Potency of biofertilizer to control damping off disease and stimulate plant growth on *Japansche Citroen* Seedling

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**Abstract.** Damping off is one of the diseases caused by soil-borne pathogens resulting in the death of seedling in a short time. Control of this disease is quite difficult. The study aimed to examine the potential of biofertilizers and the time of application to damping off disease intensity and plant growth on *Japansche citroen* seedling. This study used a Factorial Completely Randomized Design with two factors: 1). Biofertilizer namely AzzoFor (P1), liquid biofertilizer (P2), a secondary metabolite of *Trichoderma asperellum* (P3), the mixture of three biofertilizers (P4), and control (P5), 2). The time of application, namely the treatment of biofertilizers first then followed by the inoculation of *F. oxysporum* (A1) and the treatment of *F. oxysporum* first followed by treatment of biofertilizer (A2). The results showed that the lowest disease intensity was found in A1P3 treatment (0%), while in control reached 41.3%. The treatment of A1P2, A2P1, A2P2 and A2P4 showed the same disease intensity. The highest disease control effectiveness found in A1P2, A1P3, A2P1, A2P2 and A2P4 treatments and this was significantly different compared to A1P4, A2P3 and control treatments. The most significant percentage of seeds germinate at 21 DAS was shown in A1P1 treatment (26.7%) and A2P3 treatment (25.3%), while after 28 DAS, it was in A2P4 treatment (73.3%). The lowest plant height at 28 DAS was A1P4 treatment and control. Biofertilizer is useful for controlling the damping-off disease if applied separately.

1. **Introduction**

Citrus is one of the horticulture plants that can grow well and has high economic value in Indonesia. The productivity of citrus in Indonesia must be supported by providing quality citrus seeds that are free of disease and a reasonable and appropriate cultivation system, especially in handling diseases [1].

Citrus species are almost universally propagated by bud-ding on to seedling rootstocks. One of the disorders in citrus plants is damping off causes plant death a short time. Damping-off of seedlings in nursery bed is a widespread problem of citrus industry and frequently occurs in citrus orchards where phytosanitary conditions are difficult to maintain.

The characteristics of the affected plants are decay in the seeds or roots and stems of plants, causing plants to collapse and die [2]. Caused by several soil-borne pathogens (*Fusarium, Rhizoctonia, Pythium, Phytophthora*), the damping off disease is a common disease that attacks the rootstock of citrus plants seedlings during germination and after emergence when the seedling tissue is succulent. Typical symptoms of damping-off result when the soil or seed-borne fungus penetrates the stem just above the soil line and causes the seedling to topple. Infected seedlings are killed rapidly when moisture is abundant and temperatures are favorable for fungal growth. Plants usually become resistant to damping-off once true leaves have emerged and the stem tissue at the soil line has matured.
Using soil that already contained an inoculum as a seedling medium will cause continuous pathogenic infections. Some methods have been used to control damping off the disease on various plants such as using essential oil of Artemisia herba-alba as a biological control agent [3], seed treatment by combination fungicide and Trichoderma [4], fungicides seed treatment [5]. Control of this disease is quite tricky, so control measures must be taken before a pathogen attack occurs. One potential for controlling this disease is through the use of biofertilizers.

Biofertilizer is a substance which contains living microorganism, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant [6]. Biofertilizers have an essential role in plant nourishment as well as in the enhancement of soil fertility by means of several mechanisms like biological nitrogen fixation, bioavailable phosphorous for plant uptake, acquisition of essential macro and micronutrients with mineralization of organic manures/organic matter, lowering plants ethylene levels, production of plant growth promoting substances, disease control by suppression of soilborne pathogens and acceleration of other microbial activities in the soil [7-10]. The purpose of this study was to identify pathogens that cause damping-off disease on citrus seedlings, the potency of biofertilizer and the time of biofertilizer application to control of damping off disease and growth of Japansche citroen rootstock.

2. Materials and Methods

2.1. Damping off pathogen identification

The pathogen that causes damping off was isolated and identified. The pathogen on infected plants were isolated using PDA (Potato Dextrose Agar) media. Damping off infected seedling was sterilized by alcohol 70%. Individual parts of infected seedling were then plated onto petri dishes containing PDA and incubated for 3 to 5 days at 22°C. Approximately 15 putative Fusarium spp. colonies were subculture onto dishes containing PDA until pure cultures were obtained. Pure cultures confirmed to be F. oxysporum were transferred to slants containing 10 ml PDA. Pathogen morphology was analyzed descriptively through comparative literature study. Pathogenicity test to prove that the fungus was the cause of seedling disease in citrus plants was conducted by soaking the citrus seeds (Japansche citroen) in the inoculum pathogen suspension for 30 minutes. After that the seeds were planted in the seedbed. The seeds were placed in the wet condition and covered with plastic mulch and sprayed once a day.

2.2. Biofertilizer treatments

Biofertilizer treatments used factorial Completely Randomized Design with 2 factors, namely the type of biological fertilizer (P): AzzoFor (production of Indonesian Institute of Science) (P1), liquid biofertilizer (P2), a secondary metabolite of Trichoderma asperellum (P3), combination of three biofertilizer (P4) and control (P5). AzzoFor is a solid inoculant consisted of peat indigenous bacteria isolates i.e. Rhizobium, Azotobacteria, Azospirillum, and Phosphat Soluble Bacteria (PSB) [11]. Liquid biofertilizer is liquid inoculant containing endophytic bacteria isolated from citrus leaf. The second factor was the time of application (P), namely P1 = Application of biofertilizer 3 days before the inoculum of the pathogen (10^6) and P2 = Application of pathogenic inoculum (10^6) three days before biofertilizer application. Giving inoculum of Fusarium oxysporum (10^6) in the rootstock Japansche citroen seedling without biofertilizer treatment was used as a control. Each treatment used 25 seed per seedbed, with three replications. Biofertilizer was applied every three days. The observation focus on:

2.2.1. Disease intensity. The disease intensity is calculated from seeds that show symptoms of disease from the whole seed germinating percentage. Observations were made up to 28 days after seedling. The disease intensity data were used to measure the percentage of disease inhibition based on the value of Area Under Disease Progress Curve (AUDPC). According to [12], AUDPC was used to
summarise the disease severity. It was calculated by the formula to measure the severity of the disease that was evaluated, the severity of the disease during each evaluation and epidemic term. The value of AUDPC in each treatment was obtained by the following formula [13]:

\[
AUDPC = \sum_{i=1}^{n-1} \left( \frac{y_i + y_{i+1}}{2} \right) (ti + 1 - tt)
\]

Note: \( y_i \) is an assessment of disease (percentage) at the \( i \)th observation, \( ti \) is time (in days) at the \( i \)th observation, and \( n \) is the total number of observations

2.2.2. The percentage of seeds germination. The number of growing seeds was observed by calculating the number of seeds that can germinate vigorously from the total number of seeds planted in each replication. Observations were made up to 28 days after seedling.

2.2.3. Plant height. Plant height was measured on the last day of observation.

Data were analysed using Analysis of Variance (ANOVA) through calculations using SPSS program

3. Results and Discussion

3.1. Damping off pathogen identification

Pathogen isolates causing damping off on citrus seedling was identified as *Fusarium oxysporum*. *F. oxysporum* is a pathogenic fungus that causes wilt in various plants (Fig. 1). This disease is characterized by necrosis of plant tissue and is followed by leaf wilt due to the attack of pathogens in plant vascular tissue. This fungal attack can cause plant death in a few weeks [14]. *Fusarium* sp. produces several toxins including fusaric acid and fumonisin which can worsen disease infections. The initial symptoms of the disease are marked by color changing in the shoots of the affected plant to reddish brown, and then the part will become wilted. Weeping plants can occur gradually in several leaves and will develop into all parts of the plant. Symptoms of severely attacked plants are characterized by plants wither and die quickly. The root of the sick plant decays.

![Image](image1.jpg)

**Figure 1.** Symptoms and pathogen of damping off disease. Visual symptom of damping off on *Japanske citroen* seedling (left), isolate of *Fusarium oxysporum* (middle), microconidia of *F. oxysporum* (right).

The source of infection comes from contaminated soil for several years, garbage, infected plants or agricultural equipment. This pathogen often attacks during the rainy season, especially in high humidity and wet climates. The transmission through the flow of pathogen-contaminated water spread the disease and its coverage area more widely [15]. It covers cold climates to tropical climates and can live well in arid regions with annual rainfall <250 mm to wet areas with rain above 1000 mm per year.
In Indonesia, only six Fusarium species have been reported, one of which is the cause of damping off disease on citrus plants [16]. *F. oxysporum* also causes fusarium wilt of citrus trees in Tunisia [17]. There is a correlation between *Fusarium* wilt disease affecting citrus seedlings and dry root rot disease observed on scaffold roots of trees [18]. Another *Fusarium* species causes dry rot disease in California citrus production (*F. solani*). The pathogen is a weak pathogen on citrus and can infect only when a tree is under stress [19].

3.2. Biofertilizer effect on damping off disease on *Japansche citroen* seedling

3.2.1. Damping off disease intensity. The success of *F. oxysporum* transmission in citrus seedling related to the incubation period, namely the speed at which symptoms appeared after the infection was carried out. The level of symptoms showed that the seedling in each replication depends on the influence of the environment, the virulence of the pathogen and the inhibition due to biofertilizer treatment. Damping off due to infection by *F. oxysporum* showed visual symptoms of fallen sprouts and wilting sprouts dried and brownish at 28 DAS. Damping off disease intensity on Japansche citroen rootstock is shown on Fig. 2.

![Figure 2](image)

**Figure 2.** Damping off disease intensity at 28 days after seeding (DAS). P1: AzzoFor, P2: liquid biofertilizer, P3: a secondary metabolite of *T. asperellum*, P4: Mixture of three biofertilizers, and P5: control. A1: the treatment of biofertilizers first then followed by the inoculation of *F. oxysporum*., A2: the treatment of *F. oxysporum* sp. first followed by treatment of biofertilizer.

The application of a combination of three biofertilizers before the seed was planted (A1P4) and application *T. asperellum* secondary metabolite after inoculation of pathogen (A2P3) showed different disease intensity compared to other treatments except for untreated control (P<0.05). Combination of biofertilizers was known to have good nitrogen-fixing bacteria in one biofertilizer mixture. The actual nitrogen-fixing bacteria produce high levels of nitrogen in the soil. Nitrogen levels that are too high are the right growth place for the pathogen [20]. It is also known that the combination of biofertilizer contains a variety of microbes that creates competition between microbes, so that microbes’ function as antagonists for pathogens become weak and pathogen infection become higher [21]. Damping off disease has not occurred on *Japansche citroen* rootstock seedling applied by *T. asperellum* before inoculation of *F. oxysporum*. According to Mbarga et al. [22], strains of *T. asperellum* present omising biocontrol potential against *Pythium myriotylum* causing root rot in cocoyam cultivation. *T. asperellum* (NVTA2) also reduced approximately 69% stem rot incidence (*Sclerotinia sclerotiorum*)
on carnation with root dip, followed by soil drenching [23]. A biological control agent is influenced by abiotic and biotic factors such as disease pressure and competition from the indigenous microflora. The results indicate that microorganism content in biofertilizer and time of application might play an important role in considering the biological control method in the field.

3.2.2. The effectiveness damping off disease control on Japansche citroen seedling. The effectiveness of damping off disease control using biofertilizer is calculated from the accumulation of disease intensity through the value of the AUDPC (Figure 3). The value of control effectiveness calculated based on the development of disease intensity shows that the application of Trichoderma secondary metabolites with the time of application of the biofertilizer before the seeds are planted is the most effective in reducing the attack of damping off disease with the smallest AUDPC value obtained in the treatment that is equal to 0. This show that the treatment has increased resistance to pathogens compared to other treatments. According to Novita [24], the application of Trichoderma to the growing media will inhibit the growth and development of Fusarium sp. so that the viability of the Fusarium sp. become reduced. According to Wu Q et al. [25] mycelium of T. asperellum GDFS1009 exhibits a high growth rate, high sporulation capacity, and strong inhibitory effects against pathogens that cause cucumber fusarium wilt and corn stalk rot. T. asperellum strain BHUT8 also has an effect on biopriming seed on plant growth promotion and induced systemic resistance in tomatoes by producing high phenylpropanoid activities and lignification [26].

![Figure 3](image_url)

**Figure 3.** AUDPC value until 28 days after seeding (DAS). P1: AzzoFor, P2: liquid biofertilizer, P3: a secondary metabolite of T. asperellum, P4: Mixture of three biofertilizers, and P5: control. A1: the treatment of biofertilizers first then followed by the inoculation of F. oxysporum, A2: the treatment of F. oxysporum first followed by treatment of biofertilizer.

3.3. Biofertilizer effect on seedling growth
3.3.1. Percentage of germination. Seed germination can be influenced by the environment and fertiliser application. All biofertilizer treatments did not show significantly differences on percentage of seed germination (P<0.05). There were no JC rootstock seedlings that had grown at the first observation at 7 DAS. The initial seed germination occurred at 14 DAS at the treatment of T. asperellum (P2) secondary metabolite before planting with 8% germination. Trichoderma is known as an antagonist agent and is also a Growth Promoting Rhizobacteria Plant (PGPR) which can improve
plant growth and accelerate seed growth. According to Palad et al. [27], three times application of 4 gr/l T. asperellum provides the most effective results for growing cocoa seeds. Singh et al. also suggested that T. asperellum T42 can be used as plant growth promoting fungus similar to other Trichoderma species. Inoculation cacao seedling with T. asperellum and Arbuscular Mycorrhizal Fungi (AMF) alone was essential for the promotion of plant growth. A significant increase in plant height, root and shoot fresh weights, as well as phosphorous uptake, was recorded in comparison to non-inoculated control plants [29]. Trichoderma is also able to improve plant conditions due to abiotic stress in plants [30]. The percentage of Japansche citroen seed germination at 21 DAS increased in biofertilizer treatment compare than control, except on application of mixture biofertilizers (Figure 4). The rapid increase was shown in the treatment of AzzoFor biofertilizer before the seeds were planted with a percentage of 26.7%. The administration of AzzoFor fertilizer can increase plant growth especially in plant height because AzzoFor contains a various of bacteria which have the task of fixing nitrogen and phosphate bacteria needed in the plant growth phase. The percentage of Japansche citroen seed germination at 28 DAS increased, especially in mixed biofertilizer treatment (P4) at the time of application after planting. Although slow in giving effect, the mixture of biofertilizer had the best impact because the mix of several biofertilizers could improve the physical, chemical and biological properties of the soil and could increase the level of good bacteria for growth [21]. Biofertilizer mixtures have an excellent impact on plant growth but are less useful for damping off disease control.

![Figure 4. Seeds germinate (%) at 7 DAS; 14 DAS; 21 DAS and 28 DAS on biofertilizer treatment. DAS: Day after seedling. P1: AzzoFor, P2: liquid biofertilizer, P3: a secondary metabolite of T. asperellum, P4: Mixture of three biofertilizers, and P5: control. A1: the treatment of biofertilizers first then followed by the inoculation of F. oxysporum., A2: the treatment of F. oxysporum. first followed by treatment of biofertilizer.]

3.3.2. Plant height. The growth of citrus seedlings applied with F. oxysporum inoculum has satisfying growth with a different average of plant height, although not significantly different between treatments at P< 0.05 (Figure 5). The highest plant height was shown in the AzzoFor biofertilizer treatment in the application after planting. The content in AzzoFor biofertilizer is a group of bacteria that function as nitrogen and phosphorus fasting bacteria. These bacteria include Azospirillum, Azotobacter, and Rhizobium. The mixture of bacteria has a great influence on plant growth because each bacterium has a feature that is beneficial to farmers. Azotobacter has a lot used as biological fertilizer on agriculture and its use is recommended for enrichment soil nitrogen and maintain soil quality [31]. Azotobacter
can act as an antagonist plant pathogen, besides being able to act as biological fertilizers. *Azotobacter vinelandii* produce inhibiting antifungal *F. oxysporum* causes wilt on various plants [32]. Fertilization carried out can influence plant growth. Fertilization is used as a source of nutrients needed to overcome nutrient deficiencies in the soil. Biofertilizers often function to help absorb nutrients, to increase plant growth (bio stimulant) and to control disease in plants (bioprotectant). The lowest plant height was shown in application of mixture biofertilizer followed by inoculation of *F. oxysporum* (A1P4). There are different results with treatment of triple microbe (*Pseudomonas fluorescens*, *T. asperellum*, *Rhizobium* sp.) that increase seed germination and seedling growth in *Cicer arietinum* and *Phaseolus vulgaris* [33].

**Figure 5.** Plant height of Japansche citroen rootstock at 28 days after seeding (DAS). P1: AzzoFor, P2: liquid biofertilizer, P3: a secondary metabolite of *T. asperellum*, P4: Mixture of three biofertilizers, and P5: control. A1: the treatment of biofertilizers first then followed by the inoculation of *F. oxysporum*, A2: the treatment of *F. oxysporum* first followed by treatment of biofertilizer.

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

4. Conclusion
Having examined the disease and the impact of applying biofertilizer, it can be concluded that: A single application of biofertilizer before pathogenic inoculum that causes damping off occurs can reduce the intensity of the disease, while the use of biofertilizer after the presence of inoculum in the soil is still able to reduce the severity of damping off except the application of *T. asperellum* secondary metabolite. Applying biofertilizer can increase the germination percentage of Japansche citroen seed compared to untreated control. Applying AzzoFor biofertilizer after planting causes higher increment than other treatments.

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