



## Application of *Trichoderma harzianum* T10 Liquid Formula Based on Soybean Flour Against Cucumber Seedlings Damping-Off (*Pythium* sp.)

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### ABSTRACT

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(Proper concentration of soybean flour in the liquid formulation for *Trichoderma harzianum* T10 growth, was investigated to improve suppression efficiency on damping-off and growth of cucumber seedlings. A randomized completely design was used in *in vitro* test with five treatments and five replications. The treatments were *T. harzianum* T10 cultured in Potato Dextrose Broth (control), in liquid information based on soybean flour at concentration 1, 2, 3 or 4%. A randomized block design was used in *in planta* test with six treatments and five replications. Variables observed were conidia density, incubation period, disease incidence, area under the disease progress curve (AUDPC), maximum potentially growth, germination percentage, crop height, root fresh weight, shoot fresh weight, and root length. The results of the experiment showed that the best liquid formulation for *T. harzianum* T10 growth was the use of soybean flour at concentration of 2% resulting in conidia density as 67,10% compared to PDB. The formulation could suppress the disease incidence, decreased AUDPC value, increased root fresh weight, shoot fresh weight, and root length as 67%, 66%, 57%, 44%, and 42%, respectively, as compared to those in control.

### INTRODUCTION

Cucumber (*Cucumis sativus* L.) is a plant that produces fruit, which can be eaten both fresh and further processed (Andrie *et al.*, 2015). Cucumber plants can be cultivated in temperate and tropical environments. Moreover, it is said that cucumber is to be native to many regions of the world (Mortimore, 2015).

Based on data from the Central Statistics Agency (2017), the development of cucumber production in Indonesia in 2013 reached 491,636 tons / year, but in 2014-2016 the production of cucumber plants decreased respectively,

namely 477,989, 447,696 and 430,218 tons/year. One of the causes of the decline is due to of disease in cucumber plants. According to Sutton *et al.* (2006), barriers in cucumber cultivation include damping-off, which often harms cucumber planters. Efforts to control plant pathogens have been carried out, both with the use of resistant plants although not always available (Koike *et al.*, 2000; Weller *et al.*, 2002) or with synthetic pesticides; but the impact is not good for the environment or humans (Aktar *et al.*, 2009; Zaidi *et al.*, 2011).

One alternative to anticipate these impacts is through biological control, by utilizing biological

control agents (Shafique *et al.*, 2016). One of the biological agents is *Trichoderma* sp. that has been widely used to control plant pathogenic microbes (Kumar *et al.*, 2017). *Trichoderma* sp. has antagonistic properties to pathogens in the form of space and nutrition competition, mycoparasites and antibiosis (Benítez *et al.*, 2005).

Biofungicide *Trichoderma* sp. is widely developed in the form of solid substrates to be applied in the field, but liquid form might be more efficient. Solid substrate form is still not practical and efficient for large plantation scale, so it needs to be formulated according to the needs in the field (Nurhidayati *et al.*, 2015), such as liquid form. The formulation aims to facilitate application, transportation, determination of concentration, production costs, storage capability, environmental conditions, and efficacy (Gašić and Tanović, 2013).

Growth of *Trichoderma* sp. require food ingredients, such as carbohydrates as an energy source during their growth and development (Urailal *et al.*, 2012). Soybean flour is a semi-finished material that can be used as a carrier and as an ingredient that can enrich nutrients such as carbohydrates (Gozalli, 2015). The use of soy flour as the main ingredient in making liquid formulas for *T. harzianum* propagation is still very rarely done.

The research objective was to determine the right concentration of soy flour in the preparation of liquid formula for the growth of *T. harzianum* T10, its effect on suppression of seedling diseases, and on the growth of cucumber plants.

## MATERIALS AND METHODS

### Preparation of *T. harzianum* T10

*T. harzianum* T10 isolates (Soesanto *et al.*, 2019b) were propagated on the PDA medium aseptically and incubated for 5-7 days. Subsequently the fungus was propagated on broken corn medium and incubated at room temperature for 10-12 days (Heydari and Pessarakli, 2010).

### Preparation of *Pythium* sp.

*Pythium* sp. was isolated from symptomatic chilli seedlings. The symptomatic plant was removed and the affected part was cut and then

surface sterilized and grown on a PDA. Following the growth of the fungi, they were microscopically observed then purified and propagated into a new PDA and incubated at room temperature for 2-5 days (Sutton *et al.*, 2006).

### Preparation of liquid media based on soybean flour and Potato Dextrose Broth (PDB)

Liquid medium was made to five treatments. The control treatment used PDB, the soy flour treatment with 10, 20, 30, and 40 g of soy flour with each concentration treatment added 10 g/L of sugar in 1 liter of water. Flour liquid medium was made by mixing soy flour and sugar according to treatment, then heating it until it boils (Soesanto *et al.*, 2015). Medium PDB was made by peeling potatoes and weighed 200 g, followed by boiled in 1000 mL water. The boiled water was put into a beaker and the volume was returned to 1000 mL. After that, 20 grams of dextrose was put into a beaker and cooked again until it boiled. Each medium, as much as 100 mL that has been made was put into a 140 mL bottle, covered with aluminum foil and then coated with paper and tied using a rubber band. All mediums were then sterilized using an autoclave at 121°C, 15 psi pressure for 30 minutes.

### Development formula

The liquid medium of soy flour and sterile PDB was added with 1 mL *T. harzianum* T10 with a density of  $10^6$  conidia mL<sup>-1</sup>. Furthermore, each treatment was shaken with a 150 rpm orbital shaker at room temperature for 7 days (Soesanto *et al.*, 2019a).

### In vitro test

*In vitro* testing was carried out using randomized completely design with five treatments and five replicates so that there were 25 experimental units. The treatments consisted of *T. harzianum* T10 in PDB, in liquid formula containing 1, 2, 3, and 4% of soy flour.

### In planta experiment

*In planta* testing was carried out using randomized block design with six treatments

and five replicates so that there were 30 experimental units, each with four potted plants, so that there were 120 experimental pots. The treatments consisted of control, *T. harzianum* T10 in PDB, in liquid formula of soy flour 1, 2, 3, and 4% soy flour. The experiment was carried out by applying liquid formula to plants in the experimental pot. The planting media in the polybag was made a planting hole, placed one cork drill (12.7 mm diameter) culture of *Pythium* sp. then covered with thin soil and on top of it was placed cucumber seeds (Inácio *et al.*, 2017). A total of 10 mL *T. harzianum* T10 with a density of  $10^6$  conidia  $\text{mL}^{-1}$  was poured after the cucumber seeds were thinly covered with soil medium and manure (Soesanto *et al.*, 2019a).

### Variable observed

Observed in this study were conidia density, pathosystem components (incubation period, disease incidence, and Area Under Disease Progress Curve (AUDPC) values, and cucumber growth components (maximum growth potential, germination capacity, plant height, roots fresh weight, shoot fresh weight, and root length).

Conidia density was calculated using a microscope with a magnification of 100 x on the haemocytometer. The calculation was repeated four times and the density was calculated using formula:  $S = [(t \times d)/(b \times 0.25)] \times 10^6$ , where S = number of conidia per gram of media culture, t = the number of conidia calculated on the count media, d = degree of dilution, n = the number of small boxes observed (i.e.  $5 \times 16 = 80$  small boxes), and 0,25 = correction factor (Akagi *et al.*, 2015).

The incidence of lodging diseases in cucumber were calculated using the following equation (Noordzij *et al.* (2010), :  $DI = [b/N] \times 100\%$ . Note: DI = percentage of shoot disease (%), n = number of plants affected by *Pythium* sp., and N = number of plants observed.

Effectiveness of disease suppression was calculated to determine the ability of treatment in suppressing seedling disease compared to those in untreated control. The formula for calculating the effectiveness of disease control, according to Alves *et al.* (2015), was:

$$\text{Effectiveness} = \frac{X_{\text{control}} - X_{\text{treatment}}}{X_{\text{control}}} \times 100\%$$

Note:  $X_{\text{treatment}}$  = DI on treatment and  $X_{\text{control}}$  = DI on control.

The rate of disease progression over time was calculated using the AUDPC formula as follows (Jeger and Viljanen-Rollinson, 2001):

where: AUDPC: Disease progression curve (% -days), Y = severity of disease at time t, i = number of days after planting, time of observation to i, n = total number of measurements.

### Data analysis

Observed data were analyzed for variance (F test) at an error level of 5%. If there were significant differences, then the Honesty Significant Difference (HSD) test was further applied at an error level of 5%.

$$\text{AUDPC} = \sum_{i=0}^{n-1} \left( \frac{Y_i + Y_{i+1}}{2} \right) \cdot (t_{i+1} - t_i)$$

## RESULTS AND DISCUSSION

### Population density of *T. harzianum* T10 in soy flour liquid formula

Based on the results of the statistical analysis in Table 1, the best concentration for growth and development of *T. harzianum* T10 is at 2% soy flour concentration. These results are shown from the observation of the amount of *T. harzianum* T10 conidiat 2% flour concentration increased by 67.10% compared to PDB.

The liquid media used in the propagation of *T. harzianum* T10 was based on soy flour (GASOL), which contains carbohydrates of 6.85 g carbohydrates and 7.22 g protein . The nutritional content in soy flour is supportive for the growth and development of *T. harzianum* T10. This is in accordance to the opinion of Zehra *et al.* (2017), that it is important to consider various parameters affecting their growth and sporulation when planning the application

Table 1. Conidia density of *T. harzianum* T10

Treatment	Conidia density $\times 10^7$ conidia mL <sup>-1</sup>
PDB	3.26 a
Soy flour 1%	5.36 ab
Soy flour 2%	9.91 b
Soy flour 3%	5.66 ab
Soy flour 4%	7.94 ab

Note: Numbers followed by different letters show significant differences in Tukey test with an error rate of 5%. Data were analyzed after they were transformed to  $\sqrt{x}$

of antagonistic *Trichoderma* strains for the purpose of biological control. Uruilal et al. (2012) revealed that the growth of *Trichoderma* sp. was strongly influenced by carbohydrate content, such as monosaccharides, disaccharides and polysaccharides used as energy sources for growth.

High or low number of *T. harzianum* T10 conidia was influenced nutrient content in the form of carbohydrates and cellulose as a food source (Gusnawaty et al., 2017). The amount of conidia in the liquid formulation of soy flour 2% concentration was higher than the concentration of soybean flour 3 and 4%. At concentrations of 3 and 4% liquid formula, soy flour was very thick, so that it will affect the solubility of oxygen as an energy source in the growth and development of *T. harzianum* T 10 in liquid formulas when shaking. *T. harzianum* produces conidia during its growth and development, so that if oxygen is sufficient, then the amount of conidium produced is also great. Said (2010) states that stirring or shaking the liquid medium can increase oxygen concentration in the medium; the faster stirring speed, the higher oxygen concentration in the culture medium is .

The amount of conidia in the liquid formula 2% is more density than the 3 and 4% concentration. It is also suspected because the water in the media is bound by soy flour, so there is no available water for *T. harzianum* T10 growth. This statement is supported by Kredics et al. (2004) that, *T. harzianum* requires water in the available form on for mycelial growth and extracellular enzyme systems. Napitupulu et al. (2013), claimed that

soy flour is one of the binders that can increase the binding capacity of water in food ingredients because in soy flour there are starches and proteins that can bind water.

### Application of *T. harzianum* T10 liquid formula on pathosystem components

#### Incubation period

The analysis results in Table 2 show that all treatments were not significantly different in term of the incubation period. The absence of a significant effect of the given treatment is thought to be caused by environmental conditions such as temperatures that support the development of the fungus *Pythium* sp. so the the duration from inoculation of pathogens until symptoms appear relatively uniform.

Table 2. The effect of treatments on pathosystem components

Treatments	Incubation period (dai)	Disease incidence (%)	AUDPC value (%-days)
Control	4.6 a	90 a	1,180
PDB	3.6 a	65 ab	965
Soy flour 1%	5.2 a	50 ab	700
Soy flour 2%	6.0 a	30 b	400
Soy flour 3%	3.6 a	40 b	510
Soy flour 4%	3.8 a	40 b	530

Note: Numbers followed by the same letter in the same column show no significant difference in HSD test with an error level of 5%. The incubation period data was transformed  $\sqrt{x}$  and the disease incidence data was transformed in  $\arcsin \sqrt{\%}$  . dai = days after inoculation.

Characterising the incubation period of a pathogen-host system relies on the ability to determine and relate the relative development of host infectiousness and pathology influenced by host age or environmental variables (Leclerc et al., 2014). The temperature in the experimental field when conducting the research was 28°C. According to Nzungize et al (2012), *Pythium* can thrive at temperatures of 25-36°C. In addition, the different incubation periods are thought to be caused by the lack of application of *T. harzianum* T10 raw secondary metabolites (produced from density of  $10^6$  conidia mL<sup>-1</sup>), although it appears in the Table 2 of application that the raw secondary metabolites symptoms appear longer than control. This is consistent with the

results of the study that the two highest densities ( $10^8$  and  $10^{10}$  spore/g soil) of both *T. harzianum* and *T. viride* showed persistent and significant ( $P \leq 0.05$ ) effects (Al-Hazmi and TariqJaveed, 2016).

#### *Disease incidence*

Liquid formula of *T. harzianum* T10 significantly affected *Phytium* incidence (Table 2). Concentration of 2% soy flour in the liquid formula was the best concentration in reducing the disease incidence by 66.67% as compared to control. These results might indicate that the *T. harzianum* T10 raw secondary metabolites is able to control seedling disease in cucumber. This indication is based that under soy flour 2% formula, the number of *T. harzianum* spores produced is very large, i.e., 67.10% compared to those under PDB, so that the raw secondary metabolites of *T. harzianum* T10 produced to suppress the development of pathogens are also numerous. According to Vinale *et al.* (2014), raw secondary metabolites from *T. harzianum* in the form of antibiotics, enzymes, hormones and toxins can inhibit the development of pathogens in plant tissues, with various mechanisms. The enzymes produced include protease, cellulase, selobiase, 1,3- $\beta$ -glucanase and chitinase. Low concentrated enzymes diffuse into the pathogenic fungal cell walls and damage the pathogenic fungal cell walls. Chitinase enzyme will hydrolyze  $\beta$ -1,4 bonds in chitin and chitin oligomer. Chitin is an important component of cell walls of some pathogenic fungi. Köhl *et al.* (2019) added that the enzyme produced by *T. harzianum* was able to dissolve the pathogenic fungal cell walls.

#### **The value of the area under the development of pathogenic curve curve (AUDPC)**

The lower the AUDPC value, the more effective the treatment is in controlling plant pathogens (Hanudin *et al.*, 2013). Based on the Table 2, cucumber treated with *T. harzianum* T10 had lower AUDPC values as compared to those in control. The AUDPC value on the control was 1,180 %-days for  $\pm 3$  weeks, whereas the lowest AUDPC value was at the treatment of raw secondary metabolites in the liquid formula of soy flour 2% as 400 %-days for  $\pm 3$  weeks or decreasing 66,10% the value as compared to those in control. The low AUDPC values in all treatments compared to

controls might be mainly because the raw secondary metabolites *T. harzianum* T10 can reduce the incidence of *Pythium* seedling disease in cucumber. *T. harzianum* produced in the form of antibiotics, enzymes, hormones and toxins (Vinale *et al.*, 2014). Central to the biocontrol activity of *Trichoderma* spp. is the production of secondary metabolites, which are natural compounds that aid the producing organism in survival and basic functions such as symbiosis, competition and differentiation (Shwab and Keller, 2008).

#### **Application of *T. harzianum* T10 liquid formula on plant growth components Maximum growth potential, germination ability, and crop height**

The results showed that all treatments did not significantly affect germination, maximum growth potential, and crop height. The treatment given did not affect the germination of cucumber though it showed that PDB had a germination rate lower than other soy flour treatments except the soy flour concentration of 3%.

This is presumably because the seeds planted have good vigor. Good vigor is supported because of good aeration in control and treatment plants so that given raw secondary metabolites do not have a significant effect. These results are in line with the opinion of Finch-Savage and Bassel (2016) explained that seeds that are able to grow normally, even though natural conditions are not optimum are called seeds that have good vigor. In addition, *Trichoderma* sp. produced growth hormones. According to Jaroszuk-Ścisiel *et al.* (2019), *Trichoderma* sp. synthesized not only IAA and gibberellic acid (GA) but also the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCD) enzyme regulating ethylene production by plant in the liquid culture. It is suggested that hormones produced by *T. harzianum* T10 is low influenced by its conidia density. Phytohormone compounds produced must be in a very high microbial population density, the environmental factors influences the speed of growth, whereas antagonistic microbes grow faster in artificial media than in soil media (Egamberdieva *et al.*, 2017).

Table 3. The effect of treatments on cucumber growth components

Treatments	Maximum growth potential (%)	Germination ability (%)	Crop height (cm)	Root fresh weight (g)	Crop fresh weight (g)	Root length (cm)
Control	95 a	95 a	10.74 a	0.55 a	3.54 a	11.30 a
PDB	100 a	90 a	12.92 a	0.74 ab	4.55 ab	13.79 ab
Soy flour 1%	95 a	100 a	14.18 a	1.13 ab	6.19 ab	16.96 ab
Soy flour 2%	100 a	100 a	14.80 a	1.29 b	6.30 b	19.42 b
Soy flour 3%	95 a	95 a	13.75 a	0.75 ab	4.57 ab	15.29 ab
Soy flour 4%	100 a	100 a	13.65 a	0.99 ab	5.64 ab	16.80 ab

Note: Numbers followed by the same letter in the same column show no significant difference in HSD with an error level of 5%.

### Root and crop fresh weight, and root length

Liquid formula significantly increased root fresh weight, canopy fresh weight, and root length (Table 3). The liquid formula of 2% soy flour was the best formulation, increasing root fresh weight by 57.36%, canopy fresh weight by 43.81%, and root length 41.81% as compared to control. The increase may be due mainly to interactions between the raw secondary metabolites of *T. harzianum* T10 and plants. According to Jaroszuk-Ścisiel *et al.* (2019), *Trichoderma* sp. can produce active substances such as the auxin hormone which can stimulate the formation of lateral roots. Baihaqi *et al.* (2013) said that good root growth will support the plant's need for more nutrients, making it possible to increase the number of root and crop fresh weight. *Trichoderma* spp. significantly increased plant height (<0.001), leaf number (0.001), root length (0.007), root fresh weight (<0.001) and root dry weight (<0.001) compared to the untreated plants (control) on mustard growth (*Brassica juncea* L.) (Roslee *et al.*, 2017). It is supported by Azarmi *et al.* (2011) that seed germination rate was not affected by *Trichoderma* application, but shoot height, shoot diameter, shoot fresh and dry weight and root fresh and dry weight in tomato seedlings were interestingly ( $p \leq 0.05$ ) increased when sown in *Trichoderma* sp. T and *T. harzianum* T969 fortified soil and when compared to the control.

### CONCLUSION

The right concentration of soy flour in

making liquid formula for the growth of *T. harzianum* T10 is 2% indicated by the amount of *T. harzianum* conidia, 67.10% greater than those in PDB. *T. harzianum* T10 in the liquid formula containing 2% soybean flour suppressed the disease incidence and decrease AUDPC value at 66.67% and 66.10%, respectively, as compared to those in control. In addition, *T. harzianum* 10 in those liquid formula increased root fresh weight, crown fresh weight, and root length by 57.36%, 43.81%, and 41.81%, respectively, as compared to those control plants.

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