

Screening of soil fungi as bioremediation fungicide and its effect on growth of potato plants

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Abstract. Abadi AL, Choliq FA, Oktavianita M, Arinata N, Hadi MS, Setiawan Y. 2022. Screening of soil fungi as bioremediation fungicide and its effect on growth of potato plants. *Biodiversitas* 23: 1605-1610. The negative impact of using fungicides on the environment can lead to chemical residues in the soil. The chemical degradation process can be carried out in several ways, one of which is by biological means by using microbes such as soil fungi. This study aimed to evaluate soil fungi isolated from natural forests for bioremediation of Mancozeb fungicide commonly used in potato fields in Indonesia. Isolated soil fungi were identified based on morphological and molecular level (PCR). The inhibition assay was carried out by growing fungal isolates on PDA media containing a fungicide with an active ingredient of 80% Mancozeb. *Trichoderma harzianum* isolated as a soil fungus from natural forest could grow in the fungicide Mancozeb medium. The biodegradation assay showed that treatment of *Trichoderma harzianum* and Mancozeb fungicide did not inhibit the growth of potato plants (plant height, number of leaves, and root length) as compared to the control. Based on the results of degradation test using HPLC method, *T. harzianum* both isolates can reduce the residue of fungicide Mancozeb in soil during the vegetative growth period of potato plants.

Keywords: Bioremediation, HPLC, Mancozeb, *Trichoderma harzianum*, soil fungi

INTRODUCTION

Industrial and agricultural activities can cause environmental pollution, with various chemical properties released into the environment (Briffa et al. 2020). If these chemicals are not degraded, they will combine with soil particles and become part of an interconnected food chain (Gupta et al. 2016). Currently, one of the pollutions from agricultural activities is the application of fungicides for disease control. The negative impact of using fungicides on the environment can produce chemical residues in the soil (Zubrod et al. 2019). According to Wightwick et al. (2010), the presence of fungicides in the agroecosystem adversely affects soil organisms, such as earthworms and other microorganisms that perform important functions in the soil, such as breaking down organic matter and facilitating nutrient cycling (Roman et al. 2021). Mancozeb is a fungicide that is often used in Indonesia for plant disease control. Mancozeb is a member of the ethylenebis (dithiocarbamate) group of fungicides, which contains the related active ingredients maneb and metiram (Li et al. 2013). This fungicide was used to control diseases caused by pathogenic fungi in agricultural sector such as wheat, grapevine, potatoes, and tomatoes, as proposed by the applicants (Verasoundarapandian et al. 2022). In Indonesia, Mancozeb is widely used in potato plants to control potato late blight (*Phytophthora infestans*) (Sari et al. 2021).

The chemical degradation process can be carried out in several ways, one of which is by biological means by using microbes (Joutey 2013). An alternative way to restore the

environment contaminated with fungicides is by bioremediation. Bioremediation can be defined as the process of biological agents such as bacteria, fungi, plants, enzymes used to reduce the mass of contaminants and toxicity in soil, groundwater, and air (Fareed et al. 2017; Sharma 2021). The aim of bioremediation is to reduce the toxicity and concentration of contaminants in the soil either through organic biodegradation or metal biotransformation (Igiri et al. 2018). Bioremediation technique is an environmentally friendly approach by utilizing microorganisms to convert and degrade chemicals that contaminate or pollute the environment (Azubuike et al. 2016). However, these microbes can degrade chemical pollutants from certain systems depending on environmental conditions (Das and Chandran 2011). Microbial utilization is considered environmentally friendly, as it does not add further chemicals to the environment (Alori and Babalola 2018). Utilizing these microbes reduces or degrades chemicals with one of the mechanisms: co-metabolic degradation, polymerization and binding with natural compounds, intracellular and extracellular accumulation, mineralization, and detoxification (Ahlawat et al. 2010). Many fungi have been isolated from various types of habitats such as tropical forests, grasslands, deserts, and coastal areas (Hj Yakop et al. 2019).

Fungi are considered one of the most adaptable groups of microorganisms and an important constituent of soil microbes (Gougoulas et al. 2014). Fungi act as decomposers of organic matter such as wood, stems, and

leaves and function as plant symbionts in ecosystems, playing important roles in ecological and biogeochemical processes (Wurzburger and Clemmensen 2018; Afandhi et al. 2022). Mycoremediation is a technique where fungi remove hazardous chemical contaminants such as polycyclic aromatic hydrocarbons, harmful phenolics, dyes, heavy metals, and several others (Deshmukh et al. 2016). The combination of *Trichoderma* sp. and *Aspergillus* sp. has been reported to degrade carbendazim and Mancozeb (Ahlawat et al. 2010). Adetutu et al. (2008) also reported that soil fungal species, such as *Cladosporium* sp., *Penicillium* sp., *Fusarium* sp., and *Alternaria* sp., can degrade azoxystrobin fungicides. Therefore, the aims of this study was to evaluate soil fungi isolated from natural forests for bioremediation of the Mancozeb fungicide commonly used in potato fields in Indonesia.

MATERIALS AND METHODS

Soil sampling

The soil sampling was conducted in natural forests at UB Forest, Karangploso Sub-district, Malang District, East Java, Indonesia (7°53'35" S; 112°53'41" E). The UB tropical rain forest was located in the south side of Mount Arjuna. The soil samples were collected from five sampling points by digging 15-20 cm deep into the soil and then placed in a labeled plastic bag. Soil samples were put in iceboxes to avoid high temperatures.

Soil fungal isolation

The potato dextrose agar medium (PDA) with antibacterial (chloramphenicol) was used for the isolation of fungi (Joseph et al. 2015). The soil fungus was isolated using the dilution plate method by adding 1 g of soil into the test tube and then adding 10 mL of sterile distilled water and then shaking it until homogeneous. One mL of the homogeneous solution was transferred to the test tube and added 9 mL sterile distilled water to make 10^{-2} dilutions, then take 1 mL from 10^{-2} dilution and add 9 mL sterile distilled water to make 10^{-3} dilutions. The desired dilution was then poured into a petri dish containing media and flattened with an L-stick. The experiment was done in triplicates in laminar airflow to prevent contamination, and plates were incubated for 3-7 days at room temperature (27-28°C).

Purification

For each different fungal colony, a little inoculum was taken and re-grown on a petri dish containing solid PDA and incubated at room temperature (27-28°C) for five days. After incubation, the fungal colony was placed on a glass object that already contained a PDA medium. The medium was covered with a glass cover and incubated for 2-3 days at 28-30°C for further identification.

Inhibition assay of soil fungi on Mancozeb *in-vitro*

The inhibition assay was carried out by growing fungal isolates on PDA media containing a fungicide with an active ingredient of 80% Mancozeb with a concentration of

150 ppm. One ose of soil fungal isolates was grown on PDA media containing Mancozeb. Fungal growth was observed 10 days after inoculation (DAI). The addition of fungicides aimed to prevent the growth of soil fungi on selective media. Inhibition assays were done by inoculating the fungus in the middle of the solidified media. Inhibition experiment was carried out three times. Observations were made by measuring the diameter of the fungal colonies. Inhibition of fungus was calculated by the following formula:

$$\text{Inhibition} = \frac{C - T}{C} \times 100\%$$

Where:

C = Diameter of soil fungal colonies in control (cm)

T = Diameter of soil fungal colonies in treatment (cm)

Identification of soil fungi

Identification of soil fungi was based on morphological and molecular levels. Morphological identification was carried out by macroscopic and microscopic observations. Macroscopic morphology included shape, color, and edges of the colonies growing on PDA media. While microscopic identification was made by placing a single colony with a small amount of PDA media on a glass slide with an ose needle, then covered with a coverslip and simultaneously pressed slowly. After 7 days of incubation, mycelium, spores, and conidia of soil fungi were observed by using a compound microscope with a magnification of 400 times. Molecular identification was carried out at the Genetic and Molecular Laboratory of the Biology Study Program, Faculty of Science and Technology, State Islamic University of Maulana Malik Ibrahim Malang, Malang, Indonesia. The process of molecular identification with the PCR test consisted of several steps i.e. DNA isolation, DNA purification, quantitative measurement of DNA concentration using BioSpec-Nano, electrophoresis, PCR, and sequencing.

Mancozeb biodegradation assay on potato plants *in-vivo*

Preparation of planting media

The planting media used in this study was soil and compost with 1:1 ratio. The soil and compost mixture was sterilized using 4% formalin at a dose of 25 mL for 1 kg of soil by spraying evenly. The use of formalin 4% aimed to reduce or even kill pathogen populations. After watering 4% formalin, the mixture was then covered with plastic to incubate for 7 days and air-dried for about 7 days.

Inoculation of soil fungi on growing media

The prepared planting media was put into polybags with a size of 25×25 cm. The suspension of soil fungus isolate was made with potato dextrose broth (PDB) media (spore density 10^5 spores mL⁻¹) that was shaken with an orbital shaker for 7 days. One hundred mL per 3 kg of soil fungi suspension was inoculated in each polybag of growing media. After inoculation, soil was then covered with plastic for 24 hours. This experiment was conducted using a factorial randomized block design consisting of 10 treatments and three replications. The *in-vivo* biodegradation

test was performed in greenhouses by applying the soil fungus and Mancozeb with different concentrations.

Mancozeb fungicide toxicity test

The first application of Mancozeb fungicide was carried out after 21 days after planting (DAP) with an interval of 7 days during the planting period, this was under the recommendations on the product packaging.

Mancozeb residue analysis on soil with high-performance liquid chromatography (HPLC)

The soil samples were analyzed at the Laboratory of Soil Chemistry, University of Muhammadiyah Malang. The analysis of Mancozeb residue remaining in the soil was expressed in ppm. The following formula was used for the percentage reduction of residue:

$$\text{Residual reduction} = \frac{\text{Initial Value} - \text{Final Value}}{\text{Initial Value}} \times 100\%$$

Where:

Initial value: Residue of control treatment

Final score: Residue of Mancozeb fungicide treatment and soil fungi

Data analysis

The data obtained were analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). The difference among the data was considered significant for at least $P < 0.05$. All the data were performed using Microsoft Excel 2016.

RESULTS AND DISCUSSION

Mycelial growth inhibition assay

This study used growth inhibition assay to determine Mancozeb inhibition of actively growing fungal mycelia. Results showed that four soil fungi isolates were obtained from natural forest at UB forest, Karangploso Sub-district, Malang District, East Java, Indonesia. All four isolates were evaluated for their ability growth in selective medium with Mancozeb (Table 1). The results of inhibition growth

soil fungi by fungicide Mancozeb indicated that TD1 and TD2 isolate had the ability to grow in selective media and were significantly different. The lowest percentage of inhibition growth in selective medium with Mancozeb was TD1 isolate (35.81%) and TD2 isolate (77.11%) (Table 1).

Morphological and molecular identification of soil fungi

Results of morphological observations showed that the two isolates were round, light green to dark green in the middle, and the edges were white with a cotton-like texture (Figure 1.A and 1.B). Microscopically, both TD1 and TD2 isolates had perpendicular conidiophores and branched, phialides were short and thick, and conidia were globose and green.

Based on molecular identification of TD1 and TD2 isolates, DNA bands were firm and clear on gel when irradiated with ultraviolet light. TD1 and TD2 isolates primers produced a single DNA band of 550 base pairs (bp) (Figure 2). Molecular identification results revealed that TD1 and TD2 isolates showed 91.10% and 95.64% similarity with *T. harzianum*, respectively (Table 2).

Table 1. Percent inhibition of four soil fungi isolates by Mancozeb

Soil fungi isolates	Inhibition (%)
MC	97.10 d
TD1	35.81 b
PN	100.00 d
TD2	77.11 c
FS	100.00 d

Note: According to Duncan's test, the mean followed by the same letters in each row is not significantly different at $P < 0.05$.

Table 2. Molecular identification of TD1 and TD2 isolates based on the alignment of ITS region sequences

Fungal isolates	Similarity ID (%)	Species	GenBank
TD1	91.10	<i>Trichoderma harzianum</i>	MZ130515.1
TD2	95.64	<i>Trichoderma harzianum</i>	MZ130515.1

Table 3. Plant height, number of leaves, and root length of potato plants on soil fungi and 80% Mancozeb with a different concentration on 63 days after planting

Treatments	Plant height (cm)	Leaf number	Root length (cm)
Control	7.83	29.00 ab	38.33
<i>Trichoderma harzianum</i> TD1 + Mancozeb 75 ppm	7.83	25.67 a	30.67
<i>Trichoderma harzianum</i> TD1 + Mancozeb 150 ppm	9.50	28.33 a	40.00
<i>Trichoderma harzianum</i> TD1 + Mancozeb 200 ppm	10.83	43.67 bc	38.50
<i>Trichoderma harzianum</i> TD1 + Mancozeb 250 ppm	14.67	59.67 cd	36.33
<i>Trichoderma harzianum</i> TD2 + Mancozeb 75 ppm	16.50	63.67 de	40.67
<i>Trichoderma harzianum</i> TD2 + Mancozeb 150 ppm	21.00	79.00 e	40.67
<i>Trichoderma harzianum</i> TD2 + Mancozeb 200 ppm	19.17	63.67 de	31.67
<i>Trichoderma harzianum</i> TD2 + Mancozeb 250 ppm	21.50	72.67 de	35.50

Note: According to Duncan's test, the mean followed by the same letters within each row is not significantly different at $P < 0.05$. Control: 150 ppm (based on recommended concentration of product)



Figure 1. Morphology of *Trichoderma harzianum*: A. TD1 isolate, B. TD2 isolate; (a, f) conidia, (b, e) phialides, (c,d) conidiophore

Mancozeb biodegradation assay by the soil fungi on potato plant growth *in-vivo*

The plant height and root length on biodegradation assay showed no significant difference, but the leaves in potato plants were significantly different. The highest number of leaves was found in *T. harzianum* TD2 with 150 ppm Mancozeb (79.00) and the lowest was found in *T. harzianum* TD1 with 75 ppm Mancozeb (25.67) (Table 3).

Mancozeb residue test in soil by HPLC

The HPLC analysis showed that *T. harzianum* TD2 with 250 ppm Mancozeb had the lowest residue, while the highest residue was found in control. The percent residual reduction showed that *T. harzianum* TD2 with 250 ppm Mancozeb had highest percentage (74%) (Table 4). The HPLC chromatogram analysis showed that *T. harzianum* TD2 with 250 ppm Mancozeb had the lowest chemical compound of Mancozeb ethylenebis(dithiocarbamate), whereas the highest was found in control (Figure 3).

Discussion

In the present study, soil fungi isolated in natural forest at UB forest had the ability to grow in fungicide Mancozeb. TD1 and TD2 isolates were identified as *T. harzianum*. Based on identification, both isolates had light green to dark green in the middle, and the edges were white with a cotton-like texture. Conidiophores were perpendicular and branched, phialides were short and thick, and conidia were globose and green. According to Suada (2017), *T. harzianum* fungus has macroscopic characteristics in the form of a circle with clear boundaries, center of the colony is light green then dark green, and the edges are white like cotton. Naher et al. (2019) observed that *T. harzianum* has microscopic characteristics of erect and branched conidiophores, short and thicker phialides,

and oval or round conidia. TD1 and TD2 isolates produced a single DNA band of 550 base pairs (bp). According to Chakraborty et al. (2010), DNA bands of *Trichoderma* sp. isolates using universal primers ITS1 and ITS4 can produce 500-600 bp. *Trichoderma harzianum* had the ability to grow in selective media than other isolates found in UB Forest. Fungi of the genus *Trichoderma* are commonly found in all climatic zones and the most typical habitats of these fungi include soil (Nurbailis et al. 2019). Our findings are in accordance with Ahlawat et al. (2010) who reported that fungicide degradation of carbendazim and Mancozeb was found in *Trichoderma* sp. that was collected from soil. The genus *Trichoderma* has high genetic diversity with a number of abilities among strains (Filizola et al. 2019). *Trichoderma* is also tolerant of various pesticide pollutants, fungicides, including heavy metals, and polyaromatic hydrocarbons (Tripathi et al. 2013).

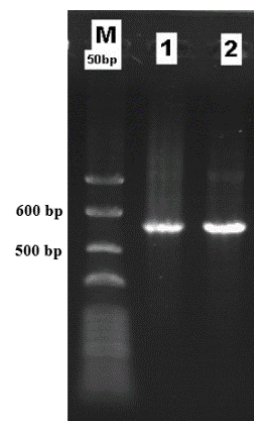


Figure 2. DNA bands from DNA electrophoresis using ITS1 universal primers; (1) TD1 isolate, (2) TD2 Isolate

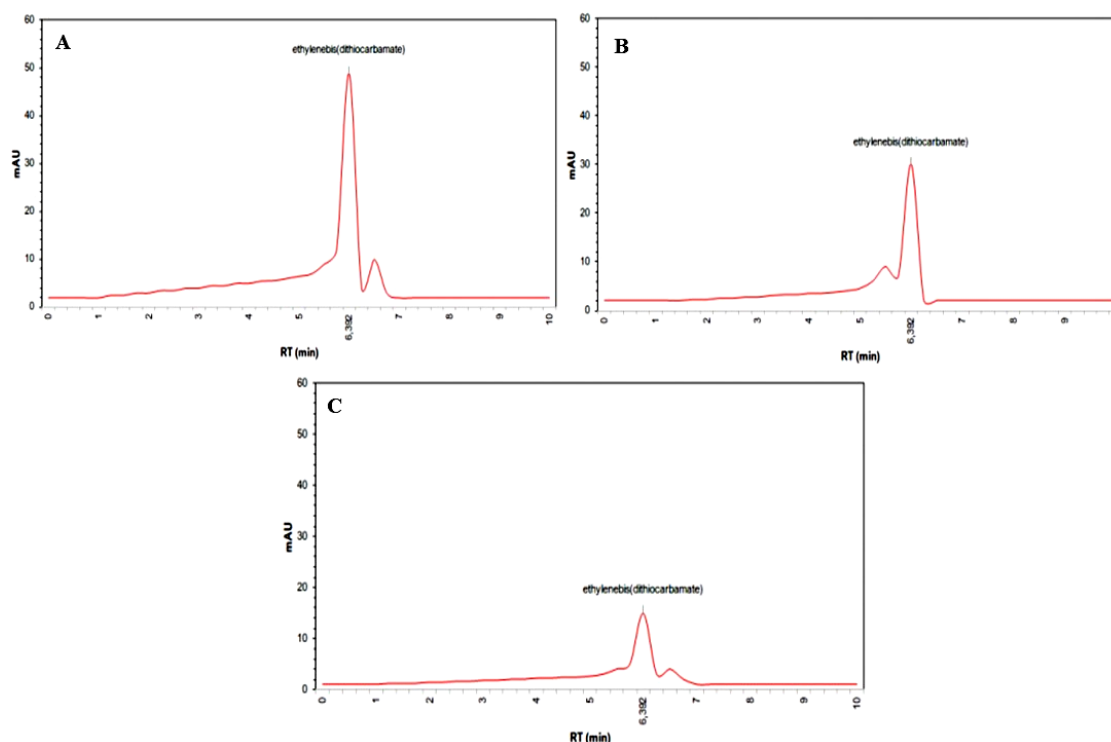


Figure 3. HPLC chromatogram analysis: A. Control, B. *Trichoderma harzianum* TD1 + Mancozeb 250 ppm C. *Trichoderma harzianum* TD2 + Mancozeb 250 ppm

Table 4. Mancozeb fungicide residue in soil

Treatments	Residue (ppm)	Percentage of residual reduction (%)
Control (Mancozeb 150 ppm)	133.73	0.00
<i>Trichoderma harzianum</i> TD1 + Mancozeb 250 ppm	78.39	41.38
<i>Trichoderma harzianum</i> TD2 + Mancozeb 250 ppm	33.94	74.62

The potato plant with application of *T. harzianum* and fungicide Mancozeb with different concentrations can grow in the highest concentration of Mancozeb than the recommended concentration (150 ppm). Tomer et al. (2018) reported that *T. harzianum* has high compatibility with Mancozeb fungicides with concentrations between 100-200 ppm. In addition, *T. harzianum* is a fungus that can help potato plant growth. *Trichoderma harzianum* can grow quickly to colonize and associate well with plant roots and significantly increase plant growth (Cai et al. 2015). Several *Trichoderma* sp. positively affect plants by stimulating plant growth, and protecting plants from fungal and bacterial pathogens (Błaszczuk et al. 2014). They are used in biological plant protection as bio-fungicides as well as in bioremediation. However, it was found that the interaction of *T. harzianum* and Mancozeb did not significantly affect plant height and plant root length. This may be because the application of *T. harzianum* is not able to increase the nutrient absorption of potato plants. According to Ojuederie and Babalola (2017), different microorganisms (bacteria, fungi, yeasts), bioremediation processes have as limiting factors O₂, temperature, humidity, nutrients (N, K, P), and the concentration and toxicity of the contaminants.

Soil fungi *T. harzianum* had the ability to degrade Mancozeb based on percentage of residue reduction. Residues produced by the fungicide Mancozeb based on the results of HPLC chromatogram analysis, namely ethylenebis (dithiocarbamate). Our finding is in accordance with Ahlawat et al. (2010), who reported that the highest degradation of Mancozeb was recorded with *Trichoderma* sp. Species of *Trichoderma* are used in biological plant protection as bio-fungicides as well as in bioremediation (Zin and Badaluddin 2020). In the process of degradation of chemical compounds by *Trichoderma* sp., this fungus produces extracellular enzymes, where the degradation process is carried out by extracellular enzymes and intracellular enzymes from bacteria must work well together so that the degradation process can occur optimally. In addition, it is suspected that other factors that influence the degradation process are environmental factors. According to Szpyrka et al. (2020), various factors can influence the degradation of fungicides in the soil such as soil type, organic and mineral content, soil pH, humidity, temperature, presence of microorganisms, and active chemical ingredients used. In conclusion, soil fungi in natural forest, namely *Trichoderma harzianum* TD1 and *Trichoderma harzianum* TD2 showed growth in selective

media with Mancozeb. *Trichoderma harzianum* isolates were able to significantly degrade the residue of Mancozeb fungicide in the soil and did not interfere with plant growth of potato plant.

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