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Remediation of chlorpyrifos-contaminated soils by crude secondary metabolites of *Trichoderma harzianum* T213 and its effect on maize growth

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Abstract. *Soesanto L, Susanti RA, Mugiastuti E, Manan A, Sastyawan MWR, Maryanto J. 2022. Remediation of chlorpyrifos-contaminated soils by crude secondary metabolites of* Trichoderma harzianum *T213 and its effect on maize growth. Biodiversitas 23: 2464-2470.* The study was conducted to determine the role of *Trichoderma harzianum* T213 crude secondary metabolites on the growth of maize plants and the reduction of chlorpyrifos on contaminated soils. A randomized block design was used in this study with four treatments. The treatments applied on chlorpyrifos contaminated soil were: without the application of secondary metabolites (0%) and the application of *T. harzianum* T213 crude secondary metabolites at the concentration of 50, 100, and 150%. Variables observed were residual levels of insecticide in maize, plant height difference, number of leaves, plant fresh weight, and root length. The results showed that the application of *T. harzianum* T213 crude secondary metabolites effectively increased plant growth, including plant height, the number of leaves, plant fresh weight, and root length compared to control on chlorpyrifos-contaminated soil. The highest percentage (99.54%) of the reduction of chlorpyrifos residue was the application of 100% *T. harzianum* T213 crude secondary metabolites. It was followed by applying 50 and 150% *T. harzianum* T213 crude secondary metabolites by 99.39 and 99.14%, respectively.

Keywords: Crude secondary metabolites, maize, organic remediation, *Trichoderma harzianum* T213

INTRODUCTION

Soils play an important role, especially in agriculture, because the life and development of plants are very dependent on soil conditions. Moreover, soils contribute to basic human needs like food, clean water, and clean air and are a major carrier for biodiversity (Keesstra et al. 2016). Therefore, land use for agriculture without improvement efforts will cause land subsidence or damage. Soil damage is the loss or reduction of the soil's function that reduces the soil's ability to produce (Gomiero 2016). In addition, soil degradation also disturbs the ecological and biological balance in the soil, especially the presence of microorganisms. Soil microbes play a vital role in decomposing organic matter and plant debris (Jacoby et al. 2017). Soil degradation and low productivity could be caused by several factors, including deforestation, farming mechanisms, fires, agricultural chemicals, and monoculture planting (Alam 2014). Agricultural chemicals can be pesticides, fertilizers, or other chemicals, such as adhesives and antibiotics. These chemicals are widely applied in inorganic agriculture because of practical and effective considerations, besides being readily available in the market (Tudi et al. 2021).

Chemical pesticides are agricultural chemicals used to eradicate plant pests and diseases (Saputra et al. 2015). Chemical pesticides are widely applied in all plants, both food crops, horticulture, plantations, and forest plants, from land to storage. After application, pesticide residues are present on plants, soils, and soil organisms (Silva et al. 2019). Chlorpyrifos is one of the most widely used insecticides (Lee et al. 2012). Chlorpyrifos is an organophosphate pesticide used widely around the world. This insecticide effectively controls pests of economically important crops because of its broad-spectrum (Hill et al. 2017; Rahman et al. 2021). These non-biodegradable compounds persist in agricultural fields after application. These substances enter the soil, enter the city's water supply and contaminate the surrounding area (Matula et al. 2020). Chlorpyrifos is moderately toxic to humans that primarily affect the nervous system by inhibiting cholinesterase (Jaiswal et al. 2017).

Chlorpyrifos contaminated soils are not cultivable, and the yield cannot be maximized (Hwang et al. 2018). Further studies on the repellent effect of chlorpyrifos under standard agricultural conditions include aerial spraying of chlorpyrifos on outdoor plants causes the pesticide to drift onto the ground and become residue in the soil (Aktar et al. 2009). In addition, chlorpyrifos may enter the environment through volatilization, spills, and the disposal of chlorpyrifos waste (Arguello et al. 2020). Chlorpyrifos from contaminated environments causes acute toxicity to aquatic organisms (especially fish). Furthermore, overexposure makes them susceptible to acute phosphorus

poisoning due to phosphorylation of acetylcholinesterase (AcHE) (Dahiya et al. 2017). Therefore, remediation of soil contaminated with pesticide residues is needed to restore. One of the methods of soil remediations is carried out by using fungi through their reduction mechanism, both inside and outside the cell (García-Hernández et al. 2017; Rigoletto et al. 2020). Several species of fungi that are commonly used as remediator agents include *Aspergillus* sp. (Jayanthi et al. 2014), *Fusarium* sp. (Sen and Dastidar 2011), *Penicillium* sp. (Jayanthi et al. 2014; Abigail et al. 2015), *Hanerochaete* sp., *Saccharomyces cerevisiae*, and *Trichoderma* sp. (Morales and Cristiani 2008). Chlorpyrifos analysis is usually performed using gas chromatography (GC) with flame luminosity detection. Previously, high-performance liquid chromatography (HPLC) was used to measure chlorpyrifos in soil (Mauldin et al. 2006).

Remediation of contaminated soil can be carried out using secondary metabolites of antagonistic fungi due to many advantages over fungal spores. However, the use of spore-based fungi for soil remediation often faces several problems, such as the nature of the organisms utilized (Ojuederie and Babalola 2017), abiotic stress factors (Azubuike et al. 2016), and stability of spore production (Hojnik et al. 2019). Fungi produce several secondary metabolites compounds, which actively work in soil remediation. Fungal secondary metabolites are organic compounds resulting from metabolism that do not play a role in the fungi' growth, development, and reproduction (Pusztahelyi et al. 2015; Braga et al. 2016). *Trichoderma harzianum* T213 is a fungal antagonist that produces several secondary metabolites compounds. This study aimed to determine the effect of the crude secondary metabolites from *T. harzianum* T213 on the absorption of chlorpyrifos residue. The growth of maize plants on the remedied chlorpyrifos contaminated soil was also observed.

MATERIALS AND METHODS

Preparation of *Trichoderma harzianum* T213

Trichoderma harzianum T213 isolated from shallot rhizosphere (Santoso et al. 2007) was cultured on PDA in Petri dishes and incubated for five days at room temperature (Kouipou et al. 2016). *T. harzianum* T213 has been widely used in the antagonism assay against plant pathogens.

Production of secondary metabolites

The growth media for producing secondary metabolites was prepared by mixing rice washing water and coconut water (4: 1, v / v) and then adding 100 g of granulated sugar and boiling. After boiling, the mixture was filtered and immediately put into a 3 L sterile jerry can (Soesanto et al. 2019). *T. harzianum* colony was cut with a cork borer (0.8 mm in diameter) as many as three pieces for every 500 mL of media, inoculated onto the media, and incubated using a modified incubator shaker at a speed of 150 rpm for seven days at room temperature (Singh et al. 2014). After incubation, the fungal population density was calculated using a hemocytometer to obtain a density of 10^7 conidia per mL. The solution was centrifugated at 9000 rpm for 3 minutes to separate the supernatant and pellet. The supernatant was filtered using Whatman filter paper no. 1 for further use in this study.

Soil media preparation

The planting soil media was collected from the shallot rhizosphere. The soil media have the continuous application of chlorpyrifos. It was collected in Serang Village, Karangreja Sub-district, Purbalingga District, Central Java, Indonesia at 1,256 m above sea level. The soil type was andisol, crumb, loose, and susceptible to erosion. Soils were collected from a depth of 20 cm in the tillage layer in the diagonal sampling method (Olson and Al-Kaisi 2015). Each polybag was filled with 4 kg of soil.

Cultivation of maize

F1 hybrid maize seeds were planted in polybags containing chlorpyrifos-contaminated soil remediated with the crude secondary metabolites of *T. harzianum* by pouring the crude secondary metabolites in the soils and keeping for two weeks, and mixed with manure (1:1, w/w). Each polybag was planted with 1 seed, and each treatment had three replications. Watering the plants was done every day in the morning and evening. Plant maintenance was done by removing weeds and controlling pests. The analysis of chlorpyrifos residue in plants was carried out evenly for each treatment with a total weight of 300 g per treatment. Plant maize was harvested at100 days after planting.

Experimental design

The experimental design used was a randomized block design with the treatment of different dosages of crude secondary metabolites of *T. harzianum* T213, namely 0% (without crude secondary metabolites), 50, 100, and 150% crude secondary metabolites. The crude secondary metabolites percentage was calculated from the *T. harzianum* population density of 10^7 conidia per mL as 100%. Each treatment had six replications. The secondary metabolites of *T. harzianum* T213 were drenched on the soils and incubated for a week in the field (Herliana et al. 2018).

The analysis of residual chlorpyrifos using HPLC

Residual chlorpyrifos was analyzed at the Surabaya Center for Seedlings and Plantation Protection using the High-Performance Liquid Chromatography (HPLC) analysis method (Hussain et al. 2020). HPLC analysis was prepared as follows. One gram of homogenized plants sample was accurately weighed into a 50 mL screw-top glass vial, followed by the addition of 5.0 mL of a premixed solution of 90% ethoxy/10% 1 mM PO4 buffer (pH 4.5). The samples were then gently mixed by hand, sonicated for 10 minutes, vortexed, then placed in a mechanical shaker (Eberbach Equalpoise, Ann Arbor, Michigan) at high speed for 15 minutes. The sample was then centrifuged at about 2000 rpm for 5 minutes; the supernatant was removed and transferred to a 10 ml tube. A second 5.0 mL premix solvent was added to the original sample, and the extraction procedure was repeated. Whenever possible, the first 5 ml of extract was carefully

returned to the original sample tube, vortexed, and centrifuged. All supernatants were returned to the 10 mL tube containing the original extract and mixed thoroughly. The aliquot of the extract was filtered through a 0.45 mm Teflon syringe filter and placed in an amber HPLC vial. Extracts were analyzed using an 1100 series HPLC (Agilent Co., Sunnyvale, CA) equipped with a UV/Vis detector (UV at 230 nm). The Phenomenex (Torrance, CA) Prodigy ODS/3, C18, 5 mm, 250 mm 4.6 mm i.d. analytical column was used with the Phenomenex SecurityGuard guard column (4 mm 2 mm, C18). The extract was chromatographed at room temperature with a premixed mobile phase of 75% ethanol/25% 1 mM PO4 (pH 4.5). One hundred µL sample was injected at a flow rate of 1 mL/min for 17 minutes, increased the flow rate to 2.5 mL/min for 13 minutes, then reduced the flow rate to 1 mL/min for 2 minutes, and stabilize the flow rate (0.500 µg/g).

Variable observed

Plant height, number of leaves, plant fresh weight, and root length were measured.

Data analysis

Data were analyzed using the analysis of variance. The level of significance was determined at P <0.01 and 0.01> P <0.05. The Honest Significantly Difference (HSD) analyzed the significant difference between treatments at the 0.01 level of confidence.

RESULTS AND DISCUSSION

The effectiveness of *Trichoderma harzianum* T213 metabolites for remediation

Maize plants treated with crude secondary metabolites of *T. harzianum* T213 at different dosages result in different content of chlorpyrifos residue (Table 1). It indicated that *T. harzianum* T213 crude secondary metabolites could absorb chlorpyrifos insecticide residues.

The level of chlorpyrifos residue in plants decreased significantly due to the application of crude secondary metabolites of *T. harzianum* T213 on the soils. Based on the results in Table 1, showed that applying crude secondary metabolites of *T. harzianum* T213 at a dose of 100% was the best dose in reducing chlorpyrifos residue in plants by 99.54%, followed by treatment dosages of 50 and 150%. All dosages of crude secondary metabolites reduced chlorpyrifos residue in the soils by more than 99%. Therefore, it might be concluded that crude secondary metabolites of *T. harzianum* T213 can remediate chlorpyrifos contaminated soils through chemical processes in the soil.

Chlorpyrifos persists in soils ranging from a few days to four years; the persistence increases in more acidic soils (Mora-Gutiérrez et al. 2021). Therefore, the insecticidal impact of chlorpyrifos could be harmful to humans and other organisms (Ang et al. 2005). The effect of chlorpyrifos could be the inhibition of the enzyme acetylcholinesterase, the buildup of the neurotransmitter acetylcholine at nerve endings, and nerve impulses' over-transmission. Due to the high risk to humans, the environment, and its long persistence in soil and agricultural products, remediation methods of chlorpyrifos residues should be carried out. Remediation of chlorpyrifos residues can be done in several methods, but remediation using microbes is easy and inexpensive (Ashraf et al. 2013; Harikumar et al. 2013). One of the microbes commonly used to remediate pesticide residues is *Trichoderma* spp.

Trichoderma spp. is a soil-borne fungus widely used as a biological control agent for plant pathogens. The *Trichoderma* spp. are generally isolated from the plant rhizosphere in soil and may contain insecticide residues. These antagonistic fungi can also remediate insecticide residues. Katayama and Matsumura (1993) reported that *T. harzianum* degrades DDT, dieldrin, endosulfan, pentachloronitrobenzene, and pentachlorophenol. They concluded that the major enzyme system responsible for the degradation of endosulfan in *T. harzianum* is an oxidative system. *Trichoderma* spp. also degrades herbicides to topramezone, a pyrazolone compound (Choudhury et al. 2019).

Table 1. The level of chlorpyrifos residue in maize plants treated with different doses of *Trichoderma harzianum* T213 metabolites

Treatments	Chlorpyrifos residue (ng g^{-1})	Remediation effectiveness (%)
Control	69.442,00	-
Sec. met. of *T.harzianum* T213 50%	423,20	99.39
Sec. met. of *T.harzianum* T213 100%	317,30	99.54
Sec. met. of *T.harzianum* T213 150%	595,00	99.14

Table 2. The effect of secondary metabolites of *Trichoderma harzianum* T213 on the growth of maize grown on remedied chlorpyrifos contaminated soils

Treatments	Plant height difference (cm)	Number of leaves	Plant fresh weight (g)	Root length (cm)
Control	5.04 c	1.62 b	1.81 c	3.99 c
Sec. met. *Trichoderma harzianum* T213 50%	6.89 ab	2.18 a	2.90 b	5.94 ab
Sec. met. *Trichoderma harzianum* T213 100%	6.43 b	2.11 a	2.92 ab	5.53 b
Sec. met. *Trichoderma harzianum* T213 150%	7.22 a	2.14 a	3.31 a	6.56 a

Note: PH: Plant Height, NL: Number of Leaves, PW: Plant Fresh Weight, RL: Root Length. Numbers with different letters in the same column significantly differ based on the HSD 0.01 after transforming the data to √x.

Trichoderma spp. has been known can produce various secondary metabolites compounds (Zeilinger et al. 2016; Khan et al. 2020). According to Khan et al. (2020), secondary metabolites could be categorized as antibiotics, enzymes, hormones, and toxins. Secondary metabolites of fungi that play a role as enzymes may help break down insecticide residues. Enzymes that can degrade organochlorinated pesticides are mainly dehydrochlorination enzymes, hydrolytic enzymes, and dehydrogenases (Javaid et al. 2016). Organochlorinated pesticides represent a target for enzymatic degradation (Matula et al. 2020). According to Qin et al. (2019), the influence of environmental persistent organic contaminants such as organochlorinated pesticides (e.g., hexachlorocyclohexanes [HCHs]) on the enzymatic degradation of extracellular DNA HCH was by enhancing DNA degradation, which is not caused by increasing deoxyribonuclease I (DNase I) activity. HCH binds to a DNA base (probably guanine) via van der Waals forces and halogen bonds. This binding increased the helicity and accumulation of DNA base pairs, resulting in a more compact DNA structure, more exposure to DNase I-sensitive sites, and accelerated DNA degradation. Microorganisms utilize these enzyme-driven degradation reactions to derive their growth and maintenance energy or detoxify pesticides (Molina et al. 2020).

Secondary metabolites of *T. harzianum* can degrade chlorpyrifos. Besides, chlorpyrifos can also be used as a sole carbon source by the conidia of *Trichoderma* sp. in liquid media (Alvarenga et al. 2015). In addition, chlorpyrifos degrades sulfur and phosphorus sources and releases free chlorine atoms (Bose et al. 2021). Sulfur and phosphor increase the abundance of microorganisms in soils, including antagonistic fungi, and could positively impact plant nutrient availability and plant growth (Fox et al. 2014). Meanwhile, chlorine is a mineral nutrient, and its deficiency causes metabolic problems that impede growth (Geilfus 2018) and affect the inhibition of soil microorganisms (Lee et al. 2019).

Impact of organic remediation on plant growth

Maize grown on remedied chlorpyrifos contaminated soils with the crude secondary metabolites of *T. harzianum* T213 had significantly different plant growth than control (insecticide contaminated soils without organic remediation) (Table 2). Plant height, the number of leaves, plant fresh weight, and root length are higher than the control treatment. In addition, chlorpyrifos insecticide residues in the soil were reduced due to applying the crude secondary metabolites of *T. harzianum* T213 (Table 1).

The effect of Trichoderma harzianum *T213 sec. met application on the plant height difference*

The difference in plant height is calculated from the plant height of the last observation and the plant height at the initial observation. The results in Table 2 showed treatment of secondary metabolites of *T. harzianum* T213 affected maize plant height difference. The treatment of crude secondary metabolites of *T. harzianum* T213 results in a significantly higher plant height difference than the control. It shows that the treatment of *T. harzianum* T213 crude secondary metabolites increases the difference height of maize plants indirectly and can degrade insecticide residues on chlorpyrifos insecticide-contaminated soil. Insecticide-contaminated soil in control treatment did not support plant growth. The degradation process of chlorpyrifos releases chlorine atoms into the soil, inhibiting plant growth. Chlorine is a micronutrient needed for plant growth, but only in very small amounts (Geilfus 2018). Chlorine may kill bacteria, so excessive amounts can adversely affect soil bacteria that benefit plants. An excessive amount of chlorine can also directly damage the roots of plants (Lee et al. 2019).

The application of 150% crude secondary metabolites of *T. harzianum* T213 increased maize plant height difference by 30.19% higher than other dosages (Table 2), but not significantly different from the application of 50% and 100% crude secondary metabolites of *T. harzianum* T213; however, it differed from the control. It is assumed that the application of crude secondary metabolites in the soil increases the absorption of nutrients in plants, thereby increasing plant height (Halifu et al. 2019). The remedied soil turns into healthy soils that can support plant growth. The crude secondary metabolites of *Trichoderma* species promote nutrient uptake by organic acids to dissolve minerals and activate nutrients in the soils (Li et al. 2015), which leads to the circulation and utilization of nutrients in the soil. *Trichoderma* is attracted by the chemical signals released by the roots of plants. Trichoderma's secondary metabolites are absorbed by the roots and distributed to all plant parts. These secondary metabolites contain extracellular enzymes such as sucrase, urease, phosphatase, and organic acids to improve soil nutrient cycling and enzyme activity (Gianfreda 2015; Adetunji et al. 2017). Plants with sufficient nutrient intake from healthy soils can grow optimally (Berkhout et al. 2019). Soil contaminated with insecticide residues reduces nutrient availability (Meena et al. 2020), so plants cannot grow properly. Therefore, remediated soils using secondary metabolites of *T. harzianum* T213 enhance the supply of nutrients. Healthy soil can support optimum plant growth (Sood et al. 2020).

Number of leaves

The application of crude secondary metabolites of *T. harzianum* T213 significantly affected the number of leaves. However, the number of leaves at the application of 50, 100, and 150% crude secondary metabolites did not differ (Table 2). Therefore, the increase in the number of leaves might be indirectly due to the influence of *T. harzianum* T213 crude secondary metabolites. The increase in the number of leaves on contaminated soil remedied with *T. harzianum* T213 secondary metabolites indicates that the soil is healthy so that plants can grow well. Healthy soil provides sufficient minerals and active nutrients in the soil (Li et al. 2015; Adetunji et al. 2017). Application of *Trichoderma* spp. secondary metabolites promote morphological characteristics of plants such as the length of root shoot, biomass, height, and the number of leaves, shoots, branches, and fruits (Halifu et al. 2019).

Fresh weight of maize plant

Based on Table 2, the application of crude secondary metabolites of *T. harzianum* T213 on chlorpyrifos contaminated soils affected the growth of maize plants. The application of 150% crude secondary metabolites of *T. harzianum* T213 results in the highest fresh weight of maize plants by 45%. However, it was not significantly different from the application of 100% and 50% crude secondary metabolites of *T. harzianum* T213. It is assumed that the application of *T. harzianum* T213 crude secondary metabolites can indirectly increase the ability of roots to absorb nutrients. Therefore, the growth of maize plants on remedied contaminated soil with chlorpyrifos insecticide increases. Remediated contaminated soil with chlorpyrifos residue using the crude secondary metabolites of *T. harzianum* T213 increases nutrient absorption by plants (Halifu et al. 2019). The absorption of nutrients by plants is accelerated by the formation of root hairs (Jones and Dolan 2012) so that they absorb more nutrients and increase the rate of photosynthesis (Jungk 2001). It improves plant metabolism and affects fresh-weight plants.

Root length

The application of 150% crude secondary metabolites *T. harzianum* T213 results in the highest root length of maize plants by 45%. But it was not significantly different from the other application of crude secondary metabolites at 100 and 50% (Table 2). Higher maize hair root growth improves the absorption of nutrients in the soil to increase plant growth. Root development can grow optimally if sufficient nutrients are available. Plant roots do not develop optimally in soil contaminated with insecticide residues (Jing et al. 2017), so plants cannot grow well. The application of *T. harzianum* T213 crude secondary metabolites increases the growth of plants (Halifu et al. 2019) and roots by producing growth regulators. The application of *T. harzianum* also increases the absorption of active minerals and other nutrients from the soil (Li et al. 2015; Aishwarya et al. 2020). *Trichoderma harzianum* stimulates plant growth due to the production of hormones such as gibberellic acid (GA3), indoleacetic acid (IAA), and benzylaminopurin (BAP) (Brenner 2003; Jaroszuk-Ściseł et al. 2019). The secondary metabolites of *T. harzianum* increase plant growth and development, mainly root length. The secondary metabolites of *T. harzianum* perform as an organic agent and decompose natural matter, which can boost the breakdown so that soil fertility will constantly be maintained.

In conclusion, the application of *T. harzianum* T213 crude secondary metabolites on remediated insecticide-contaminated soil effectively increased plant growth, including plant height, the number of leaves, plant fresh weight, and root length compared to control. In addition, the highest reduction of chlorpyrifos residue was obtained at the treatment of 100% crude secondary metabolites of *T. harzianum* T213 (99.54%) followed by 50 and 150% application that reduces chlorpyrifos residue by 99.39 and 99.14%.

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