INTRODUCTION

Soil-borne pathogen is one of the most devastating pathogens on mungbean seedling. Several phytopathogenic fungi such as Rhizoctonia solani, Sclerotium rolfsii, Fusarium sp., and Phytophthora sp., have been reported associating with damping-off, root rot, and wilting on mungbean crops in Indonesia (Rahayu, 2016). R. solani becomes an important pathogen in legumes crop due to its aggressiveness and was able to devastate soybean plantation in swampland in South Kalimantan (Rahayu, 2014), and infected more than 20% of mungbean germplasm collections cultivated in dry season 2014. The control and management of this pathogen are very difficult since R. solani has high survival capability (Veena, Priya, Raheesa, & Divya, 2014), a broad range of hosts (Zheng et al., 2013), and genetically different groups which are sensitive to different pesticides (Carling, Baird, Gitaitis, Brainard, & Kuninaga, 2002).

There are only a few reports about the successful controls of R. solani using chemical fungicides in Indonesia (Djunaedy, 2008; Muis, 2007). Biological agents are increasingly considered to reduce the negative effects of intensive fungicide applications to the environment (Meena, Swapnil, Zehra, Aamir, et al., 2017). However, the effectiveness of bio-control agents often lowers than the chemical fungicides. The use of integrated bio-control agents such as Trichoderma sp. with some...
bactericides (oxytetracycline and streptomycin sulphate or agrimycin) can be expected to increase its effectiveness to control pathogens (Soesanto et al., 2018). *Trichoderma* sp. has been known for its great potency as promising bio-control agents to suppress the growth of soil-borne pathogens including *R. solani* due to its ideal characters such as fast-growing ability, wide adaptation to various types of soil and temperature range, good root colonizer, and survival ability on extreme conditions (Mohiddin, Khan, Khan, & Bhat, 2010; Zehra, Dubey, Meena, & Upadhyay, 2017). Moreover, *Trichoderma* sp. has various action modes directly and indirectly in controlling pathogenic fungi (Nakkeeran, Vinodkumar, Priyanka, & Renukadevi, 2018). *Trichoderma* suppress pathogen growth through competition, antibiosis, production of cellulases and other hydrolytic enzymes (i.e proteases, β-1,3 glucanases, and chitinase), and mycoparasitism. *Trichoderma* sp. is also able indirectly to suppress pathogens by inducing plant resistance and promote plant growth (Nakkeeran, Vinodkumar, Priyanka, & Renukadevi, 2018). Mycoparasitism of *Trichoderma* species is a complex process as they involved both mechanical and enzymatically actions (Qualhato et al., 2013; Viterbo & Horwitz, 2010). Mycoparasitism of *Trichoderma* sp. to control *R. solani* is classified into necrotrophic mycoparasites where the parasite highly depends on saprophytic growth and caused destructive effects (Mukherjee et al., 2012).

Initially, *Trichoderma* recognizes its host by signals producing by the pathogens, one of which is the production of lectin (Monfil & Casas-Flores, 2014). Mycoparasite then penetrates the host cell wall which can be both through physical damaging and enzymatic processes. Direct penetration through the formation of special structures such as appressoria or papillae-like structure has been recognized as the most common form of growth suppression from *Trichoderma* species besides the formation of hooked branches and coiling around host hyphae (Viterbo & Horwitz, 2010). Those processes are influenced by the production of hydrolytic enzymes which helped the host cell wall degradation and the production of antibiotics (Mukherjee et al., 2012). Chitin and β-1,3 glucan are the main compounds of fungal cell wall including *R. solani*. During mycoparasitism, *Trichoderma* use cellulase, chitinase, and glucanases for the enzymatic process (Monfil & Casas-Flores, 2014; Vipul et al., 2015). Other enzymes such as chitinases, β-1,3 glucanases, β-1,6-glucanases, α-1,3-glucanases, and proteases were also reported having strong antifungal activities against plant pathogens (Contreras-Cornejo, Macias-Rodriguez, Herrera-Estrella, & Lopez-Bucio, 2014; Wu et al., 2017). The ability to produce hydrolytic enzymes is a crucial property for mycoparasitic fungi. Therefore, an efficient hydrolytic strain is necessary to be explored for developing a promising bio-control agent to control soil-borne pathogens.

Recently, many studies have been focused on the development of new approaches for controlling the pathogens by utilizing local or indigenous microorganisms commonly found. *T. virens* is one of the most common species found around the rhizosphere of various crops in East Java besides *T. asperellum* and *T. harzianum* (data not shown). *T. virens* has been studied intensively for its potential biological control (Angel et al., 2016; Angel, Sundram, Ping, Yusof, & Ismail, 2018) and mycoparasitism has been reported as the most common strategies to control pathogen (Strakowska, Blaszczyk, & Chelkowski, 2014). *T. virens* also reported induced systemic resistance that effective against a broad range of pathogens (Wang, Borrego, Kenerley, & Kolomiets, 2020). Our previous study showed that seven indigenous *T. virens* strains from forty indigenous *Trichoderma* isolates were effective to suppress the growth of different soil-borne pathogens such as *Rhizoctonia solani* (R.s1), *R. solani* (R.s2), and *Fusarium* sp. on dual culture assay (Yusnawan, Inayati, & Baliadi, 2019). The volatile organic compounds produced by indigenous *T. virens* also inhibited the growth of *R. solani* through its antifungal activity ([Inayati, Sulistyowati, Aini, & Yusnawan, 2019]. During the study, there was variation in inhibition capability among *T. virens* strains against a certain pathogen, for example, strains Tv6 showed high antagonist activity on dual culture assay, however not showed growth inhibition on *R. solani* growth on double plate assay indicating a specific action mode from *T. virens* strains on their mycoparasitism. Therefore, this study aimed to identify potential mycoparasitism of indigenous *T. virens* against *R. solani* through their physical and chemical processes as a basis for further studies of selected indigenous *T. virens* as a promising bio-control agent on mungbean.
MATERIALS AND METHODS

The research was conducted at the Centre laboratory of Indonesian Legumes and Tuber Crops Research Institute (ILETRI), Malang from August to November 2018. All T. virens strains and R. solani isolates were obtained from ILETRI’s culture collection as isolated by Yusnawan, Inayati, & Baliadi (2019).

Inhibition Growth Test on Dual Culture and Culture Filtrate Assay

Dual culture assay was carried out using a method performed by F. Zhang et al. (2016). A 5 mm disk of T. virens and R. solani were transferred to PDA media and placed in the opposite position, then incubated for 5 days. R. solani grown on PDA without Trichoderma was used as the control. The inhibition percentage was calculated by the following formula:

\[
\% I = \left(1 - \frac{R_2}{R_1}\right) \times 100
\]

Where: I, R1, and R2 are growth inhibition, radial growth of pathogen without Trichoderma, and radial growth of pathogen challenged with Trichoderma, respectively.

Growth inhibition of R. solani by non-volatile metabolites on fungal filtrate produced by T. virens was carried out using the protocol described by You et al. (2016). Two mycelial disks (5 mm) taken from active-growing colonies were inoculated in a flask containing 100 ml potato dextrose broth (PDB) and then incubated for 7 days. Filtrate for the antagonistic test was prepared by filtering the Trichoderma supernatant through Whatman filter paper and then incubated on a water bath at 60°C for 30 minutes before unsolidified PDA was added at the ratio of v/v. Pure PDB was added to PDA at the same ratio, also prepared as the control. A mycelial block of R. solani was then inoculated on PDA plates for 5 days. The percentage of pathogen growth inhibition was calculated using the formula:

\[
\% I = \left(\frac{C-R}{C}\right) \times 100
\]

Where: C and R are the growth radius of R. solani in the control treatment and growth radius of R. solani in media supplemented with T. virens filtrate, respectively.

Enzyme Activity Assay

For the production of cellulases, T. virens was cultured on the same medium for the plate assay without agar. The filtrate from liquid culture was used as a crude extract. The CMC-ase activity was determined by measuring the amount of reducing sugar liberated from CMC using 3,5-dinitro salicylic acid (DNS) method (Potprommanee et al., 2017). The reaction mixture was prepared by mixing 0.5 ml of crude extract and 0.5 ml of CMC (1%) dissolved in 0.1M phosphate buffer, pH 7.0. The mixture was then incubated at 50°C for 20 minutes and the reaction was stopped by adding 2.5 ml of DNS reagent. The absorbance values were measured at 575 nm. One unit (U) of the enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose per minute.

Scanning Electron Micrograph (SEM) observation was conducted by growing both Trichoderma and R. solani on a thin layer of PDA at microscope slides. After the glass was covered by mycelia of both Trichoderma and pathogen, the object was prepared for SEM observation.

Determination of Hydrolytic Enzyme: Cellulases and Chitinases on Agar Media

The production of cellulase was determined by Congo red plate assay method (Syed, Riyaz-Ul-Hassan, & Johri, 2013; Zehra, Dubey, Meena, & Upadhyay, 2017). T. virens was grown on yeast extract peptone agar medium supplemented with 0.2% and 0.5% (v/v) of carboxymethylcellulase (CMC) and 0.2% congo red. Mycelia of T. virens was cultivated on the center of the plate and incubated for 3 days. The cellulase activity was indicated as a clear zone around the inoculated media after staining with 1% Congo red solution and washing with 1 M NaCl.

Chitinase production was observed according to Agrawal & Kotasthane (2012) with minor modifications. The medium (g/l) consisted of 0.3 g of MgSO4·7H2O, 3.0 g of (NH4)2SO4, 2.0 g of KH2PO4, 1.0 g of citric acid monohydrate, 20 g of agar, 4.5 g of colloidal chitin and 0.15 g of bromocresol purple; pH 4.7. A 5 mm of fresh culture plug of the T. virens was inoculated into the medium and incubated at room temperature. Positive chitinase production will change the color of the medium from yellow to purple.
Chitinase from *T. virens* was produced on liquid media, as proposed by Agrawal & Kotasthane (2012). Chitinase activity was determined using a colorimetric method (Modification from Qualhato et al., 2013). The reaction mixtures contained 1 ml enzyme solution and 1 ml 0.5% colloidal chitin in phosphate buffer saline (0.1 M). After incubation at 37°C for 15 minutes, a 2 ml DNS was added and the reaction was maintained at 100°C for 5 minutes. The amount of reducing sugar was determined at 540 nm. One unit (U) of enzyme activity was determined as the amount of enzyme required to release 1 mmol N-acetylglucosamine in 1 hour at 37°C.

**Statistical Analysis**

Analysis of variance of data from dual culture, filtrate, and hydrolytic enzyme assays was calculated using Microsoft Excel software. Least Significant Difference (LSD) was performed on 0.05 confidence level. The robust analysis of principal component (PCA) was calculated using R-studio statistical environment R version 3.5.3 (RStudio Team, 2015).

### RESULTS AND DISCUSSION

**Hyperparasites Potential of *T. virens***

Competition for space and nutrients were the most common mode of hyperparasitic actions of *Trichoderma* species on dual culture assay. Rapid radial growth of *T. virens* in agar media caused the reduction and inhibition of pathogen growth which was determined by the larger area occupied by the colonies of *Trichoderma* on the plate. Our study showed that all seven strains of *T. virens* had hyperparasitic ability to reduce the growth of *R. solani* starting from 3 days of incubation (doi) with different inhibition capabilities (Table 1). The Tv3 strain showed that the highest inhibition percentage was at 3 doi followed by Tv7 and Tv2. However, at 5 doi, the inhibition growth of Tv3 strain decreased while other strains indicated the increasing pattern, even though Tv3 still performed the strongest inhibition effect. All *T. virens* strains grew over the plates after 5 days of incubation displayed the domination of *T. virens* over *R. solani’s* growth. (Fig. 1). However, Tv6 and Tv7 strains showed the inhibition growth was less than 50% which represented low potential inhibition to control pathogenic fungi of *R. solani*.

### Table 1. Growth inhibition of *R. solani* by *T. virens*

| Strain | Dual culture assay* | | | | Culture filtrate* | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 3 | Day 5 | | | Day 3 | Day 5 | | |
| | 15% | 30% | 40% | 15% | 30% | 40% | | |
| Tv1 | 40.3<sup>bcd</sup> | 51.2<sup>c</sup> | 18.3<sup>cd</sup> | 35.0<sup>b</sup> | 48.8<sup>b</sup> | 22.2<sup>bc</sup> | 29.4<sup>ab</sup> | 30.6<sup>a</sup> |
| Tv2 | 41.6<sup>bc</sup> | 52.6<sup>b</sup> | 28.0<sup>a</sup> | 20.0<sup>d</sup> | 25.0<sup>c</sup> | 29.4<sup>a</sup> | 27.8<sup>b</sup> | 22.2<sup>b</sup> |
| Tv3 | 74.0<sup>a</sup> | 68.6<sup>a</sup> | 15.9<sup>e</sup> | 36.3<sup>b</sup> | 37.5<sup>bc</sup> | 21.1<sup>cd</sup> | 28.9<sup>b</sup> | 28.9<sup>b</sup> |
| Tv4 | 30.2<sup>ad</sup> | 54.1<sup>b</sup> | 26.8<sup>a</sup> | 50.0<sup>ab</sup> | 51.3<sup>e</sup> | 22.2<sup>bc</sup> | 21.7<sup>a</sup> | 23.3<sup>c</sup> |
| Tv5 | 32.5<sup>cde</sup> | 51.1<sup>b</sup> | 26.8<sup>a</sup> | 43.8<sup>ab</sup> | 55.0<sup>e</sup> | 26.1<sup>de</sup> | 21.7<sup>a</sup> | 71.1<sup>a</sup> |
| Tv6 | 31.9<sup>ad</sup> | 43.8<sup>bc</sup> | 20.7<sup>de</sup> | 27.5<sup>e</sup> | 75.0<sup>d</sup> | 15.0<sup>c</sup> | 27.2<sup>a</sup> | 38.3<sup>b</sup> |
| Tv7 | 44.2<sup>b</sup> | 49.6<sup>ab</sup> | 23.2<sup>ab</sup> | 47.5<sup>a</sup> | 48.8<sup>b</sup> | 27.8<sup>ab</sup> | 30.6<sup>a</sup> | 38.3<sup>b</sup> |

Remarks: *Numbers in the same column followed by the same letter are not significantly different based on the LSD test at α = 0.05
Culture filtrate assay showed that all strains had the capacity to suppress pathogen growth. It was observed that culture filtrate at a minimum concentration of 15% (v/v) had an inhibitory effect on pathogen growth. This study showed that there was a positive correlation between the increase of filtrate concentration and pathogen suppression, even though; this trend did not always correlate to the time of exposure (Fig. 2). Tv6 strain showed the most significant growth inhibition at 3 doi at a concentration of 40% (v/v) in which the filtrate could inhibit R. solani growth by up to 75%. However, the inhibitory effect decreased to 38.3% at 5 doi. The decrease of inhibitory effect at 5 doi was also observed on other strains except for Tv5. The Tv5 showed an increase in inhibitory effect from 55% at 3 doi to 71.7% at 5 doi. In contrast to the result from dual culture assay, Tv3 strain showed the lowest inhibition growth on culture filtrate assay and so did Tv2. This result suggested that each strain had an action mode to inhibit the growth of the pathogen.

Trichoderma spp. has been reported in many studies to secrete non-volatile metabolites which diffused into the liquid medium. The metabolites had the ability to inhibit the growth of pathogens. More than 300 non-volatile compounds produced by Trichoderma sp. which had numerous functions including antifungal and antimicrobial activities (Li, Li, & Zhang, 2019; Meena, Swapnil, Zehra, Dubey, & Upadhyay, 2017). At least four toxins have been isolated from T. virens ITC-4777 i.e. gliotoxin, dimethyl gliotoxin, viridian, and viridiol which were effective to control soil-borne pathogens such as R. bataticola, M. phaseolina, Phytium deharyanum, P. aphanidermatum, S. rolfsii, and R. solani (Li, Li, & Zhang, 2019). Other studies also reported that T. harzianum and T. viride produced D-Glucose, 6-O-α-D-galactopyranosyl- and 17-Octadecynoic acid which had antimicrobial activities (Meena, Swapnil, Zehra, Dubey, & Upadhyay, 2017).

Mycoparasitic behavior of T. virens to pathogenic fungi of R. solani was observed at the cellular level which was conducted at 3 doi using an electron microscope. Microscopic inhibitory effects of T. virens on dual culture and culture filtrate assay showed that the exposure of T. virens caused morphological alteration and abnormality of the R. solani hyphae (Fig. 3). The SEM figures showed that the first inter-fungal interaction from the hyperparasitic action was physical contact between T. virens and its host of R. solani. T. virens started growing parallel to the host hyphae continuing with the penetration of knob-like structure from Trichoderma to the host (Fig. 3a). R. solani's hypha was wider compared to that of T. virens which had thin and dense hyphae.
These structures facilitated the penetration process as mentioned by Dennis & Webster (1971) which wider host hyphae are more susceptible and easier to be penetrated by thinner and solid hyperparasite hyphae. T. virens then grew intercellularly inside the host hyphae (Fig. 3b), or tightly coiling of the host (Fig. 3c). Mycoparasitism of T. virens also appeared as the degradation of the cell wall of host mycelia (Fig. 3d), and abnormal growth of the host such as swollen on mycelial tips (Fig. 3e). These processes indicated that necrotrophic mycoparasitism of T. virens was preceded by the destruction of host both intercellular and extracellular. Coiling, lyses and abnormal growth of host hyphae were influenced by direct contact and affected by biochemical stimulus during hyperparasitism. Moreover, many studies proved the presence of hydrolytic enzyme and the production of antibiotics involved in hyperparasitism of Trichoderma sp. (Mukherjee et al., 2012; Smitha, Finosh, Rajesh, & Abraham, 2014; S. Zhang, Gan, & Xu, 2014).

**Determination of Hydrolytic Enzymes Produced by T. virens Strains**

T. virens produced lytic enzymes that could help penetration and inhibition to the host. This current study showed that there were lyses on the mycelial host who was associated with the production of cellulase and other hydrolytic enzymes, namely chitinase. The observation of enzymes related to mycoparasitism in this study was conducted by plate assay representing a qualitative approach and a colorimetric method to examine the activity and the number of enzymes produced by the microorganism.

![Fig. 2. The R. solani growth inhibition by T. virens filtrate (K = control treatment, R. solani without T. virens filtrate) (a) growth inhibition capability of Tv3 strain in different filtrate concentration, from the highest (right) to the lowest (left), (b) growth inhibition capability of Tv4 strain in different filtrate concentration, from the highest (right) to the lowest (left)](image-url)
Cellulase

In vitro assay showed that all *T. virens* strains had the ability to produce cellulase, one of the important enzymes responsible for cell wall degradation. The clearing zone formed around the culture presented cellulase activity from *T. virens* to break down cellulose in the medium (Fig. 4a). The diameter of the clearing zone formed around all strains was relatively small, ranging from 5.83 to 16.67 mm, indicating low cellulase activities of all strains. Two isolates of Tv2 and Tv3 produced relatively stable enzyme activities at two times of examination at 3 days of incubation. Another study conducted by Shahririnour, Abd Wahab, Ariff, & Mohamad (2011) showed that *T. viride* isolated from palm trees displayed a larger diameter of clearing zone up to 31 mm at 14 days of incubation. The different exposure time of *T. virens* to the substrate, strains, age of culture, and other environmental conditions may affect the cellulase production. The cellulase production increased with the increase in the incubation period (Olaniyi & Oyesiji, 2015). In this study, *T. virens* isolates had fully grown over the agar plates after 5 days making the difficulty to be observed further for the cellulase activity. The study of the specific basal medium composition appropriate for cellulase assay for *T. virens* may be needed to obtain better results. Other factors such as optimum time of exposure, inoculum size, pH value, and optimum temperature also important to be optimized since all those factors influence the cellulase activity of *Trichoderma* (El-Hadi, El-Nour, Hammad, Kamel, & Anwar, 2014; Strakowska, Blaszczyk, & Chelkowski, 2014).

Cellulolytic enzymes were also produced by *T. virens* when the fungi were cultured in liquid media supplemented with CMC as cellulase substrate. The histogram generated from this study showed

---

**Fig. 3.** Scanning electron micrographs (SEM) shows hyphal interaction between *R. solani* and *T. virens* on dual culture plate assay: (a) knob like-structure penetrates host mycelia, (b) *T. virens* penetrates and grow intercellularly in host cell, (c) coiling hyphae, (d) lysis and (e) shrinkage and swollen tips of host hyphae.
that all *T. virens* strains were able to produce CMC-ase, the enzyme responsible for hydrolyzing cellulase, the essential compound of *R. solani* cell wall (Fig. 4b). The amount of cellulase produced by *Trichoderma* on liquid media was measured as the release of reducing sugar (glucose) from the substrate. Analysis of variance of cellulase activity showed that there was no significant difference in cellulase production among strains as well as cellulase concentration (Table 2). All strains showed a similar capability to synthesize cellulase in the media. The cellulase enzyme activity ranged from 2.82 U/ml to 3.25 U/ml and the cellulase production varied between 17.0 μg/ml to 19.5 μg/ml. Strain of Tv4 showed the lowest cellulase activity while Tv6 strain showed the highest cellulase activity.

Cellulolytic activity of *T. virens* on plate assay had a positive correlation with the inhibition of *R. solani* growth on dual culture assay (R = 0.63), showing hyperparasitic behavior of *T. virens* correlated with the activity of cellulase. However, no positive correlation was observed between cellulase production and activity in liquid media and the pathogen suppression on culture filtrate assay. Numerous studies reported a positive association of lyses on host hyphae with the activity of cellulase. However, the differences of methods and techniques employed leading to the different cellulase activity performance (Florencio, Couri, & Farinas, 2012). Our study showed that *T. virens* was thought to perform higher cellulase activity when cultured on liquid media which contained CMC as a substrate compared to the medium containing glucose (culture filtrate). As mention by Abou-Taleb, Mashhoor, Nasr, Sharaf, & Abdel-Azeem (2009), the addition of CMC can induce CMC-ase cellulase production.

**Fig. 4.** (a) The cellulase activity of *T. virens* on yeast extract peptone agar media supplemented with 0.2% of CMC at 3 days of incubation (b) Diameter of clearing zone represents cellulase produced by *T. virens* strains

**Table 2.** Cellulase activity of *T. virens* strains

| Strains | Cellulase concentration (μg/ml glucose) | Cellulase activity (U/ml) |
|---------|----------------------------------------|--------------------------|
| Tv1     | 18.0 ± 0.4ns                           | 3.00 ± 0.07ns            |
| Tv2     | 18.3 ± 1.7                             | 3.04 ± 0.29              |
| Tv3     | 17.0 ± 0.3                             | 2.83 ± 0.04              |
| Tv4     | 17.0 ± 0.5                             | 2.82 ± 0.08              |
| Tv5     | 18.9 ± 1.4                             | 3.15 ± 0.24              |
| Tv6     | 19.5 ± 1.6                             | 3.25 ± 0.26              |
| Tv7     | 17.2 ± 0.5                             | 2.86 ± 0.09              |

Remarks: ns = not significantly difference
Chitinase

The chitinolytic activity of *Trichoderma* in chitin-containing media and bromocresol purple showed the breakdown of chitin into N-acetyl glucosamine (Qualhato et al., 2013). This reaction was represented by the change of media around the colony from yellow to purple. All strain tested showed positive chitinase activity since a day of incubation. The difference in color development and the intensity of the purple color zone showed the various levels of chitinase synthesized among strains (Fig. 5). After 3 days of incubation, most strains could change the color of the medium, although every strain required different time to initiate the chitinase activity (Table 3). Analysis of variance of chitinase activities indicated that the production of chitinase was significantly influenced by the time of incubation and the strains. Strains of Tv4 and Tv5 showed low chitinase activity for 2 days of incubation and took longer time to hydrolyze chitin from the medium, in contrast with Tv3 strains which showed rapid and intensive color changes since the first day of incubation.

![Fig. 5. The chitinase activity assay of *T. virens* strains on media supplemented with colloidal chitin (0.5%). (A) The diameter of the purple color zone at 2 days of incubation, (B) The diameter of the purple color zone at 3 days of incubation, and (C) The diameter of the purple color zone at 4 days of incubation. Tv1 – Tv7 = strains *T. virens* No. 1 - 7](image)

| Strains | 2 Days          | 3 Days          | 4 Days          |
|---------|-----------------|-----------------|-----------------|
| Tv1     | 1.30 ± 0.17 c   | 4.00 ± 0.44 bc  | 7.23 ±0.06 a    |
| Tv2     | 1.67 ± 0.45 bc  | 5.27 ± 0.06 b   | 7.17 ±0.06 a    |
| Tv3     | 2.77 ± 0.25 a   | 7.03 ± 1.07 a   | 7.20 ±0.10 a    |
| Tv4     | 0.00± 0.00 e    | 2.07± 0.98 e    | 6.50±0.10 b     |
| Tv5     | 0.63± 0.12 d    | 3.33 ± 0.15 d   | 4.70 ±0.10 c    |
| Tv6     | 1.47 ± 0.42 bc  | 4.57± 0.06 bc   | 7.18 ±0.03 a    |
| Tv7     | 1.90± 0.26 b    | 4.67± 0.06 bc   | 7.20 ±0.10      |

Remarks: Numbers in the same column followed by the same letter are not significantly different based on the LSD test at $\alpha = 0.05$.
This current study also showed a positive correlation between the chitinase activity (purple zone diameter) and the inhibition growth of the pathogen in dual culture assay at 3 days of incubation ($R^2 = 0.67$). The Tv3 strains which displayed the most intensive and larger purple color zone had the highest inhibition growth of \textit{R. solani} on dual culture assay. This result demonstrated that chitinase was considered to have an important contribution to mycoparasitism of \textit{T. virens}. This also in line with a study conducted by Baek, Howell, & Kenerley (1999) which showed significantly decreased in the bio-control activity of \textit{T. virens} strains KO in which their chitinase genes (cht42) were disrupted compared to the wild-type strains.

Chitinase was also synthesized by \textit{T. virens} on liquid medium supplemented with chitin substrate from colloidal chitin. Concentration and chitinase activity from all 7 strains are shown in Fig. 6. The amount of chitinase synthesized was not different among strains. The chitinolytic activity was expressed as the concentration of N- acetyl glucosamine (Gl-Nac) (mg/ml) released in media supplemented with colloidal chitin per minute (Agrawal & Kotasthane, 2012). The concentration of Gl-Nac released ranged from 0.61 mg/ml to 1.12 mg/ml while the chitinase activity was recorded from 0.28 to 0.51 U/minute.

This present study showed the complex mycoparasitism process of \textit{T. virens} against \textit{R. solani}. \textit{R. solani} has a unique character because besides having cell wall which contains chitin, it also produces cell wall-degrading enzymes including polygalacturonase (Cattelan, Hartel, & Fuhrmann, 1999), polymethyl-galacturonase (PMG), cellulase (Cx) and β-glucosidase which are potential to inhibit the growth of its antagonist such as \textit{Trichoderma} (Xue, Zhou, Li, Xiao, & Fu, 2018). On the other hand, \textit{T. virens} was one of the potential \textit{Trichoderma} species which is very efficient in degrading cell walls of cellulases (Strakowska, Blaszczyk, & Chełkowski, 2014). This fact was difficult to conclude whether the suppression of pathogen growth directly correlated with the mycoparasitic properties from antagonistic fungi. For example, strains Tv2 which showed the highest chitinase activity, had relatively high suppression ability, on the other hand, Tv3 which showed relatively low chitinase activity performed stronger growth inhibition. This suggested that there were other antifungal compounds produced by \textit{T. virens} which were also involved in mycoparasitism besides the defensive action from pathogens. Hyperparasitic action such as coiling and penetration of hyphae suggested the action mode preceded the production of the lytic enzyme. This was proved by the formation of a knob-like structure and parallel growth of \textit{Trichoderma} hyphae prior to penetration and coiling the host hyphae at 24 hours of incubation. However, some strains like Tv2 showed early lytic enzymes activity which allows them to degraded host cell walls and parasite them for their own growth. The researcher’s previous study showed that volatile organic compounds produced by \textit{T. virens} strains could inhibit the growth of \textit{R. solani} up to 72% (Inayati, Sulistyowati, Aini, & Yusnawan, 2019) which supported the fact that other compounds of sesquiterpenes and fatty acids involved in the mycoparasitism of \textit{T. virens}.

Overall, the mycoparasitism variable showed insignificant direct single-factor comparison. Therefore, the principal component analysis (PCA) was performed to help the interpretation of the data variability. PCA analysis showed that \textit{T. virens} strains widespread across the PCA chart, thus confirming the high natural variability of \textit{T. virens} mycoparasitism. These differences were related to the inhibition growth and the production of hydrolytic enzymes. Two principal components (PC1 and PC2) explained 57.62% of the data variability which confirmed the mycoparasitism of \textit{T. virens} was influenced by both direct growth inhibition as well as hydrolytic enzyme production (Fig. 7). Four out of the seven strains (Tv2, Tv3, Tv4, and Tv5) separated along PC1 quadrant indicating that direct growth inhibition through hyperparasitism on dual culture as well as through non-volatile compounds produced on culture filtrate, and the production of cellulase and chitinase were the major factor of the \textit{T. virens} mycoparasitism strains against \textit{R. solani}. PC1 had a positive correlation with growth inhibition on dual culture assay, the production of cellulase on plate agar, and chitinase activity, and fairly correlated negatively with inhibition growth on culture filtrate. Therefore, PC2 had a positive correlation with the growth suppression on culture filtrate at a concentration of 30% and the production of chitinase on plate assay, however, having negative correlation with the production of cellulase which was shown by the characteristic of mycoparasitism of Tv1, Tv7, and Tv6 strains. Explanation of variability on this principal component could be related to the variability of \textit{T. virens} mycoparasitism.
Fig. 6. Chitinase concentration releases in media and chitinase activity of *T. virens* strains

Fig. 7. Principal Component Analysis (PCA) of *T. virens* mycoparasitism against *R. solani*
An important finding of this study was the indigenous strains of *T. virens* isolated from several rhizosphere crops cultivated in East Java had potential biocontrol agents through its mycoparasitic action. Although the enzyme productions were not directly correlated with the growth suppression, its showing synergistic action with the hyperparasitic ability such as physical destruction, which increased the potency of *T. virens* to control *R. solani*. The maximum inhibition by selected strains of *T. virens* was due to hyperparasitism as well as the production of both volatile and non-volatile antimicrobial compounds. *T. virens* mycoparasitism against pathogenic fungi of *R. solani*, showed the synergistic actions of both hyperparasitism and the production of cell wall degrading enzymes, especially cellulase and chitinase.

**CONCLUSION**

*T. virens* strains had different mycoparasitic ability against pathogenic fungi of *R. solani*. Synergism in the production of hydrolytic enzyme productions with the hyperparasitic ability is the mode of action of *T. virens* for controlling *R. solani*. Tv3 strain which had the highest growth inhibition and showed high cellulase and chitinase activities could be promoted as a promising strain to control *R. solani*.

**ACKNOWLEDGEMENT**

The author would like to thank Indonesian Agency for Agricultural Research and Development for funding this research.

**REFERENCES**

Abou-Taleb, K. A. A., Mashhoor, W. A., Nasr, S. A., Sharaf, M. S., & Abdel-Azeem, H. H. M. (2009). Nutritional and environmental factors affecting cellulase production by two strains of cellulytic *Bacilli*. *Australian Journal of Basic and Applied Sciences*, 3(3), 2429–2436. Retrieved from http://www.ajbasweb.com/old/ajbas/2009/2429-2436.pdf

Agrawal, T., & Kotasthane, A. S. (2012). Chitinolytic assay of indigenous *Trichoderma* isolates collected from different geographical locations of Chhattisgarh in central India. *SpringerPlus*, 1, 73. https://doi.org/10.1186/2193-1801-1-73

Angel, L. P. L., Sundram, S., Ping, B. T. Y., Yusof, M. T., & Ismail, I. S. (2018). Profiling of anti-fungal activity of *Trichoderma virens* Gv29-8 involved in biocontrol assay of *Ganoderma boninense*. *Journal of Oil Palm Research*, 30(1), 83–93. https://doi.org/10.21894/jopr.2017.0009

Angel, L. P. L., Yusof, M. T., Ismail, I. S., Ping, B. T. Y., Mohamed Azni, I. N. A., Kamarudin, N. H., & Sundram, S. (2016). An in vitro study of the antifungal activity of *Trichoderma virens* 7b and a profile of its non-polar antifungal components released against *Ganoderma boninense*. *Journal of Microbiology*, 54, 732–744. https://doi.org/10.1007/s12275-016-6304-4

Baek, J. M., Howell, C. R., & Kenerley, C. M. (1999). The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*. *Current Genetics*, 35, 41–50. https://doi.org/10.1007/s002940050431

Carling, D. E., Baird, R. E., Glaitis, R. D., Brainard, K. A., & Kuninaga, S. (2002). Characterization of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*. *Phytopathology*, 92(8), 893–899. https://doi.org/10.1094/PHYTO.2002.92.8.893

Cattelan, A. J., Hartel, P. G., & Fuhrmann, J. J. (1999). Screening for plant growth-promoting rhizobacteria to promote early soybean growth. *Soil Science Society of America Journal*, 63(6), 1670–1680. https://doi.org/10.2136/sssaj1999.6361670x

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Herrera-Estrella, A., & López-Bucio, J. (2014). The 4-phosphopantetheinyl transferase of *Trichoderma virens* plays a role in plant protection against *Botrytis cinerea* through volatile organic compound emission. *Plant and Soil*, 379, 261–274. https://doi.org/10.1007/s11104-014-2069-x

Dennis, C., & Webster, J. (1971). Antagonistic properties of species-groups of *Trichoderma*: I. Production of non-volatile antibiotics. *Transactions of the British Mycological Society*, 57(1), 25–39. https://doi.org/10.1016/S0007-1536(71)80077-3

Djunaedy, A. (2008). Aplikasi fungisida sistemik dan pemanfaatan mikoriza dalam rangka pengendalian patogen tular tanah pada tanaman kedelai (*Glycine max* L.). *Embryo*, 5(2), 149–157. Retrieved from https://scholar.google.co.id/scholar?hl=en&as_sdt=0,5&cluster=11437794908234141220

El-Hadi, A. A., El-Nour, S. A., Hammad, A., Kamel, Z., & Anwar, M. (2014). Optimization of cultural and nutritional conditions for carboxymethylcellulase production by *Aspergillus hortai*. *Journal of...*
Mukherjee, M., Mukherjee, P. K., Horwitz, B. A., Zachow, C., Berg, G., & Zeilinger, S. (2012). Trichoderma-plant-pathogen interactions: Advances in genetics of biological control. *Indian Journal of Microbiology*, 52(4), 522–529. https://doi.org/10.1007/s12088-012-0308-5

Nakkeeran, S., Vinodkumar, S., Priyanka, R., & Renukadevi, P. (2018). Mode of action of *Trichoderma* spp. in biological control of plant diseases. In *Biocontrol of Soil Born Pathogens and Hematodes* (pp. 81–95). New Delhi, IN: Jeya Publishing house. Retrieved from https://www.researchgate.net/publication/329177943_Mode_of_Action_of_Trichoderma_Spp_in_Biological_Control_of_Plant_Diseases

Olaniyi, O. O., & Oyesii, Y. V. (2015). Stimulatory effect of physicochemical factors on the expression of cellulase by *Trichoderma viride* NSPRT23. *Microbiology Journal*, 5, 58–67. https://doi.org/10.3923/mj.2015.58.67

Potprommanee, L., Wang, X. Q., Han, Y. J., Nyobe, D., Peng, Y. P., Huang, Q., … Chang, K. L. (2017). Characterization of a thermophilic cellulase from *Geobacillus* sp. HTA426, an efficient cellulase-producer on alkali pretreated lignocellulosic biomass. *PLoS ONE*, 12(4), e0175004. https://doi.org/10.1371/journal.pone.0175004

Qualhato, T. F., Lopes, F. A. C., Steindorff, A. S., Brandão, R. S., Jesuino, R. S. A., & Ulhoa, C. J. (2013). Mycoparasitism studies of *Trichoderma* species against three phytopathogenic fungi: Evaluation of antagonism and hydrolytic enzyme production. *Biotechnology Letters*, 35, 1461–1468. https://doi.org/10.1007/s12088-013-0308-5

Rahayu, M. (2014). Identification and pathogenicity of pathogen responsible for aerial blight disease of soybean. *Journal of Experimental Biology and Agricultural Sciences*, 2(2S), 279–285. Retrieved from http://www.jebas.org/wp-content/uploads/2014/09/Rahayu-JEBAS.pdf

Rahayu, M. (2016). Patologi dan teknis pengujian kesehatan benih tanaman aneka kacang. *Buletin Palawija*, 14(2), 78–88. Retrieved from http://balitkabi.litbang.pertanian.go.id/wp-content/uploads/2017/11/5-Mui-rahayu-14_2.pdf

RStudio Team. (2015). *RStudio: Integrated development for R*. Boston, MA: RStudio, Inc.. Retrieved from https://support.rstudio.com/hc/en-us/articles/206212048-Citing-RStudio

Shahriarinour, M., Abd Wahab, M. N., Ariff, A., & Mohamad, R. (2011). Screening, isolation and selection of cellulolytic fungi from oil palm empty fruit bunch

Alfi Inayati et al.: Mycoparasitic of *Trichoderma virens* against *Rhizoctonia solani*..........................

Radiation Research and Applied Sciences, 7(1), 23–28. https://doi.org/10.1016/j.jrras.2013.11.003

Florencio, C., Couri, S., & Farinas, C. S. (2012). Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by *Trichoderma* strains. *Enzyme Research*, 2012(793708), 1–7. https://doi.org/10.1155/2012/793708

Inayati, A., Sulistyowati, L., Aini, L. Q., & Yusnawan, D. (2012). Metabolites from cellulolytic fungi from oil palm empty fruit bunch. *Enzyme Research* (pp. 395–432). Singapore: Springer. https://doi.org/10.1007/978-981-10-6593-4_16

Meena, M., Swapnil, P., Zehra, A., Dubey, M. K., & Upadhyay, R. S. (2017). Beneficial microbes for disease suppression and plant growth promotion. In D. Singh, H. Singh, & R. Prabha (Eds.), *Plant-Microbe Interactions in Agro-Ecological Perspectives* (pp. 395–432). Singapore: Springer. https://doi.org/10.1007/978-981-10-6593-4_16

Li, M. F., Li, G. H., & Zhang, K. Q. (2019). Non-volatile metabolites from *Trichoderma* spp. *Metabolites*, 9(3), 58. https://doi.org/10.3390/metabo9030058

Meena, M., Swapnil, P., Zehra, A., Aamir, M., Dubey, M. K., Goutam, J., & Upadhyay, R. S. (2017). Antagonistic assessment of *Trichoderma* spp. by producing volatile and non-volatile compounds against different fungal pathogens. *Archives of Phytopathology and Plant Protection*, 50(13–14), 629–648. https://doi.org/10.1080/03235408.2017.1357360

Mohiddin, F. A., Khan, M. R., Khan, S. M., & Bhat, B. H. (2010). Why *Trichoderma* is considered super hero (super fungus) against the evil parasites? *Plant Pathology Journal*, 9(3), 92–102. https://doi.org/10.3923/ppj.2010.92.102

Monfil, V. O., & Casas-Flores, S. (2014). Molecular mechanisms of biocontrol in *Trichoderma* spp. and their applications in agriculture. In V. K. Gupta, M. Schmoll, A. Herrera-Estrella, R. S. Upadhyay, I. Druzhinina, & M. G. Tuohy (Eds.), *Biotechnology and Biology of Trichoderma* (pp. 429–453). Elsevier. https://doi.org/10.1016/B978-0-444-60576-8.00032-1

Muis, A. (2007). Pengelolaan penyakit busuk pelepah (*Rhizoctonia solani* Kuhn.) pada tanaman jagung. *Buletin Litbang Pertanian*, 26(3), 100–103. Retrieved from http://203.190.37.42/publikasi/p3263072.pdf

Copyright © 2020 Universitas Brawijaya
Mycoparasitic of Trichoderma virens against Rhizoctonia solani.........................................................

Smitha, C., Finosh, G. T., Rajesh, R., & Abraham, P. K. (2014). Induction of hydrolytic enzymes of phytopathogenic fungi in response to Trichoderma viride influence biocontrol activity. *International Journal of Current Microbiology and Applied Sciences*, 3(9), 1207–1217. Retrieved from https://www.ijcmas.com/vol-3-9-C.Smitha, et al.pdf

Soesanto, L., Mugiastuti, E., Rahayuniati, R. F., Manan, A., Dewi, R. S. (2018). Compatibility test of four *Trichoderma* spp. isolates on several synthetic pesticides. *AGRIVITA Journal of Agricultural Science*, 40(3), 481-489. https://doi.org/10.17503/agrivita.v4i03.1126

Strakowska, J., Blaszczyk, L., & Chełkowski, J. (2014). The significance of cellulolytic enzymes produced by *Trichoderma* in opportunistic lifestyle of this fungus. *Journal of Basic Microbiology*, 54(S1), S2–S13. https://doi.org/10.1002/jobm.201300821

Syed, S., Riyaz-Ul-Hassan, S., & Johri, S. (2013). A novel cellulase from an endophyte, *Penicillium* sp. NFCCI2862. *American Journal of Microbiological Research*, 1(4), 84–91. https://doi.org/10.12691/ajmr-1-4-4

Veena, D., Priya, H. R., Raheesa, M. K., & Divya, J. (2014). Soilborne diseases in crop plants and their management. *Research & Reviews: Journal of Agriculture and Allied Sciences*, 3(2), 12–18. Retrieved from http://www.rojij.com/open-access/soilborne-diseases-in-crop-plants-and-their-management.php?aid=33851

Vipul, K., Mohammad, S., Mukesh, S., Anuradha, S., Sonika, P., & Manoj, K. M. (2015). Screening of *Trichoderma* species for virulence efficacy on seven most predominant phytopathogens. *African Journal of Microbiology Research*, 9(11), 793–799. https://doi.org/10.5897/ajmr2014.7342

Viterbo, A., & Horwitz, B. A. (2010). Mycoparasitism. In K. Borkovich & D. J. Ebbole (Eds.), *Cellular and Molecular Biology of Filamentous Fungi* (pp. 679–693). Washington, DC: ASM Press. Retrieved from https://books.google.co.id/books?id=ftwSsBAAAQBAJ

Wang, K.-D., Borrego, E. J., Kenerley, C. M., Kolomiets, M. V. (2020). Oxylinips other than jasmonic acid are xylem-resident signals regulating systemic resistance induced by *Trichoderma virens* in maize. *The Plant Cell*, 32(1), 166-185. https://doi.org/10.1105/tpc.19.00487

Wu, Q., Sun, R., Ni, M., Yu, J., Li, Y., Yu, C., … Chen, J. (2017). Identification of a novel fungus, *Trichoderma asperellum* GDFS1009, and comprehensive evaluation of its biocontrol efficacy. *PLoS ONE*, 12(6), e0179957. https://doi.org/10.1371/journal.pone.0179957

Xue, C. Y., Zhou, R. J., Li, Y. J., Xiao, D., & Fu, J. F. (2018). Cell-wall-degrading enzymes produced in vitro and in vivo by *Rhizoctonia solani*, the causative fungus of peanut sheath blight. *PeerJ*, 6, e5580. https://doi.org/10.7717/peerj.5580

You, J., Zhang, J., Wu, M., Yang, L., Chen, W., & Li, G. (2016). Multiple criteria-based screening of *Trichoderma* isolates for biological control of *Botrytis cinerea* on tomato. *Biological Control*, 101, 31–38. https://doi.org/10.1016/j.biocontrol.2016.06.006

Yusnawan, E., Inayati, A., & Baliadi, Y. (2019). Isolation of antagonistic fungi from rhizospheres and its biocontrol activity against different isolates of soil borne fungal pathogens infected legumes. *Biodiversitas Journal of Biological Diversity*, 20(7), 2048–2054. https://doi.org/10.13057/biodiv/200735

Zehra, A., Dubey, M. K., Meena, M., & Upadhyay, R. S. (2017). Effect of different environmental conditions on growth and sporulation of some *Trichoderma* species. *Journal of Environmental Biology*, 38(March), 197–203. https://doi.org/10.22438/jeb/38/2/MS-251

Zhang, F., Ge, H., Zhang, F., Guo, N., Wang, Y., Chen, L., … Li, C. (2016). Biocontrol potential of *Trichoderma harzianum* isolate T-aleo against *Sclerotinia sclerotiorum* in soybean. *Plant Physiology and Biochemistry*, 100, 64–74. https://doi.org/10.1016/j.plaphy.2015.12.017

Zhang, S., Gan, Y., & Xu, B. (2014). Efficacy of *Trichoderma longibrachiatum* in the control of *Heterodera avenae*. *BioControl*, 59, 319–331. https://doi.org/10.1007/s10526-014-9566-y

Zheng, A., Lin, R., Zhang, D., Qin, P., Xu, L., Ai, P., … Li, P. (2013). The evolution and pathogenic mechanisms of the rice sheath blight pathogen. *Nature Communications*, 4, 1424. https://doi.org/10.1038/ncomms2427