

# APPLICATION OF RAW SECONDARY METABOLITES FROM TWO ISOLATES OF *Trichoderma harzianum* AGAINST ANTHRACNOSE ON RED CHILI PEPPER IN THE FIELD

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## ABSTRACT

*Application of raw secondary metabolites from two isolates of Trichoderma harzianum against anthracnose on red chili pepper in the field.* Anthracnose on red chili pepper is one of the highly damaging diseases that difficult to control by pesticides. This study aimed to determine the effect of raw secondary metabolites isolated from two isolates of *Trichoderma harzianum* towards anthracnose as well as the growth and yield of red chili pepper plants in the field. The research used a randomized block design with five treatments and seven replicates. The treatments tested were control, raw secondary metabolites of *T. harzianum* T10 and T213, and their combination. The observed variables were incubation period, disease intensity, the late population density of *T. harzianum*, plant height, root length, fresh and dry weight of the plant and root, flowering time, number of fruits per plant, fresh weight of fruit, and phenolic compound content analysis. The result showed that the raw secondary metabolites from the combination of the two *T. harzianum* isolates were the best treatments that could lengthen the incubation period and decrease the disease intensity as 30.2 and 87.05%, respectively. However, these applications could not increase the growth components; while for the yield components, the application could improve the number of fruits per plant and fresh weight of fruit as 15.33 and 34.53%, respectively.

**Key words:** anthracnose, red chili pepper, raw secondary metabolites, *Trichoderma harzianum*

## INTRODUCTION

Red chili pepper (*Capsicum annum* L.) is the horticultural plants that has a high selling value and needed in daily life for Indonesian. The need for red chili pepper is always increase along with the population growth, so that the business opportunity is still promising (Malen *et al.*, 2011). The productivity of red chili pepper in Indonesia has increased from 2010 to 2014. In 2013 the productivity of red chili pepper had reached 8,16 tons/ha and became 8,37 tons/ha in 2014 (BPS, 2015).

One of the major concerns in the production of red chili pepper is anthracnose (patek, local name). According to Pakdeevaporn *et al.* (2005) and Semangun (2007), anthracnose caused by *Colletotrichum capsici* (Syd.) EJ Butler & Bisby. The yield loss due to anthracnose could reach 60% or more (Pakdeevaporn *et al.*, 2005), it can destroy crops up to 90% especially during the rainy season (Agromedia, 2007). Typical anthracnose symptoms on red chili pepper were sunken necrotic tissues, with concentric rings of acervuli. The visible symptoms on

red chili pepper could decrease its selling point (Manandhar *et al.*, 1995).

The effective disease management strategy of anthracnose on red chili pepper is ongoing research; however the commercial cultivars that are resistant to anthracnose have not yet been developed (Than *et al.*, 2008). The alternative control to overcome anthracnose infection was by utilizing microorganisms or using antagonistic agents, which known to inhibit the development of pathogens (Aktar *et al.*, 2009).

Two isolates of *Trichoderma harzianum*, the ginger and shallot isolates (Soesanto *et al.*, 2011) were isolated and identified to be used in this study. Latifah *et al.* (2011) reported that the treatment of shallot isolate before the inoculation of *Fusarium* sp. showed the effect on growth with the number of roots reaching 192.33 or an increasing of 57.45% than the control. While the treatment of ginger isolates before inoculation showed the effect on root length reached 26.29 cm or increased by 17.67% compared to control.

The use of raw secondary metabolites isolated from *T. harzianum* is one of the alternative that can be

used to overcome anthracnose on red chili pepper (Soesanto *et al.*, 2015). This study aimed to understand the effect of raw secondary metabolites application from two *T. harzianum* isolates, both single and combined to anthracnose as well as their effects on the growth and yield of red chili pepper plants in the field.

## MATERIALS AND METHODS

**Research Site.** This research was carried out in a red chili pepper field with altitude 225 m above sea level and ultisol soil type in Banteran village, Sumbang, Banyumas. The study was conducted from September to December 2014.

**Preparation of *T. harzianum* Isolates.** Two *T. harzianum* isolates used in this study were *T. harzianum* T10 isolated from the ginger rhizosphere (Soesanto *et al.*, 2005) and *T. harzianum* T213 isolated from the shallot rhizosphere (Santoso *et al.*, 2007). The isolates were cultured on the potato dextrose agar (PDA) and propagated in broken maize grains and chicken manure medium. Propagation in broken maize grains medium was conducted by adding two cork borrers (6 mm in diameter) of *T. harzianum* culture to 50 g of broken maize grains medium, then incubated at room temperature. While propagation in chicken manure was conducted by adding 50 g of *T. harzianum* T10 and T213 culture in broken maize grains medium each to 1 kg of chicken manure medium, then stirred and incubated in a bucket under closed conditions (Soesanto *et al.*, 2014).

**Preparation of *Colletotrichum capsici*.** *Colletotrichum capsici* was isolated and identified from red chili pepper showing anthracnose symptom. The *C. capsici* was cultured on the PDA and incubated for seven days at room temperature (Waller *et al.*, 2001). The conidia then harvested by adding 10 mL of sterile water in the culture and the density was calculated to  $10^6$  conidium/mL using haemocytometer (Herwidyarti *et al.*, 2013). Spraying was done one day before the treatment of raw secondary metabolites.

**Preparation of *T. harzianum* Raw Secondary Metabolites.** Raw Secondary Metabolites of *T. harzianum* were isolated by using a mix medium of rice water and coconut water in a ratio of 8 : 2, then added with 10 g/L sugar (Soesanto *et al.*, 2014). The medium then boiled, filtered, and sterilized using autoclave at 121 °C for 30 min. After the medium was reached the room temperature, two mycelia plug of each

isolates were added separately, then shaken using orbital shaker for seven days at room temperature. The population density of *T. harzianum* was calculated using haemocytometer to determine the ability of raw secondary metabolites produced. Furthermore, the solution of *T. harzianum* was filtered using Whatman filter paper 42 to separate the fungal propagules.

**Preparation of Red Chili Pepper Seedlings.** The seeds of Astina F1 variety (Panah Merah) were grown in seedling media filled with 100 g of soil inside a polybag. After the red chili pepper seedlings were 14 days old, the NPK fertilizer was given around 2 g per polybag and 30-day-old seeds ready to be transplanted.

**Land Preparation.** The land used was 110 m<sup>2</sup> with 10 beds measuring 1 × 10 m. The distances between beds were 1 m and the height of beds were 50 cm. Spacing used were 50 × 50 cm. The beds were covered with black silver plastic mulch, perforated with a planting hole diameter of 10 cm. Before transplanting, the application were carried out according to the specified treatment. The use of each *T. harzianum* isolate in chicken manure medium was 50 g/planting hole as basic fertilizer according to treatment. After being treated, the seeds were planted in each planting hole according to treatment.

**Experimental Design.** The study was conducted based on a randomized block design consisting of four treatments: (1) control (sterile water), (2) raw secondary metabolite of *T. harzianum* T10, (3) raw secondary metabolite of *T. harzianum* T213, (4) combined raw secondary metabolites of *T. harzianum* T10 and T213. Each treatment was repeated seven times. Each treatment consisted of six plants, so that 168 experimental plants were used.

**Application of *T. harzianum* Raw Secondary Metabolites in the Field.** The application was conducted by spraying the raw secondary metabolites once a week starting since the flowering of red chili pepper plants. Each *T. harzianum* isolate and its combination were given 50 mL/plants by spraying it on the flower and all plants. Furthermore, the culture of *T. harzianum* was sprayed 5 mL/plants using handsprayer that on the flowered and fruited plants.

**Observation.** The observed variables on this study were the pathosystem component, plant growth, yield, and tissue analysis. The pathosystem component were consisted of incubation period and disease intensity, while

the growth component consisted of plant height, root length, fresh plant weight, fresh root weight, dry plant weight, and dry root weight. The yield component consists of flowering time, number of fruits per plant, and fresh fruit weight, while tissue analysis consists of tannins, saponins, and glycosides (Chairul, 2003). The intensity of disease was calculated by the following formula:

$$DI = \frac{\sum(n \times v)}{Z \times N} \times 100\%$$

DI = intensity of anthracnose

n = number of damaged fruits in each attack category

v = the value of each attack category

Z = highest damage category value

N = number of observed fruits

The scale value of anthracnose was based on the scale of spots on red pepper attacked by pathogen as follows (Herwidarti *et al.*, 2013):

0 = healthy fruit

1 = Spots on red pepper 1–20%

2 = Spots on red pepper 21–40%

3 = Spots on red pepper 41–60%

4 = Spots on red pepper > 60%

The tannins test was identified by reacting  $\text{FeCl}_3$  to extract 10 g of fruit with 80% ethanol, and given 5 drops of 1% NaCl solution then was observed the level of color change compared to control.

The saponins test is froth test using merak as a control. Procedures for testing were 2 mL of plant extract was put into a test tube then 10 mL of water was added, covered, and shaken for 30 sec, then left for 30 min. Observations were made on the height of the froth formed compared to control.

The glycosides test using Keller Kiliani reagent was extracts of plant material (fruit) were dried with a water bath, then washed with hexane and added 3 mL of  $\text{FeCl}_3$  reagent, stirred and dropped 1 mL of concentrated sulfuric acid solution, then left for a

minutes until the color changes. Observations were made on the level of color density compared to control. Observation of the level of sensitivity and high froth were qualitatively written with the symbol + (a little), ++ (rather a lot), and +++ (a lot).

**Data Analysis.** Data were analyzed by anova and followed by Duncan's multiple range test (DMRT) at 5%.

## RESULTS AND DISCUSSION

**Pathosystem Component.** Based on the statistical analysis, the treatment of *T. harzianum* raw secondary metabolites had a significant effect on the disease incubation period and intensity in red chili pepper compared to control (Table 1). In this case, the treatment of *T. harzianum* raw secondary metabolites namely T10, T213 or a combination could delay the incubation period.

The increase of incubation period by *T. harzianum* T10 was 23.1%, while *T. harzianum* T213 and the combination were 29.2% and 30.2% compared to controls. This was due to the many complexities contained in raw secondary metabolism that can inhibit the growth of pathogens. Some of these compounds are the chitinase enzyme and  $\beta$ -1,3-glucanase. This is consistent with Sulistiyono's research (2014), that all *T. harzianum* isolates tested produced chitinase,  $\beta$ -1,3-glucanase, protease, and cellulase enzymes among 0.171–0.335; 8.868–10.256; 9.738–12.952 and 5.504–5.924 U/mL, respectively.

Elad *et al.* (1982) showed that isolates of *T. harzianum* were found differently in the level of the hydrolysis enzyme produced. Glucanase activity increases to 67% when fungi were grown on a mixture of laminarin and glucose (3:1, v/v). Kumar *et al.* (2012) also reported that the activities of chitinase and  $\beta$ -1,3-glucanase were found in all *Trichoderma* isolates in the growing medium. *T. viride* was noted to have the highest chitinase activity while *T. harzianum* had the

Table 1. Treatments of *T. harzianum* raw secondary metabolites on the pathosystem component

Treatments	Incubation period (DAI)	Disease intensity (%)	The effectivities of disease intensity (%)
Control	41.53 b	15.99 a	-
<i>T. harzianum</i> T10	54.00 a	3.08 b	80.74
<i>T. harzianum</i> T213	59.26 a	2.91 b	81.80
Combination of <i>T. harzianum</i> T10 and T213	59.46 a	2.07 b	87.05

The numbers in the same column followed by the same letters are not significantly different based on DMRT at the 5% level; DAI: day after inoculation.

highest  $\beta$ -1,3-glucanase activity. The most important thing is *Trichoderma* spp. has the ability of antagonists to be efficiently higher by secreting lysis enzymes in the form of chitinase and  $\beta$ -1,3-glucanase extra-cells. The lysis enzyme breaks down the polysaccharide cell wall into short oligomers and facilitates hyperparasite for penetration into the cytoplasm of the target fungal pathogen (de la Cruz *et al.*, 1995; Viterbo *et al.*, 2002; Markovich & Kononova, 2003).

Based on Table 1, the raw secondary metabolites of two *T. harzianum* isolates namely T10 and T213 could increase the incubation period compared to controls. In this case proved that the combination of two or more antagonistic isolates gave better results when applied in the field than control. This is consistent with Thangavelu & Gopi's (2015) that the combined application of endophytic isolates and the rhizosphere of *Trichoderma* sp. could suppress Fusarium wilt and improve the growth of banana plants in the greenhouse. Sandheep *et al.* (2013) also reported that the combination of *T. harzianum* and *Pseudomonas fluorescens* could increase the growth of vanilla plants better than a single application.

Data on disease intensity (Table 1) showed that there were a decrease in anthracnose intensity in red chili pepper due to the treatment of the raw secondary metabolite *T. harzianum*. The combined treatment of *T. harzianum* was able to reduce the disease intensity of anthracnose up to 87.05% compared to control. This condition was in line with an increase in the incubation period (Table 1), which was thought because of more complete ability of the two *T. harzianum* raw secondary metabolites isolates, both by their own mechanism and by increasing plant resistance.

**Component of Growth.** Based on statistical analysis, it showed that the components of plant growth treated by *T. harzianum* T10 showed results that were not different compared to control. This indicated that the treatment had no significant effect on plant growth; while the application of *T. harzianum* T213 and the combination showed a negative effect on plant growth components. This can be seen from the significant decrease in plant growth components after being treated with T213 and their combination (Table 2). According to Vinale *et al.* (2012), raw secondary metabolites of *Trichoderma* at low concentrations had optimal activity as auxin which can stimulate growth, whereas at high doses it can act as an inhibitor.

Application of *T. harzianum* raw secondary metabolites can suppress anthracnose development on red pepper, but had not been able to increase growth

Table 2. The effect of *T. harzianum* raw secondary metabolites application towards anthracnose disease on the red chili pepper growth factors

Treatments	Plant height (cm)	Root length (cm)	Fresh plant weight (g)	Fresh root weight (g)	Dry plant weight (g)	Dry root weight (g)
Control	37.09 a	17.18 a	29.08 ab	4.08 a	6.19 a	1.06 a
<i>T. harzianum</i> T10	35.53 a	15.98 a	31.93 a	4.67 a	6.98 a	1.22 a
<i>T. harzianum</i> T213	29.28 ab	14.06 ab	19.11 bc	2.92 b	3.83 b	0.74 b
Combination of <i>T. harzianum</i> T10 and T213	25.98 b	13.80 b	16.63 c	2.79 b	3.97 b	0.79 b

The numbers in the same column followed by the same letters are not significantly different based on DMRT at the 5% level.

components (plant height, root length, fresh plant weight, dry plant weight, fresh root weight, and dry root weight) compared to control (Table 2). This is presumably due to the lack of growth hormone content in the raw secondary metabolite of *T. harzianum*.

The ability of the raw secondary metabolite *T. harzianum* is highly dependent on the *T. harzianum* in nature. This is in accordance with Saravanakumar *et al.* (2017), that *Trichoderma* strains vary based on environmental impacts, host-pathogen specificity and stability. *Trichoderma* spp. reported can produce IAA hormones and indole compounds such as indole-3-ethanol, indole-3-acetaldehyde, and indole-3-carboxaldehyde as part of its metabolism (Contreras-Cornejo *et al.*, 2009; Contreras-Cornejo *et al.*, 2011). It was further reported by Contreras-Cornejo *et al.* (2016), that some *Trichoderma* strains impacted root branching and increased plant canopy biomass as a result of cell division and expansion due to the presence of tub-auxin fungal compounds. According to Heddy (1986), the presence of auxin was greatly influenced by the presence of sunlight. The presence of sunlight with high intensity can inhibit and damage the work of auxin. Vandebussche *et al.* (2003) reported that the decrease in light intensity along with an increase in hormone production including ethylene.

**Component of Yields.** Based on Table 3, the application of *T. harzianum* raw secondary metabolites could increase the yield of red pepper. Although the flowering time of red chili pepper treated with *T. harzianum* raw secondary metabolites was longer compared to control, but the treatment of *T. harzianum* raw secondary metabolites could increase the number of fruits per plant and fresh fruit weight.

Combined treatment of *T. harzianum* T10 and T213 raw secondary metabolites showed a longer flowering time compared to control and a single treatment. This was consistent with Table 2 that the inability of *T. harzianum* isolates produced the cytokinin

hormone that affected flowering. In addition, the presence of high sunlight intensity in the field can affect hormone production including flowering hormones (Heddy, 1986).

Although the flowering time was longer, the application of *T. harzianum* raw secondary metabolites, both single and combined could increase the number of fruits per plant and the fresh weight of fruit (Table 3). The highest increase in number of fruits in the treatment of *T. harzianum* T10 was 26.2% compared to controls. Meanwhile, the highest increase in fresh fruit weight in combined treatment was 34.54% compared to controls

This proved that the raw secondary metabolites from combination of two *T. harzianum* isolates could increase the yield of red chili pepper which was in line with the highest decrease in disease intensity (Table 1). In this case, it was thought because of the lack of anthracnose pathogens in red pepper plants due to the application of the *T. harzianum* raw secondary metabolite.

The results of this study were supported by reports that *T. harzianum* was conventionally able to control several plant diseases, including rhizome rot in ginger (Amalia *et al.*, 2004; Soesanto *et al.*, 2005), leaf blight on rice (Susilo *et al.*, 2005; Waluyo *et al.*, 2005), rhizome rot in aromatic ginger (Prabowo *et al.*, 2006), Fusarium wilt in chili (Utami *et al.*, 2019), moler in red onion (Santoso *et al.*, 2007), and Fusarium wilt in gladiolus corms (Wardhana *et al.*, 2009). In addition, the application of *T. harzianum* raw secondary metabolites can directly influence plant pathogens (Vinale *et al.*, 2008) or indirectly on plants mediated by increased plant nutrition (Shoresh & Harman, 2008).

**Qualitative Tissue Analysis.** Based on the results of a qualitative tissue analysis (Table 4), the application of *T. harzianum* raw secondary metabolites can increase the content of phenol compounds in the red pepper plant tissue. Almost all treatments of *T. harzianum* raw secondary metabolites, both single and combined isolates

Table 3. The effect of *T. harzianum* raw secondary metabolites application towards anthracnose on the red chili pepper yields

Treatments	Flowering time (day)	Number of fruits per plant	Fresh weight of fruit (g)
Kontrol	28.00 a	3.81 bc	23.71 bc
<i>T. harzianum</i> T10	30.56 ab	5.23 a	34.70 ab
<i>T. harzianum</i> T213	31.72 ab	3.98 abc	25.53 abc
Combination of <i>T. harzianum</i> T10 and T213	33.93 b	4.50 ab	36.22 a

The numbers in the same column followed by the same letters are not significantly different based on DMRT at the 5% level.

Table 4. The content of phenol qualitatively red pepper plants in testing the raw secondary metabolite *T. harzianum* to control anthracnose

Treatments	Glycosides	Saponins	Tannins
Controls	++	+	++
<i>T. harzianum</i> T10	++	+++	++
<i>T. harzianum</i> T213	+++	+++	+
Combination of <i>T. harzianum</i> T10 and T213	++	++	+++

+ = a little, ++ = rather a lot, dan +++ = a lot.

were able to increase the content of phenol compounds qualitatively compared to controls. This is in line with the results of anthracnose intensity (Table 1).

The increase in phenol compounds in red pepper plants qualitatively will affect the increase in plant resistance to pathogen attack. Phenol compounds in plants play a role in the mechanism of plant resistance to disease. If phenol compounds is high, then the plant has a high resistance to disease infection. The low content of tannins in the treatment of *T. harzianum* T213 was thought to have the presence of compounds that decomposed tannins. According to DeRito & Madsen (2009), some *Trichoderma* spp. was able to degrade phenol compounds. In general, all treatments had ability to increase total phenol compounds (combined glycosides, saponins, and tannins). This is in line with Nawrocka *et al.* (2018), that the application of *T. atroviride* showed a remarkable increase in the concentration of 23 phenols which included hydroxybenzoic acid, cinnamic acid, catechin, flavonol, flavone, and flavanone. Brotman *et al.* (2012) and Contreras-Cornejo *et al.* (2016) stated that *Trichoderma* spp. can control the roots of mono- and dicotyledonous plants, which can cause real changes in plant metabolism, hormone content, soluble sugars, phenol compounds and amino acids.

Phenol compounds act as defense when stressed to the environment, such as light, low temperatures, pathogen infections, herbivores, and nutrient deficiency, which can cause increased production of free radicals and other oxidative species in plants (Lattanzio, 2013). Lin *et al.* (2016) said that phenol compounds can act as antioxidants, structural polymers (lignin), attractants (flavonoids and carotenoids), UV protectors (flavonoids), signal compounds (salicylic acid, flavonoids), and defense compounds (tannins, phytoalexin). Vallad & Goodman (2004) also said that raw secondary metabolite, such as alkaloids, phenols, flavonoids, glycosides, and phytoalexins are toxic and inhibit the growth of pathogens so that can affect plant resistance. This is in line with Wachjadi *et al.* (2013),

that phenol compounds in plants was directly related to the level of plant resistance to disease infection. Soesanto & Rahayuniati (2009) also stated that application of *T. koningii*, *T. harzianum* and *Gliocladium virens* could increase the glycoside, tannin and saponin in banana seedlings qualitatively.

## CONCLUSION

Raw secondary metabolites from the combination of the two *T. harzianum* isolates were the best treatments for controlling anthracnose on red pepper, which was shown by a longer incubation period and reduced disease intensity by 30.2 and 87.05%, respectively. However, the application of raw secondary metabolites had not been able to increase the growth component; while for the yield component, the treatments were able to increase the number of fruits per plant and fresh weight of fruit respectively by 15.33 and 34.54%.

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