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Evaluation of Effervescent Tablet Formulation of *Trichoderma harzianum* Raw Secondary Metabolites Toward Fusarium Wilt on Pepper

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ABSTRACT

Fusarium oxysporum f.sp *capsici*, which causes chili-fusarium wilt disease, may be regulated by a secondary metabolite of *Trichoderma harzainum*. Effervescent tablets are used because liquid formulations have some drawbacks. The purpose of this study is to qualitatively determine *T. harzianum*'s best crude secondary metabolites, growth and yield, and phenolic compound content in chili crops in foamed tablet formulations against *F. oxysporum in vitro*. The *in vitro* study used 6 replicates, a completely randomized design, and 4 treatments consisted of controls and 4, 6, and 8 tablets. Under in vivo conditions, the experiment used a randomized block designs with 4 replicates, eight treatments consisting of controls, fungicides (benomyl), and four, six, or eight tablets per day before or after inoculation. The variables observed were antagonist testing, incubation time, disease intensity, disease incidence, AUDPC, germination rate, plant height, root fresh weight, and qualitative phenolic composition. The results of the study showed that the best dose of *T. harzianum*'s crude secondary metabolite in vitro was 4 tablets. Medications in in-plant studies delayed the incubation period by 64.11%, suppressed disease outbreaks by 58.34%, reduced disease intensity by 80.45%, increased plant height by 50.4%, and harvested phenols (saponins, tannins, hydroquinone). The content of the compound has been qualitatively increased.

INTRODUCTION

Chili (*Capsicum annuum* L.) is widely cultivated crop. Production and harvested area of chili from 2016 to 2020 continued to increase. The average national chili productivity has only reached 3.76 %, while the potential for chili production can reach 10.9 t/ha. It can be assumed that chili production can still be increased up to 20.12% of the potential production (Rizaty, 2021). The high production of chili is usually constrained by limited land, bad weather, and pest attacks. The reduction of chili production of chili was due to different pathogens, e.g., fungi, viruses, bacteria, and nematodes (Parisi, Alioto, & Tripodi, 2020). One of the pathogenic fungi that reduces chili production is *Fusarium oxysporum* f.sp *capsici*, which causes fusarium wilt. Fusarium wilt in chili can reduce production by up to 100 percent, especially if the disease occurs when the plants are still in the vegetative stage (Shafique, Asif, & Shafique, 2015).

Fusarium wilt in chili is generally controlled with synthetic fungicides such as carbendazim (Bashir et al., 2018). The unwise use of pesticides causes the emergence of pest resistance to pesticides and the death of natural enemies (Hasyim, Setiawati, & Lukman, 2015). In addition, the continuous and unwise use of pesticides can cause soil and water pollution (Bisht & Chauhan, 2020), health problems for farmers and consumers (Damalas & Koutroubas, 2016; Nicolopoulou-Stamati, Maipas, Kotampasi, Stamatis, & Hens, 2016), reduce soil fertility (Aktar, Sengupta, & Chowdhury, 2009),

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and increase production costs (Gagic, Holding, Venables, Hulthen, & Schellhorn, 2021). Therefore, to overcome chili disease, alternative control that is safe and environmentally friendly is needed.

An alternative control that is environmentally friendly is using the biological agent of the antagonist fungus *Trichoderma harzianum*. *T. harzianum* is a soil-borne antagonist fungus that is widely used to treat plant diseases (Puyam, 2016; Srivastava et al., 2015), included fusarium wilt (Barari, 2016; Perveen & Bokhari, 2012). This fungus is capable of producing secondary metabolites (Vinale et al., 2014). Applications of *T. harzianum* are generally still based on the conidia produced (Mulatu, Alemu, Megersa, & Vetukuri, 2021). The use of conidium which is applied in the field often encounters biotic and abiotic factors (Köhl, Kolnaar, & Ravensberg, 2019).

The use of antagonistic fungi in the form of formulas has begun to be developed. For example, the liquid formula (Soesanto, Solikhah, Mugiastuti, & Suharti, 2020; Tyśkiewicz, Nowak, Ozimek, & Jaroszuk-Ściseł, 2022). The liquid formula has several drawbacks including: when exposed to direct sunlight (Lahlali et al., 2022), it affects the levels of secondary metabolites, and leads to less effective and interferes with the delivery process, but solid formulations in the form of tablets are still rarely found (Köhl, Kolnaar, & Ravensberg, 2019). Based on this, it is necessary to manufacture a solid formula in the form of effervescent tablets (Patel & Siddaiah, 2018).

The objective of this study was to determine the best raw secondary metabolites of *T. harzianum* in effervescent tablet formulation towards *F. oxysporum in vitro*, growth and yield of chili *in planta*, and content of phenolic compounds qualitatively in chili crop due to the treatment.

MATERIALS AND METHODS

Exploration and Identification of *F. oxysporum* f.sp. *capsici*

The fungus *F. oxysporum* f.sp. *capsici* was obtained from chili plant samples at Gandatapa Village, Sumbang District, Banyumas Regency. Sampling was taken on May 2019 based on plants affected by fusarium wilt then the sample was isolated, purified, and identified macroscopically (color or mycelium growth) and microscopically (false head, macroconidia, or microconidia) according to Leslie & Summerell (2006).

Preparation of Raw Secondary Metabolites

Pure culture of *T. harzianum* that had grown in PDA was drilled with cork, put the 20 cork drill bits in 450 ml of PDB, then shaken for 2 weeks at speed of 150 rpm in room temperature (Hudson, Waliullah, Ji, & Ali, 2021). The next step was the calculation of the conidia density into 10^8 conidia m/l. The conidia was then centrifuged at high speed of 9000 rpm for 2 minutes to separate the conidia from secondary metabolites (Shehata, Badr, El Sohaimy, Asker, & Awad, 2019).

Preparation of Effervescent Tablets

Ingredients were weighed based on the specified ingredient formulation. The ingredients consisted of 45 g of Na bicarbonate and 3 g of citric acid, 112.5 g of sucrose, 1 g of gelatin and 150 ml of raw secondary metabolite from *T. harzianum*. The mixture was then separated dried, mixed all the ingredients, re-drying and tablet printing (Srinath et al., 2011).

Antagonist Inhibition Testing (*In Vitro*)

The test was done on PDA using a completely randomized design, with four treatments, namely control, 4 tablets, 6 tablets and 8 tablets of the secondary metabolite *T. harzianum* and repeated six times. Filter paper with a diameter of 0.5 cm which had been sterilized by autoclave was put into a suspension of effervescent tablets in each treatment for 15 minutes and then planted in a Petridish containing PDA. *F. oxysporum* f.sp. *capsici* was taken out using a 0.5 cm diameter cork borer. The pathogen isolate was placed in the middle while the filter paper was placed around the pathogen at the same distance (Passera et al., 2019).

In Planta Test of Secondary Metabolite Tablets of *T. harzianum*

Randomized block design was used in the test of raw secondary metabolites from *T. harzianum* in effervescent tablets and fungicides as a comparison. The treatment consisted of control and 4, 6, and 8 tablets a day before or after inoculation, and benomyl. Chili seedlings were transplanted after 2 weeks sowing, then the roots were injured and soaked in Fusarium suspension. When transplanting, the application was carried out according to the treatment, on the 1st, 2nd, and 3rd applications, the plants were treated with 50 ml of raw secondary metabolite tablets that had been dissolved in 1 L of water per plant, the 4th and 5th

applications were 100 ml and 6th as much as 150 ml. Applications were made every 5 days.

Variables and Measurements

Antagonistic test *in vitro*. Pathogen growth was recorded and growth inhibition was calculated based on the formula 1 by Vincent (1947).

$$GI = \frac{R1-R2}{R1} \text{ x } 100\ \% \quad \text{.....1)}$$

Where: GI = growth inhibition (%), R1 = growth of pathogenic radius that does not lead to raw secondary metabolites of *T. harzianum*, and R2 = growth of pathogenic radius that leads to raw secondary metabolites of *T. harzianum*.

Germination ability. Germination ability was calculated with formula 2 by Gairola, Nautiyal, & Dwivedi (2011).

$$GA = \frac{\text{Numbers of seeds germinated}}{\textit{Numbers of total seeds}} \text{ x } 100\% \quad \text{.....2)}$$

Incubation period; disease incidence calculated with formula 3.

$$D = a / (a + b) \text{ x } 100\% \quad \text{.....3)}$$

Where: D = Disease incidence (%), a = Number of plants affected by the disease, and b = Number of healthy plants.

The intensity of fusarium wilt in chili plants was calculated using formula 3 by Wongpia & Lomthaisong (2010).

$$DI = \frac{\sum(n\ x\ v)}{Z\ x\ N} \text{ x } 100\% \quad \text{.....3)}$$

Where: DI = disease intensity (%), n = number of plants with disease symptoms with a certain scale, v = value of the measurement results of the unit of observation, Z = highest numerical value in the category of damage, N = number of plants. The fusarium wilt intensity scale of chili according to Wongpia & Lomthaisong (2010), which is: 0 = no wilting symptoms, 1 = slightly stunting compared to control, 2 = slightly stunted and chlorosis (yellowing of the leaves), 3 = 10% leaf chlorosis or 10 % of wilted plants, 4 = 11-25% of wilted plants, 5 = 26-50% of wilted plants, 6 = 51-100% withered or dead plants.

AUDPC calculations were carried out to determine the relationship between disease intensity and time. AUDPC was calculated using formula 4 (Paraschivu, Cotuna, & Paraschivu, 2013):

$$\text{AUDPC} = \sum_{i}^{n-1}\left[\frac{y_i + y_{i+1}}{2}\right](t_{i+1} - t_i) \quad \text{.....4)}$$

Where: AUDPC = Disease progression curve, y_{i+1} = i+1 observation data, y_i = 1st observation data, t_{i+1} = i+1 observation time, t_i = 1st observation time.

The effect of effervescent tablets on plant growth included plant height, crop fresh weight, and root fresh weight.

The effect of effervescent tablets on the content of phenolic compounds in plants which included saponins, tannins and hydroquinones. These was quantitavely measured according to Fahrunnida & Pratiwi (2015), Rahmania, Herpandi, & Rozirwan (2018), and Simamora, Basyuni, & Lisnawita (2021).

Data Analysis

Observational data were analyzed using a descriptive approach and data of growth inhibition, germination ability, pathosystem and growth components were analyzed using analysis of variance (ANOVA) at an error rate of 5%. If there is a significant and very significant difference, it is continued with the DMRT (Duncan's Multiple Range Test) at an error level of 5% using DSAASTAT.

RESULTS AND DISCUSSION

Growth Inhibition

Growth inhibition of Fusarium by antagonists under *in vitro* condition (Table 1) showed that the effervescent tablets with concentrations of 4, 6, and 8 tablets affected differently from the control. This was presumably because all concentrations contained antibiotic compounds and had the same inhibitory ability in inhibiting the growth of *F. oxysporum* f. sp. *capsici*. In accordance with the opinion of Živković et al. (2010) that *T. harzianum* produce antibiotic compounds, which will enter pathogenic cells and cause mycolysis. Supported by Keswani, Mishra, Sarma, Singh, & Singh (2014) and Khan, Najeeb, Hussain, Xie, & Li (2020) that *Trichoderma* sp. produce beneficial diverse activities of secondary metabolites to agriculture.

Table 1. Inhibition test of *F. oxysporum* f. sp. *capsici* and germination ability of chili seeds caused by *T. harzianum* raw secondary metabolites tablets formulated

Treatments	Growth inhibition (%)	Germination ability (%)
Control	0 a	96.67 a
4 tablets	51.85 b	93.33 a
6 tablets	49.99 b	96.67 a
8 tablets	46.00 b	98.33 a

Remarks: Numbers in the same column followed by different letters differ significantly under DMRT (α ≤ 5%)

Germination Ability

Table 1 shows that all treatments were not significantly different from the control. This is presumably because the seeds of Lembang-1 chili are SNI-standard seeds. Based on the results of the germination test, the percentage of germination is quite high, because it is above SNI standard (85%) (Waluyo, 2016). This is reinforced by the opinion of Kildisheva et al. (2020), who said that high-quality seeds had a viability of more than 90 percent. All treatments of effervescent tablets were not significantly different. This is presumably due to the influence of secondary metabolites in the form of the hormone auxin. This is in accordance with the opinion of Shuai et al. (2016), auxin will break seed dormancy and will stimulate the seed germination process.

Effect of *T. harzianum* Secondary Metabolites Effervescent Tablets Formulation on Pathosystem Components

Incubation period of all secondary metabolites and fungicide treatments (Table 2) had a significant effect compared to control. The control treatment developed disease more quickly, while the treated plants began to be attacked an average 3 19 days after planting or could delay as 61.14% compared to control.

The treatment of effervescent tablets could delay the period of incubation. All plants treated with effervescent tablets and fungicides had a significant effect on suppressing disease incidence, when compared to controls. This shows that effervescent tablets have almost the same ability as fungicides. The delay is thought to be due to the content of secondary metabolites. In accordance with the opinion of Noronha & Ulhoa (1996), that *Trichoderma* sp. known as fungi that can produce 1,3-β glucanase. This enzyme can degrade and hydrolyze the mycelium cell wall of plant pathogenic fungi during the mycoparasite process, thus play a role in the defense mechanism against pathogens. Supported by Cuervo-Parra, Ramírez-Suero, Sánchez-López, & Ramírez-Lepe (2011), that *T. harzianum* VSL291 is capable of producing lytic enzymes, namely 1,3-glucanases, chitinases, proteases, xylanases to inhibit pathogens.

In line with the incubation period, the treatment that had the highest suppression of disease incidence was 4 tablets compared to others (Table 3). This is presumably due to the large carbohydrate content contained in effervescent tablets 6 and 8, so that *F. oxysporum* f.sp. *capsici* get a source of energy. In accordance with the opinion of de Oliveira Costa & Nahas (2012) that the high carbohydrate content in the media plays an important role in the fungal metabolism process. This statement is supported by the opinion of Reischke, Rousk, & Bååth (2014) that hyphae will absorb simple molecules such as glucose directly.

Table 3 also shows that all plants treated with effervescent tablets and fungicides had a significant effect on suppressing disease intensity. Treatment of 4, 6, and 8 tablets a day before or after inoculation, and benomyl gave suppression of 77.14, 77.14, 60, 65.7, 60, 74.29, and 77.14%, respectively, but among the treatments were not significantly different. This indicates that the effervescent tablets had the ability equivalent to fungicides in suppressing the growth of *F. oxysporum* f. sp. *capsici* and support plant growth. This is presumably due to the fungus *T. harzianum* has the ability of antagonistic mechanisms against pathogens and can affect plant resistance so that it can reduce disease intensity. According to the opinion of Köhl, Kolnaar, & Ravensberg (2019), that protection by secondary metabolites of biological control agents is from within the plant and plays important role of increasing phenolic compounds in plants (Table 3), which functions in plant resistance to pest attack. Khatri, Tiwari, & Bariya (2017) and Loc, Huy, Quang, Lan, & Ha (2020) added that the chitinase and endokinase enzymes in *Trichoderma* sp. is an enzyme that has the highest lytic and antifungal activity compared to other types of chitinase enzymes.

The results of the AUDPC variable (Table 2 showed significant differences in treatment of 4, 6, and 8 tablets a day before or after inoculation, and benomyl which had average values of 24.30, 20.83, 34.73, 27.77, 36.46, 30.38, and 1.15%, respectively, lower than the control. The high and low intensity of the disease that occurs affects the AUDPC value. The intensity of the disease that continues to increase makes the AUDPC value also increase. This statement is in accordance with Jeger & Viljanen-Rollinsen (2001), that the AUDPC value indicates the accumulated value of disease severity for several weeks of observation. The higher the severity of the disease would make higher AUDPC value.

Table 2. Effect of effervescent tablet formulated secondary metabolites of *T. harzianum* on pathosystem components

Treatments	Incubation Period (dai)	Disease Intensity (%)	Disease Incidence (%)	AUDPC (%-days)
Control	7.5 a	48.61 a	100 a	145.83 a
4 tablets, before	20.92 bc	11.11 b	41.66 b	24.30 b
4 tablets, after	20.9 bc	11.11 b	58.33 ab	20.83 b
6 tablets, before	20 b	19.44 b	75.00 ab	34.73 b
6 tablets, after	21.25 bc	16.67 b	58.33 ab	27.77 b
8 tablets, before	19.3 b	19.44 b	75.00 ab	36.46 b
8 tablets, after	21.58 bc	12.5 b	58.33 ab	30.38 b
Benomyl	23.92 c	11.11 b	41.67 b	12.15 b

Remarks: dai = days after incubation; Numbers in the same column followed by different letters differ significantly under DMRT ($\alpha \leq 5\%$)

Table 3. Effect of secondary metabolites effervescent tablets formulated of *T. harzianum* on chili growth components

Treatments	Crop Height (cm)	Crop Fresh Weight (g)	Root Fresh Weight (g)
Control	11.16 a	0.07749 a	0.01416 a
4 tablets, before	22.50 b	0.14167 a	0.01833 a
4 tablets, after	23.25 b	0.11609 a	0.01792 a
6 tablets, before	18.83 b	0.08267 a	0.02042 a
6 tablets, after	18.75 b	0.07675 a	0.01458 a
8 tablets, before	18.16 b	0.07291 a	0.01375 a
8 tablets, after	20.33 b	0.09166 a	0.01500 a
Benomyl	22.41 b	0.12918 a	0.01709 a

Remarks: Numbers in the same column followed by different letters differ significantly under DMRT ($\alpha \leq 5\%$)

Table 4. Results of tissue analysis qualitatively

Treatments	Saponins	Tannins	Hydroquinons
Control	+	+	+
4 tablets, before inoculation	+ + +	+ + +	+ +
4 tablets, after inoculation	+ +	+ +	+ +
6 tablets, before inoculation	+ + +	+ +	+
6 tablets, after inoculation	+ +	+ + +	+ + +
8 tablets, before inoculation	+ + +	+ +	+ + +
8 tablets, after inoculation	+ + +	+ + +	+ + +
Benomyl	+	+	+

Remarks: + = a little, ++ = enough, and +++ = a lot.

Effect of Effervescent Tablets Formulation Containing *T. harzianum* Secondary Metabolites on Chili Growth

The the treatment of effervescent tablets and fungicides (Table 3) had an effect on increasing plant height. The application of 4, 6, and 8 tablets a day before or after inoculation, and benomyl gave an average plant height of 22.5; 23,25; 18.83; 18.75; 18.16; 20.33; and 22.41 cm, respectively. While, in control treatment, it is only 11.16 cm.

This is presumably because the secondary metabolites of *T. harzianum* contain auxin hormone (IAA) which functions to stimulate growth, especially in plant height, so that cell enlargement, cell division, and tissue differentiation can be stimulated. In accordance with the opinion of Halifu, Deng, Song, & Song (2019) and Zin & Badaluddin (2020), that *Trichoderma* sp. has the ability to produce the hormone auxin which in turn, the substance can speed up plant growth and development, thus the plant can produce healthy roots and increase root depth (deeper below the soil surface).

The fresh weight of crop crowns and roots (Table 3) were not significantly different between the plants treated and the control, this was presumably due to the high intensity of rain so that the secondary metabolites and nutrients in the soil in the root area were not available to plants and increase humidity. According to the opinion of Yao, Dai, Gao, Gan, & Yi (2021), that the increase in rainfall can result in an increased risk of surface runoff and leaching of nutrients and soil particles. Losses due to surface runoff and leaching can cause biological agents and secondary metabolites to be carried to deeper soil layers or elsewhere.

Effect of Effervescent Tablets Formulation Containing *T. harzianum* Secondary Metabolites on Phenolic Compound Content Qualitatively

The treatment of effervescent tablets gave more positive reaction to the presence of phenolic compounds in the tested plants (Table 4).

The effervescent tablets gave more positive reaction to the presence of saponins in the tested plants viewed from the foam produced more than the control treatment (Table 4). Saponins are glycosides, which are secondary metabolites found in some plants. Saponins work by disrupting the integrity of pathogenic cells causing lysis of pathogenic cells and ultimately causing cell death (Podolak, Galanty, & Sobolewska, 2010). Saponins generally will cause foam when mixed with water (Faizal & Geelen, 2013). Supproted by Kharkwal et al. (2012) that saponins are foam glycosides containing low molecular weight phytochemicals containing either tetracyclic steroids or pentacyclic triterpenoid aglycones with one or more sugar chains.

From the tannins test (Table 4), the treatments of 4, 6, and 8 tablets had the highest tannins content compared to the control reflected from the formation of blackish brown color in the tested plant samples. These results are in line with the pathosystem components (Table 2). Decreasing disease intensity, disease incidence, and AUDPC value are not only caused by the raw secondary metabolites but by inducing resistance systemically of the crops as well because of phenolic compound production (Soesanto, Mugiastuti, Suyanto, & Rahayuniati, 2020). Tannins have an important role in cultivated plants, such as against microbial pathogens and harmful insects (Carvalho et al., 2018).

Table 4 indicated that the treatment of 6 tablets a day after inoculation, and 8 tablets a day before or after inoculation had the highest content of hydroquinone compounds compared to the control. Ma et al. (2019) stated that hydroquinone is a compound which contains phenols and has antibacterial and antifungal properties. Hydroquinone could degrade the bacterial cell wall and membrane, increase permeability, cause leakage of intracellular substance affect synthesis of protein, and influence expression of genes (Jeyanthi, Velusamy, Kumar, & Kiruba, 2021; Ma et al., 2019).

CONCLUSION

The highest level of inhibition to *Fusarium oxysporum* f.sp *capsici in vitro* was found at the four effervescent tablets containing raw secondary metabolites of *T. harzianum* by 51.85%. Under planta test, the best effervescent tablet dosage for growth was 4 tablets. This dosage can prolong the period of incubation by 64.11%, decrease the incidence of fusarium wilt by 58.34%, reduce the intensity of the disease by 80.45%, improve plant height by 50.4%, and increase the content of saponins, tannins, and hydroquenone compounds qualitatively in plants compared to control.

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