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Effectiveness of biofungicide formula on rhizome rot disease of red ginger and its plant growth

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Abstract. *Marwan H, Hayati I, Mulyati S. 2023. Effectiveness of biofungicide formula on rhizome rot disease of red ginger and its plant growth. Biodiversitas 24: 2143-2148.* Rhizome rot disease is a serious problem in red ginger cultivation in Indonesia. The development of organic red ginger cultivation to meet the demand for phytopharmaca raw materials requires a method of controlling rhizome rot disease without synthetic pesticides. The application of microbial biopesticides is one of the disease control methods that can be used to control rhizome rot disease in red ginger plants caused by *Fusarium oxysporum*. The purpose of this study was to determine the effect of biofungicide formula containing *Trichoderma* sp. TBP1 and *Bacillus* spp. (PBC25 and PBC32 isolates) on plant growth and disease incidence in red ginger. The results of in vitro testing showed that *Trichoderma* sp. (TBP1) isolate was able to inhibit the growth of *F. oxysporum* by 83%. The inhibition of *F. oxysporum* by *Bacillus* spp. isolates, namely PBC25 and PBC32 were 53.3% and 50%. It was observed that application of several biofungicide formulas containing *Trichoderma* sp. and *Bacillus* spp. on ginger rhizome before planting and 30 days after planting enhanced the growth of ginger plants and suppressed the development of rhizome rot disease between 78.57 - 93.3%.

Keywords: *Bacillus* sp., biofungicides formulation, *Fusarium oxysporum*, red ginger rhizomes, *Trichoderma* sp.

INTRODUCTION

Red ginger is a medicinal plant and is widely cultivated today because its rhizome contains many phenolic antioxidant compounds, vitamins, minerals, and other compounds that are beneficial to health (Mao et al. 2019). The development of organic ginger cultivation to meet the needs of biopharmaceutical industry requires biological methods to control plant diseases. Rhizome rot is an important disease of red ginger caused by *Fusarium oxysporum* f.sp. *zingiberi* (Li et al. 2014). Pathogens can attack rhizome plants both in nurseries and in croplands, causing rhizome rot and plant death. Rhizome rot disease decreases ginger production and reduces the yield by 50% to 90% (Acharya et al. 2016).

Efforts have been made to control rhizome rot disease through several approaches such as using integrated disease management (Shim et al. 2015), bio-organic fertilizer (Zang et al. 2017) organic soil amendments (Hosain et al. 2018), biocontrol agent (Liang et al. 2022), and chemical fungicides (Ghimire et al. 2022). The use of biopesticide to control rhizome rot disease is one way to produce healthy red ginger rhizome that is free from synthetic pesticides. Biopesticide is the general name given to microorganisms (microbial pesticides) and naturally occurring compounds that possess the ability to control plant diseases (Roger and Keinath 2010). Biopesticides are microbial products generated from microbes, plants, certain minerals, and other biological organisms aiming to control plant pests (Seenivasagan and Babalola 2021; EPA 2022). Biofungicides are one type of biopesticide formulations of living organisms that are used to control the activity of plant pathogenic fungi. The concept of biofungicides is based on observations of natural processes where beneficial microorganisms, usually isolated from soil, hinder the activity of plant pathogens.

The microbial group that is widely used in biocontrol plant pathogens are *Trichoderma* sp. and *Bacillus* spp. The influence of environmental factors, such as ultraviolet radiation (Costa et al. 2016), relative humidity (Swaminathan et al. 2016), temperature (Domingues et al. 2016), and storage conditions (Locatelli et al. 2018) are often inhibiting factors in the application of biocontrol agents in the field. Formulation development is needed to overcome the influence of environmental factors and improve the consistency of microbial performance as biocontrol agents in the field (Kumar et al. 2014).

The formulation of microbial bio-inoculants includes liquid formulations, solid formulations, polymeric formulations, and metabolite formulations (Chaudhary et al. 2020). Several strains of *Trichoderma* and *Bacillus* formulations have been studied to control plant diseases. *Trichoderma* sp. can be formulated in the form of talc powder, vermiculate, oil, press mud, paste granules, black bran and peat soil (Kumar et al. 2014; Adan et al. 2015). *Bacillus* spp. can be formulated on rice bran, rice husk powder, talc powder, skimmed milk (Gotor-Vila et al. 2017; An et al. 2019; Ahmad et al. 2019).

Some research results show that the combination of *Trichoderma* sp. and *Bacillus* spp. is able to control bacterial wilt disease caused by *Ralstonia solanacearum* (Kariuki et al. 2020; Zhou et al. 2021), ginger soft rot

caused by *Pythium myriotylum* (John et al. 2016), tobacco damping-off caused by *Pythium aphanidermatum*, and cucumber damping-off (caused by *Rhizoctonia solani*) diseases is more effective than using only *Bacillus* sp. or *Trichoderma* sp. (Yobo et al. 2011).

The aim of study was to: (1) determine the inhibition ability of *Trichoderma* sp. (TBP1) and *Bacillus* spp. (PBC25 and PBC32 isolates) against *F. oxysporum*; (2) to develop a biofungicide formula containing *Trichoderma* sp. and *Bacillus* spp. effective for increasing plant growth and controlling rhizome rot disease in red ginger plants.

MATERIALS AND METHODS

Inhibition test of *Trichoderma* sp. (TBP1) and *Bacillus* spp. (PBC25 and PBC32) isolates against *Fusarium oxysporum*

Trichoderma sp. was isolated from the rhizosphere of soybean plant and *Bacillus* spp. was isolated from the rhizosphere of pulai tree. *Fusarium oxysporum* was isolated from red ginger rhizome showing symptoms of rhizome rot disease. *Fusarium oxysporum* and *Trichoderma* sp. TBP1 was cultured on Potato Dextrose Agar (PDA) and incubated for 7 days until the fungal mycelium covered the PDA surface. *Bacillus* spp. (PBC25 and PBC32 isolates) were cultured on Tryptic Soy Agar (TSA) media and incubated for 2 days. TBP1, PBC25 and PBC 32 isolates were tested for their ability to inhibit the growth of *F. oxysporum* on PDA media using dual culture method. TBP1 and *F. oxysporum* isolates (0.5 cm in diameter) were placed 3 cm apart on the same PDA Petri dish. PBC25 and PBC 32 isolates were streaked lengthwise with a distance of 3 cm from *F. oxysporum* isolate on PDA Petri dish. Cultures were incubated at room temperature for 7 days. The diameter of *F. oxysporum* colonies was observed for up to 14 days. Percentage of inhibition of *F. oxysporum* by *Trichoderma* sp. and *Bacillus* spp. isolates was calculated using the following formula (100 × (CT)/C), where C = the radius of the mycelium of *F. oxysporum* towards the edge of Petri dish; T = radius of *F. oxysporum* towards *Trichoderma* sp. or *Bacillus* spp. isolates (Ting and Jioe 2016; Slama 2019).

Characterization of *Bacillus* spp. (PBC25 and PBC32 isolates)

Characterization was performed to determine some of the physiological properties of *Bacillus* spp. (PBC25 and PBC 32 isolates) against *F. oxysporum* which was related to biological control agents and plant growth promoters, such as nitrogen fixation, phosphate solvents, and chitinolytic activity. The ability to fixation nitrogen was tested by growing bacterial cultures on Biological Nitrogen Fixation (BNF) media in test tube (Harca 2015), the ability as a phosphate solvent was tested by growing bacteria on Pikovskaya media (Paul et al. 2016), and chitinolytic activity of isolates was tested using the Lingappa and Lockwood method (Zou et al. 2002).

Preparation of microbial solutions and biofungicide formula

For this, *Fusarium oxysporum* was cultured on Potato Dextrose Broth (PDB) medium. Five mycelial plugs (5 mm in diameter) of individual fungal colonies were transferred into 250 mL erlenmeyer flask containing 150 mL of PDB medium. The culture solution was shaken at a speed of 100 rpm for seven days at room temperature (Supriyanto 2020). Isolates of *Bacillus* spp. (PBC25 and PBC 32) was transferred into a 250 mL erlenmeyer flask containing 150 mL of coconut water and peptone solution. The culture solution was shaken at a speed of 100 rpm for two days at room temperature.

Formula A was made by mixing the culture solution of *Trichoderma* sp. (TBP1) with the culture solution of *Bacillus* spp. (PBC25 and PBC32 isolates) with a volume ratio (1:1:1). Formula A1 contained a mixture of metabolites of *Trichoderma* sp. (TBP1) and *Bacillus* spp. (PBC25 and PBC32 isolates). The metabolite solution was obtained by centrifuging *Trichoderma* sp. (TBP1) and *Bacillus* spp. for 5 minutes at 4000 rpm to separate the mycelium of *Trichoderma* sp. and viable cells of *Bacillus* spp. from the culture solution. The centrifuged solution containing the metabolites of each microbe was mixed in a volume ratio (1:1:1). Formula A2 originated from formula A, added with 2.5 grams of KNO_3 and 2.5 grams of Si_3 per liter of solution, while formula A3 was from formula A1 added with 2.5 grams of KNO_3 and 2.5 grams of Si_3 per liter of solution.

Application of biofungicides formula on red ginger plants

This experiment was performed in a randomized block design with six treatments and six replications, each replication consisting of five polybags. The treatments were biofungicide formula (Formula A, A1, A2, A3), positive control (without formula application), negative control (*F. oxysporum* inoculation), and commercial fungicide ((Propinep 70%). Formula application was carried out on ginger rhizome before planting and one month after planting. Biofungicide solution was prepared by mixing twenty milliliters of biofungicide suspension in one liter of water. Sprouted ginger rhizomes were soaked in biofungicide solution for one hour, then planted in medium planting consisting of a mixture of humic soil, manure, and roasted husks (volume ratio 3:1:0.5). Each polybag contains 5 kg of planting media. Inoculation of pathogen was carried out one week after the second application (one month after planting) by pouring 5 mL of *F. oxysporum* suspension on injured red ginger rhizome.

Observations were made on the growth variables of red ginger plants (plant height, number of leaves, number of shoots), and incidence of rhizome rot disease. Observations on the growth of red ginger plants were carried out 2 months after planting, while observations of disease incidence were carried out one month after inoculation of *F. oxysporum*. Data on plant growth and disease incidence were analyzed for variance (ANOVA) and Duncan Multiple Range Test at 5% level.

RESULTS AND DISCUSSION

Inhibition ability of *Trichoderma* sp. (TBP1) and *Bacillus* spp. (PBC25 and PBC32 isolate) against *Fusarium oxysporum*

The results of inhibition test showed that isolate of *Trichoderma* sp. (TBP1) was able to inhibit the growth of fungus *F. oxysporum* by 83%. Isolates of *Bacillus* spp. (PBC25 and PBC32) showed the ability of antibiosis against the growth of *F. oxysporum* colonies with inhibition of 53.3% and 50% (Figure 1). This shows that *Trichoderma* sp. (TBP1), and *Bacillus* spp. (PBC25 and PBC32) isolates can be used as active ingredients in biofungicide formula to control the pathogenic fungus *F. oxysporum* on red ginger plants.

Trichoderma has three main mechanisms for suppressing pathogens, namely parasitism, antibiosis, and competition for space and nutrients. It can secrete antibiotic compounds that function as antifungals in inhibiting growth and even becoming pathogenic fungal microparasites. According to Vey et al. (2021), *Trichoderma* strains produce volatile and non-volatile toxic metabolites, including harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-pentyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid, trichodermin, dermadins, and others. It can also parasitize other fungi by forming an appressorium structure that functions to penetrate the host tissue. *Trichoderma* attaches to pathogens with cell wall carbohydrates that bind to pathogenic lectins, then bind to pathogens and form appressoria (Howell 2001).

The inhibitory ability of *Bacillus* spp. (PBC25 and PBC32 isolates) presumably, because the bacteria produce antibiotics and enzymes, this can be seen by the presence of a clear zone in the medium. Antibiotic compounds and enzymes produced by *Bacillus* spp. diffused in the media thereby inhibiting the growth of *F. oxysporum* hyphae approaching bacterial colonies. According to Slepecky and Hemphill (2006) and Chowdhury et al. (2015), *B. subtilis* was able to produce 68 types of antibiotics, and *B. brevis* produced 23 types of antibiotics. *Bacillus* spp. produce hydrolytic enzymes (protease, glucanase, chitinase) which play a role in inhibiting the growth of *Fusarium verticillioides* which causes stalk and ear rot of maize (Douriet-Gámez et al. 2018).

The characterization results of *Bacillus* spp. (PBC25 and PBC32 isolates) showed that both isolates could fix nitrogen (+) and phosphate solvents, but did not show chitinolytic activity (Table 1). These characters can support the role of *Bacillus* spp. in the formula to enhance growth and induce plant defense against pathogens.

The effect of biofungicide application on the growth of red ginger plants

The experimental results showed that application of biofungicide formula had a significant effect on the height, number of leaves, and number of shoots of red ginger plants aged two months after planting (Table 2 and Figure 2). The best growth in height and number of plant leaves was observed in the formula A, namely which was 51.27 cm and 12.27 leaves, while the highest (3.63) number of shoots was found in the formula A3.

The increase in the growth of red ginger plants in this experiment could be due to the ability of *Bacillus* spp. isolates to fix nitrogen and dissolve phosphate (Table 1). *Bacillus* spp. can act as biofertilizers or biostimulators either by facilitating the uptake of certain nutrients from the environment (nitrogen fixation, phosphate solubilization), or by providing the plant with a compound (Borriss et al. 2011).

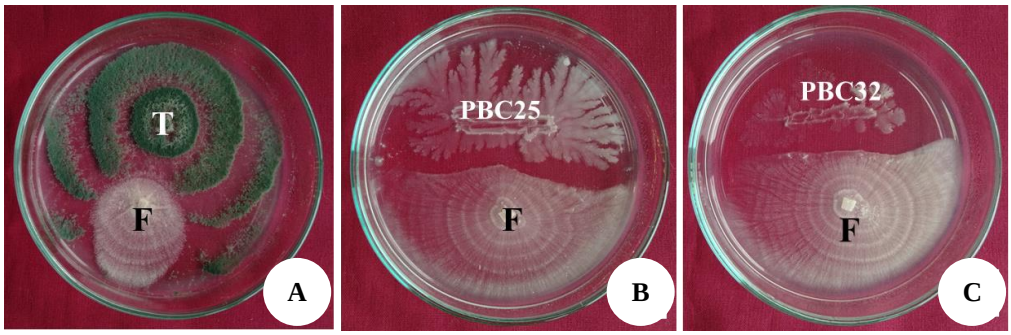

Figure 1. Inhibition of *F. oxysporum* by TBP1, PBC25 and PBC32 isolates: A. *Trichoderma* (T) vs *F. oxysporum* (F); B. *Bacillus* spp. PBC25 vs *F. oxysporum* (F); C. *Bacillus* spp. PBC32 vs *F. oxysporum* (F)

Table 1. Characteristics of *Bacillus* spp. (PBC25 and PBC32 isolates)

Isolates	Nitrogen fixation	Phosphate solubility (SI) [1)]	Chitinolytic activity (CI)[2)]
Bacillus spp. PBC25	+	2.23	0.00
Bacillus spp. PBC32	+	2.66	0.00

Note: [1)] SI = Solubilization index [2)] CI = Chitinolytic index

Table 2. Effect of biofungicide application on red ginger plant growth at two months after planting

Treatments	Plant height (cm)*	Number of leaves *	Number of shoots *
Formula A	51.27 a	12.27 a	3.27 ab
Formula A1	45.40 b	10.70 b	2.63 b
Formula A2	41.93 bc	9.50 cd	3.43 ab
Formula A3	42.90 b	10.10 c	3.63 a
Fungisida (Propineb 70%)	35.03 cd	8.77 e	2.73 b
Positive control (without formula application)	38.20 cd	9.80 cd	3.07 b

Note: *The numbers followed by the same letter are not significantly different at $p \leq 0.05$ DMRT

Nitrogen is essential for plant growth although partially unavailable in its atmospheric form. Biological nitrogen fixation (BNF) is carried out by several groups of microorganisms that can absorb elemental nitrogen from the atmosphere and form compounds that serve as plant nutrients (Musk et al. 2016). The microorganisms produce enzyme nitrogenase to catalyze the conversion of molecular dinitrogen (N2) to ammonia (NH3) which is afterward taken by plant roots and assimilated in amino acids. *Bacillus* spp. can decrease chemical fertilizer-N use and increase plant growth and yield through symbiotic nitrogen fixation. Other research by Kuan et al. (2016) stated that *B. pumilus* can fix atmospheric N2 and significantly increase the total N content and dry biomass of maize.

Besides nitrogen plants also need phosphate for growth. More than 80% of phosphate is fixed in the soil and is unavailable for plant uptake (Ogeh and Rukeme 2014). Phosphate solubilization microorganisms can dissolve inorganic P and mineralize organic P that is insoluble in the soil. Mechanisms of inorganic phosphate solubilization by microorganisms involve the production of organic and inorganic acids siderophores, protons, hydroxyl ions, and CO2 which chelate cations or reduce pH in order to release Phosphate (Sharma et al. 2013). Mineralization of organic phosphate occurs due to the synthesis of extracellular enzymes such as phosphatases, phytases, and phospholipases (Richardson et al. 2011).

Based on observations of plant growth (plant height, number of leaves, and shoots) after two applications of biofungicide formula (one month after the second application), the application of each formula showed a different effect on the three growth variables. Formulas A and A2 containing of *Trichoderma* sp. mycelium and *Bacillus* spp. viable cells showed different effects on the height and number of leaves but were not different in the number of red ginger plant shoots. The same results were also seen in the application of formulas A1 and A3 which both contained metabolites from *Trichoderma* sp. and *Bacillus* spp. Application of formula A1 showed better growth in height and number of leaves than formula A3, but on observing the number of tillers the application of the A3 formula showed better results than A1 formula. Differences in the composition of formulas A and A2 and A1 and A3 were found in the addition of KNO_3 and Si_3 compounds to the formula A2 and A3 solutions. Further observation was needed to determine the effect of adding KNO_3 and Si_3 compounds in formulas A2 and A3 on the growth of ginger plants.

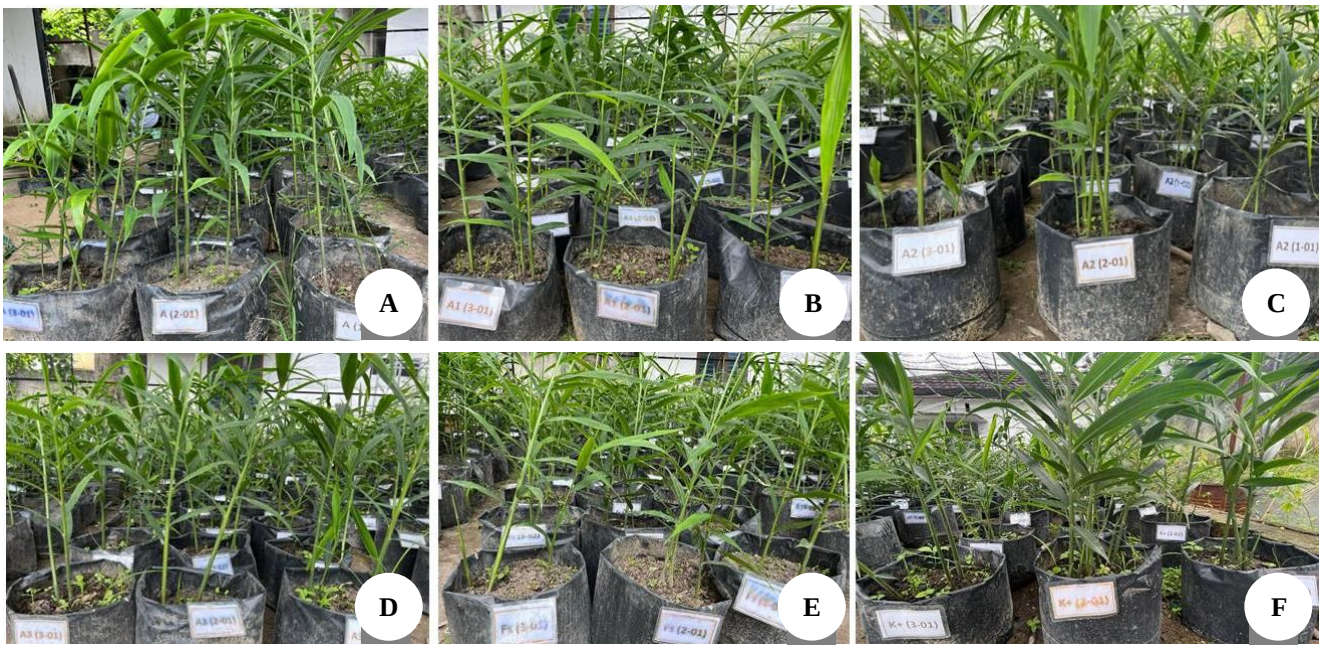

Figure 1. Performance of red ginger plants two months after planting (twice application of formula): A. application of formula A; B. application of formula A1; C. application of formula A2; D. application of formula A3; E. Fungicide application; F. Control (without formula application)

The effect of biofungicide application on rhizome rot disease of red ginger plant

The results of observations on the incidence of rhizome rot disease showed that treatment of the biofungicide formula had a significant effect on the incidence of rhizome rot disease in red ginger plants (Table 3). Formula A showed the lowest incidence of rhizome rot disease compared to other biofungicide formula treatments. The incidence of rhizome rot disease in the treatment of each formula ranged from 8.89-20% with an emphasis on disease incidence ranging from 78.57-90.48%. Formula A treatment showed the highest suppression of disease incidence, while the lowest suppression of disease incidence was found in formula A3.

Inoculation of *F. oxysporum* on red ginger root caused changes in the morphology and color of the leaf blade. In the early stages of inoculation, leaves of red ginger plant curl and followed by a change in the color of leaves to turn yellow (Figure 3). The first symptoms appeared on the injured ginger rhizome stem during inoculation of pathogens. Red ginger can be protected from *F. oxysporum* infection by applying a biofungicide formula to ginger rhizomes before planting and one month after planting. Application of formulas A and A2 containing *Trichoderma* sp. and *Bacillus* spp. cells and their metabolites were able to suppress disease incidence higher (90.48 and 85,71%) than the application of formulas A1 and A3 (83.33 and 78.57%) which only contained metabolites from the three microbial isolates. Mycelium and viable cells from *Trichoderma* sp. (TBP1) and *Bacillus* spp. (PBC 25 and PBC 32) were able to colonize red ginger rhizomes and roots, thereby protecting the surface of rhizomes and roots from *F. oxysporum* infection. Direct contact between *Trichoderma* and pathogenic fungal mycelium can cause lysis of pathogenic fungal mycelium (Safari-Motlagh and Samimi 2013). According to Miljakovic et al. (2020), the antagonistic activities of *Bacillus* spp. against fungal pathogens are frequently related to the production of secondary metabolites with antibiotic properties, involve peptides with a low molecular weight that are generated ribosomally (bacteriocins) or non-ribosomally (lipopeptides. peptides. polyketides). Competition between biocontrol agents and fungal pathogens may involve the acquisition of organic substrates released roots as well as micronutrients such as soluble iron which is often in limited amounts in soil (Kamilova et al. 2005).

Table 3. Effect of application of biofungicide formula on the incidence of rhizome rot disease in red ginger plants

Treatments	Rhizome rot incidence (%)*	Suppression of disease incidence (%)
Formula A	8.89 d	90.48
Formula A1	15.56 c	83.33
Formula A2	13.33 c	85.71
Formula A3	20.00 c	78.57
Fungicide (Propineb 70%)	51.11 b	45.24
Negative control (*F. oxysporum* inoculation)	93.33 a	

Note: * The numbers followed by the same letter are not significantly different at $p \leq 0.05$ DMRT

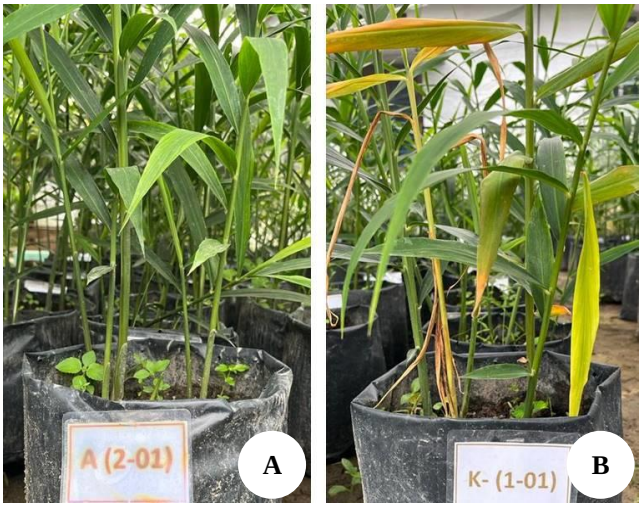

Figure 3. Effect of *F. oxysporum* inoculation on red ginger plants: A. No symptoms appeared on red ginger plants treated with biofungicide formula; B. Symptoms of yellowing and wilting appear on red ginger leaves due to *F. oxysporum* infection in ginger rhizomes

The research concluded that the application of a biofungicide formula containing *Trichoderma* sp. TBP1 and *Bacillus* spp. (PBC25 and PBC32 isolates) was able to increase the growth of red ginger plants and inhibit the incidence of rhizome rot disease by 78.57-90.48%. The addition of KNO_3 and Si_3 compounds to biofungicide formula did not show any significant effect on increasing plant growth and suppressing the incidence of rhizome rot disease in red ginger plants.

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