. Morphology of the pathogen was analysed descriptively through comparative literature study. Hypha and conidia of fungi produced by this pathogen confirmed the identification as *Pestalotiopsis* sp. that predict as causal of disease was transfer in agar slant.

2.2. In-vitro antagonist test of *Trichoderma harzianum* against *Pestalotiopsis* sp.

Antagonism test was carried out using the dual culture method between *T. harzianum* and *Pestalotiopsis* sp. The isolate was taken with a cork borer (5 mm diameter). Each isolate was grown side by side with a distance of 3 cm in a petri dish. Cultured fungi were incubated at 27°C and measured the diameter of the colony for 8 days. Each treatment was made 3 replications with control (isolate pathogens without biological control agents).
The effect of antagonist fungi of *T. harzianum* on the pathogens was observed based on percentage inhibition of radial growth (PIRG) and microscopy observations. PIRG calculation uses the formula [25].

\[
\text{PIRG} \% = \frac{R_1 - R_2}{R_1} \times 100
\]

PIRG: Percentage inhibition of radial growth (%)
R1: Pathogen isolate diameter without antagonist fungi
R2: Pathogen isolate diameter with antagonist fungi

Microscopic observations were carried out to observe the antagonistic mechanism that occurs between hyphae of *T. harzianum* fungi and pathogenic fungi *Pestalotiopsis* sp. Preparations are made by taking pieces of isolates that are on the growing border between pathogens and antagonists, placed on a glass deck and stained with methylene blue. Microscopic observation of antagonistic mechanism was observed under a light microscope with 400 x magnification.

2.3. Potency biological control agents to control crown rot disease on strawberry

Biological control agents used in the treatments were consortium bacteria and *T. harzianum*. Consortium bacteria was made using consortium endophytic bacterial isolates collected by Phytopathology Laboratory, ICSFRI (B. *subtilis*, B. *albus*, B. *cereus*, and *Pseudomonas knackmusii*). The isolate was propagated on coconut water as a growth medium and then fermented using an aerator for 7x24 hours. *T. harzianum* was produced on PDA (Potato Dextrose Agar).

Application of biological control agents being carried out on three varieties of strawberry namely Rosalinda, Dorit, and the hybrid of Early Bright and Tokii. The study was conducted using a Randomized Block Design. Tests are carried out with three treatments, namely: P1: Control (pathogen only), P2: Application of consortium endophytic bacteria and inoculation of the pathogen. Pathogen inoculation was done 3 days after the bacteria consortium application, and P3: Application of *T. harzianum* and inoculation of the pathogen. Pathogen inoculation was done 3 days after Trichoderma application. Treatments were done with three replications. Application of bacteria consortium, *T. harzianum* and inoculation of pathogen by drenching method on strawberry growth media (15 ml per polybag).

The intensity of the disease is calculated by scoring. Scoring is used according to research conducted by Dwiastuti et al. [26] that has been modified (Table 1).

Table 1. Scoring of crown rot disease symptoms on strawberry

| Score | Disease symptoms |
|-------|------------------|
| 0     | healthy (no symptoms of a pathogen attack) |
| 1     | ≥ 10%, mild pathogen attack (number of leaves attacked and attack on each leaf is small) |
| 2     | 11–25%, moderate attack (number of leaves attacked and number of attacks on each leaf is rather high) |
| 3     | 26-50%, heavily attacked (number of leaves attacked and number of attacks on each leaf appearing large and followed by wilting of the leaves) |
| 4     | ≤ 50%, plants die (all leaves wilt and no signs of life) |

The scoring value is used to calculate the percentage of disease intensity in plants with the following formula [27]:

\[
\text{Disease intensity} \% = \frac{\sum (n \times v)}{Z \times N} \times 100
\]

Note:
I: Disease intensity
N: Number of symptomatic leaves withered
V: The score on each leaf affected by a pathogen
Z: Highest score
N: Number of leaves observed in one polybag
Observations were made starting 3 days after treatment, every 3 days for five leaves in each plant. Results were analyzed by single ANOVA, continued by Tukey HSD at a significance level of 5%.

2.4. The crown rot disease resistance of strawberry varieties. The increasing of resistance strawberry varieties to crown rot disease on biological control treatments was determined based on disease intensity on each treatment as follows: Very resistant (0-5%), resistant (>5-20%), moderate resistant (>20-40%), susceptible (>40-60%) and very susceptible (>60%) [28].

3. Results and Discussions
3.1. Crown rot pathogen identification
The typical symptoms observed from diseased strawberry were the drying begins from the edge of the leaves. Lesions appear first on the leaves and spread down the crown. The severely infected plants had completely dried leaves and flowers, then turned dark in color, stems and roots are black. Plants will be stunted if there is a severe attack without disease control management (Fig. 1).

Figure 1. Visual symptoms and pathogen of strawberry crown rot disease. A. Rotting of leaves, B. Stunting, C. Orange-brown coloured of tissue, D and E. Mycelium growth of Pestalotiopsis sp. on Potato Dextrose Agar from above and below respectively, D. Typical conidial morphology of Pestalotiopsis sp.

3.2. In vitro antagonistic test of T. harzianum against Pestalotiopsis sp.
Testing T. harzianum as an antagonist to control the pathogenic fungus Pestalotiopsis sp. at the age of 8 days after treatment is shown in Fig. 2. Percentage inhibition of radial growth (PIRG) to know the potency of T. harzianum controlling Pestalotiopsis sp. shows that the presence of T. harzianum inhibition against pathogens (Fig. 3).

The results of antagonistic tests showed that the percentage of inhibition T. harzianum against Pestalotiopsis sp was low (only 32.2% at 8 days after both isolates laid out on PDA at the same time). According to Dwiastuti et al [26] the application of Trichoderma spp as biological control agents should
be carried out before soil borne pathogens infect the plant (preventive treatment). The application of Trichoderma for soil borne diseases control, together with the onset of the disease or after the onset of the disease is often ineffective.

![Antagonism between Trichoderma harzianum and Pestalotiopsis sp.](image)

**Figure 2.** Antagonism between *Trichoderma harzianum* and *Pestalotiopsis* sp. A. Control, B. Antagonism treatments.

![Percentage radial growth inhibition of Pestalotiopsis sp. by T. harzianum](image)

**Figure 3.** Percentage radial growth inhibition of *Pestalotiopsis* sp. by *T. harzianum*

Microscopic observation of antagonistic mechanism shows that hypha of *Pestalotiopsis* sp. shorter than normal hyphae size (Fig. 4). Hyphae *T. harzianum* performs a variety of mycoparasitic mechanisms by twisting around its hyphae and analyzing its wall by an enzyme produced by its, piercing, and attaching hyphae to pathogen so that the growth of pathogen is inhibited. *T. harzianum* produces an antibiotic that negatively affect the growth of pathogenic fungi. *T. harzianum* produces *trichodermin*, *trichidermol* and *harzianolide* antibiotic compounds. Trichoderma also produces enzymes such as *glucanase* and *chitinase* which are able to destroy the hypogenic cell wall of pathogenic fungus, including *Pestalotiopsis* sp. by degrading the polysaccharides and chitin on its cell wall [29-31].

Trichoderma have potency as a biological control agent which acts as a fungal antagonist. The rapid growth ability of Trichoderma is very suitable for use in controlling biological fungal pathogens in plants [32]. In general, in the soil, there are antagonistic soil fungi and pathogens that live together. So naturally, the interaction between the two fungi will produce a balance of soil ecosystems if there is no ecosystem disturbance caused by one of them using fungicides. According to Ahmed [33] faba bean treated with the biotic inducer (*B. subtilis* and *T. album*) showed an increase of chlorophyll, phenols and flavonoids, thus reducing the chocolate spot disease (*Botrytis fabae*) incidence and severity.
3.3. Potency of biological control against crown rot disease

There are decreasing of crown rot disease causing biological control application based on means of crown rot disease intensity on three varieties (Fig. 5). Plants infected with Pestalotiopsis sp. which was controlled by Trichoderma had the lowest crown rot disease intensity (18.2%) although it was not significantly different (P<0.05) from those controlled by biofertilizer (19.11%). Plants infected with crown rot disease without biocontrol have significantly different on disease intensity from those controlled by Trichoderma and biofertilizer (37.43%).

![Figure 5](image)

**Figure 5.** Effect of biological control agents applied on three strawberry varieties against crown rot disease.

There is the influence of varieties on the disease intensity attacks on all treatments. The variety that has the highest plant resistance among three varieties is the crossing varieties between Early Bright and Tokii (disease intensity 17.76%), then Dorit variety (25.45%), and the most susceptible variety is Rosalinda variety (31.87%).
3.4. Effect of biological control on crown rot disease resistance of three strawberry varieties

The experiments showed that Dorit and Rosalinda varieties are susceptible strawberry varieties to crown rot disease. Biocontrol treatments using consortium endophytic bacteria and Trichoderma will improve the resistance of three strawberry varieties to crown rot disease (Table 2). In different treatments, Trichoderma is unable to control fusarium wilt disease (*Fusarium* sp.) in California and Santung varieties [26]. In this study, all off the treated plants only showed wilting and leaf rot symptoms. No discoloration was observed in the crowns of the control and treated plants on all cultivars. This may indicate that resistance of varieties and the environmental condition affects the presence of disease.

**Table 2.** Resistance level of strawberry varieties against crown rot disease based on biological control treatments

| Variety             | Treatments                              | Resistance level   |
|---------------------|-----------------------------------------|--------------------|
| Dorit               | Positive control (*Pestalotiopsis* sp.) | Susceptible        |
|                     | Pathogen+Consortium endophytic bacteria | Resistant          |
|                     | Pathogen+*Trichoderma* sp.              | Resistant          |
| Rosalinda           | Positive control (*Pestalotiopsis* sp.) | Susceptible        |
|                     | Pathogen+Consortium endophytic bacteria | Moderate resistance|
|                     | Pathogen+*Trichoderma* sp.              | Moderate resistance|
| Hybrid (Early Bright and Tokii) | Positive control (*Pestalotiopsis* sp.) | Moderate resistance|
|                     | Pathogen+ Consortium endophytic bacteria | Resistant          |
|                     | Pathogen+*Trichoderma* sp.              | Resistant          |

The experiment showed that application of biological control agents can be used as plant disease protecting and plant growth promoting for sustainable agricultural practices compared untreated control. Therefore, it could help minimize the rampant use of chemical fertilizers for improving agricultural and horticultural practices thus improving the sustainability of agriculture.

4. Conclusion

*Trichoderma* has the potential to reduce the strawberry crown root disease intensity (*Pestalotiopsis* sp.). The consortium endophytic bacteria are also found to be effective to protect strawberry from crown rot disease. Both the biological control agent’s ability can be maximized through preventive applications. This result can be seen in Dorit and Rosalinda which were known to be susceptible to crown rot disease. In this research, both varieties are found to be more resistant to crown rot infection after treated with the biological control agents. Further investigations on agent’s mode of disease inhibitions and the biology of antimicrobial substance may be necessary to complement the results found in this research. In addition, further investigations on the pathogenicity of both agents to non-target organisms can also be done to ensure its safety before mass application in the field.

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