Application of Trichoderma as an Alternative to the use of Sulfuric Acid Pesticides in the Control of Diplodia Disease on Pomelo Citrus

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Abstract. This study aims to determine the ability of *Trichoderma asperellum* Tc-Pjn-02 compared to sulfuric acid pesticides in controlling the disease stem rot Diplodia on Pomelo citrus (*Citrus maxima*) caused by *Botryodiplodia theobromae* Pat. The research was conducted at Balai Penelitian Tanaman Buah Tropika Kebun Percobaan Kraton-Pasuruan, East Java Province, and the Laboratory of Microbiology and Biotechnology of Universitas Muhammadiyah Sidoarjo in February-April 2020. *Trichoderma* and sulfuric acid were applied in an aqueous paste formulation each of 10 units of diseased plants with rotten stems which were randomly selected as samples. The data from the observations were tested using the t-test at the real level of 5%. The results showed that *Trichoderma* sp. had a higher ability in suppressing pathogen attack compared to sulfuric acid which was able to heal rotten wounds on the stalks affected by 41.95% and 26.74%, respectively.

1. Introduction

Diplodia stem rot caused by the fungus *Botryodiplodia theobromae* Pat. in citrus plants is a disease that has spread widely in Indonesia and is able to reduce the level of citrus production [1]. This disease is characterized by symptoms of stem rot, especially tree branching, accompanied by the appearance of exudate in the form of "gum" and can lead to decreased production and even death of plants [2].

The pathogen that causes the Diplodia stem rot disease in citrus plants has the potential to spread the area of its attack and widen the host it attacks, not only Pamelo oranges but various other types of citrus. Efforts to control and suppress the spread of this disease from an early age have been carried out by many farmers; most of them use synthetic chemicals as their active ingredients. One of the most effective fungi toxic active ingredients for controlling this disease is sulfuric acid. This compound is corrosive and toxic to humans [3]. The use of synthetic and toxic chemicals can suppress non-target organisms and organisms that are beneficial to plants, create resistance to pesticides, and have the potential to pollute the environment [4].

One of the potential alternatives in selecting active disease control substances that are not toxic chemical compounds is to use biocontrol agents as active ingredients. *Trichoderma* sp. is one type of fungus that has the potential to be used as a biocontrol agent. Various studies have shown that *Trichoderma* not only has the ability to control soilborne disease pathogens [5-6] but is also able to
control pathogens that attack plant parts on the soil surface and which are airborne diseases [7-8]. In addition, this fungus has the ability to help plant growth because it produces extracellular compounds that act as growth regulators [9] while producing nutrients from overhauling organic matter [10].

To ensure the feasibility of this function as a biocontrol agent, especially for the pathogen of Diplodia, stem rot, it is necessary to test its effectiveness in controlling the disease and healing wounds caused by pathogens. As an alternative to using sulfuric acid, it is necessary to compare the ability and effectiveness of controlling this disease with the synthetic chemical compound sulfuric acid as the active ingredient most often used by citrus farmers.

This study aims to determine the ability of *Trichoderma asperellum* Tc-Pjn-01 compared to sulfuric acid pesticides in controlling the disease stem rot Diplodia in Pomelo citrus (*Citrus maxima*) which is caused by the pathogenic fungus *Botryodiplodia theobromae* Pat.

2. **Methods**

2.1 **Determination of sample plants**

In the pomelo plantations that were attacked by Diplodia stem rot at the Kraton Experimental Garden, Balai Penelitian Tanaman Buah Tropika, Pasuruan Regency, East Java province, several plants were randomly selected as samples. The number of sample plants for *Trichoderma* treatment and sulfuric acid treatment was 10 plants each. The plants selected were those that showed symptoms of attack in the category of mild and severe with a general appearance of rot symptoms in the form of wet wounds (Figure 1). Wounds that are undergoing the drying process or have dried up are not used as sample plants in this experiment, because this shows that the infection has stopped or the tissue of the part of the plant stem is being attacked and/or has died. The activity of determining diseased plants was an initial phase that was part of the experiment which took place from February-September 2019. Before a number of plant samples were used for this experiment, a Koch Postulate test was carried out [11] to confirm that the wound was caused by *Botryodiplodia theobromae* Pat.

![Figure 1. Diplodia stem rot attack symptomatic of wet wounds (left) and dry wounds (right)](image)

2.2 **Preparation of disease control applications**

Sulfuric acid is provided by mixing sulfur, detergent, lime (CaCO₃), with a weight composition of 50:10:40 grams. After that the sulfur and detergent are added to the water little by little until the total volume of water that is poured reaches 200 ml while stirring until dissolved; then the lime is put into the sulfur solution and stirred evenly and ready for use.

The formulation of *Trichoderma* made with the active ingredient is the biological agent *T. asperellum* Tc-Pjn-02 isolate collection of the Microbiology Laboratory, majoring in Agrotechnology, Faculty of Science and Technology, University of Muhammadiyah Sidoarjo. *Trichoderma* propagation was carried out on PDA media which was placed in a sterilized glass bottle at a temperature of 120°C for 1 hour. The results of the *Trichoderma* culture were crushed with a blender for 10 minutes and then poured into a 1,000 ml glass beaker containing 100 grams of husk flour. After diluting it and stirring it evenly, the *Trichoderma* formula is ready for use.

The population of *Trichoderma* was calculated by means of the dilution method with dilution levels of 10 at 10⁵-10⁸; one ml of the suspension was poured and leveled onto the surface of the PDA-m medium and incubated for four days. The number of colonies seen will indicate the number of live spores contained in the
diluted suspension. For the preparation of the *Trichoderma* formula, the number of spores was determined to be $10^6$ CFU.ml$^{-1}$. Thus, mixing the husk flour into the *Trichoderma* propagule suspension will produce a mixed formula with the number of spores equivalent to $10^6$ CFU.ml$^{-1}$.

Prior to the application of control, the wound or rotting part of the stem was cut about 2 mm thick using a sterile grafting knife to clean the surface of the stem from the gum and dry bark. About 15 minutes later, sulfuric acid and *Trichoderma* were applied according to the treatment until all the wound surfaces were covered with material. After the fungicide dries, a slightly dark brown and light brown color will appear for the sulfuric acid treatment and the *Trichoderma* formula, respectively. Furthermore, observation of the symptoms of injury was carried out 7-21 days after application with a time interval of seven days.

Monitoring progression to disease recovery was carried out for up to 21 days after applications (DAA); with the observation time interval every week from the day of application, the observation time obtained is $t_0$, $t_1$, $t_2$, and $t_3$. From the total time of observation, the area of the stem surface area of the disease rot symptoms from the initial observation to the development of the extent of the wound symptoms in the first, second, and third weeks.

At the beginning of the observation, each plant sample measured the area of the initial infection wound ($t_0$) and the area of the wound on the day of observation ($t_1$, -2, and -3) and compared them to obtain recovery of wound symptoms. The area of the wound can be calculated using the formula (1):

$$Y_{in} = \frac{a_i n X}{b} \quad \ldots \ldots \ldots (1)$$

with the following conditions: $Y$ = the area of the wound surface of the plant stem of sample $I$; $a_i$ = the weight of the paper which is a printed image of the wound surface of the plant sample $I$; $n$ = the time of observation, namely 0, 1, 2, and 3; $b$ = the weight of a sheet of paper with a standard size; $X$ = area of a sheet of paper with a standard size.

Wound recovery, which is characterized by drying the wound on the edge towards the center of the wound, is calculated by the formula (2):

$$\Delta Z = \left[ \left( Y_{int} - Y_{in0} \right) \left( Y_{in0} \right)^{-1} \right] \times 100\% \quad \ldots \ldots \ldots (2)$$

with the following conditions: $\Delta Z$ = percentage of wound healers, $Y_i$ = plant sample $i$, $n_t$ = observation week $t$, $n_0$ = initial observation.

### 2.3 Data analysis

In the pomelo plantations that were attacked by Diplodia stem rot at the Kraton Experimental Garden, Balai Penelitian Tanaman Buah Tropika, Pasuruan Regency, East Java province, several plants were randomly selected as samples.

## 3. Results and Discussion

### 3.1 Wound healing

The results showed that starting seven days after application (DAA), wound recovery had begun in both types of treatment. The edges of the infected wound begin to dry out and peel off. In this first week, the symptoms appear to change in the form of drying of the wound starting from the edges to the center; the drying process extends to the center of the wound surface of the stem. In 21 DAA all parts of the wound were almost dry (Figure 2). The mean percentage of wound recovery as a response of plants to *Trichoderma* application and sulfuric acid application in 7-21 DAA is shown in Figure 3.
Figure 2. The appearance of symptomatic stem sections treated with sulfuric acid (left) and *Trichoderma* (right) on 21 DAA.

Figure 3. The average effect of *Trichoderma* and sulfuric acid on the percentage of wound recovery in 7-21 DAA.

3.2 Biocontrol agent reliability test

The results of testing the reliability of *Trichoderma* as a biocontrol agent compared to the application of sulfuric acid show its ability to exceed that achieved by the application of sulfuric acid fungicides. The *t*-test results (Table 1) showed a significant difference between the application of *Trichoderma* and sulfuric acid at seven and 21 DAA.

| Recovery percentage:                        | 7 days after applications | 14 days after applications | 21 days after applications |
|--------------------------------------------|---------------------------|----------------------------|----------------------------|
| *Trichoderma* formulation                   | 12,92 %                   | 29,93%                     | 41,95%                     |
| Sulfuric acid fungicide                     | 7,20 %                    | 21,86%                     | 26,74%                     |

Results of *t*-test:

| *t*-stat | 2,27 | 1,41 | 2,18 |
| *t*-critical | 2,18 | 2,11 | 2,12 |
| Significance | Significant | No significant | Significant |

*) Performed at a test level of 5%
3.3 Discussion

The difference in residual color between the wound healing process in the sulfuric acid treatment and *Trichoderma* was caused by the color of the carrier material. The *Trichoderma* formula made from husk flour is darker than the residual color of the sulfur and lime mixture (Figure 1). The color changes to a lighter shade as the wound begins to heal so that the difference in the extent of the wound area and the wound area recovery in the weekly interval of observation can be determined.

The wound healing process in the *Trichoderma* treatment appeared to show a higher percentage at all observation times compared to the sulfuric acid treatment (Figure 2). *Trichoderma* application was significantly different from sulfuric acid application in seven and 21 DAA (Table 1). At seven days after application, it appears that in the *Trichoderma* treatment the wound recovery reached 12.92±2.34% and increased to 41.95 ± 5.73% in the following two weeks (Figure 2). This achievement was much greater than the sulfuric acid treatment, namely 7.21±0.91% and 26.74±97%, respectively. Sulfuric acid kills pathogens and simultaneously kills tissue through the destruction of lignin and saccharide molecules [12-13], both as a structural framework for cell walls and metabolites which further thicken the layers of dead plant tissue. Meanwhile, *Trichoderma* with its ability to produce antibiotics, toxins, and enzymes that inhibit pathogens (14-17). The chitinolytic extracellular compounds produced can remodel the cell walls of pathogenic fungi [18-20], but do not damage plant tissue cells that start to be attacked and healthy cells under the injured tissue.

On the other hand, *Trichoderma* utilizes the organic matter of dead plant tissue as a source of energy [21-22], as well as produces extracellular compounds that act as growth-regulating compounds [23-24] which in this case is thought to help plant growth. The extracellular compounds produced by these antagonistic fungi help protect plants [25] by increasing their resistance and ability to restore tissue cells that have begun to attack and cell regeneration, given the contribution of plant cell growth-promoting compounds produced by *Trichoderma* [26]. Wound recovery shows *Trichoderma* excellence in competing for space use [27-28] in plant tissue where *Trichoderma*-pathogen-plant cell interactions occur. This is possible because *Trichoderma* can take advantage of organic material from pathogenic cells and dead plant cells [29; 21].

4. Conclusion

*Trichoderma asperellum Tc-Pjn-02* recovered stem rot of Pomelo citrus caused by *Botryodiplodia theobromae* up to 41.95% better than wound recovery by application of sulfuric acid which was 26.74% at 21 days after application. *Trichoderma* application can not only be used as an alternative application of sulfuric acid but also as an agent for plant growth promoters.

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