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In vitro evaluation of *Trichoderma harzianum* strains for the control of *Fusarium oxysporum* f.sp. *cubense*

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Abstract

Tricoderma harzianum have been widely used as a biocontrol agent for the suppression of soil-borne pathogens. The objective of this work was to evaluate the potential of *T. harzianum* strains to control *Fusarium oxysporum* f.sp. *cubense* (Foc), a well-known soil-borne pathogen that associated with banana wilt disease. In this study, ten isolates of *T. harzianum* strain isolated from various regions in Java were evaluated through two *in vitro* antagonistic approaches, dual culture test and volatile organic compound (VOC) producing test. All strains showed antagonistic activity against Foc according to the dual culture test with various degree of antagonism from efficient antagonism to highly efficient antagonism. Observations after five days, the antagonists overgrew 63% until 88% of medium surface. Similarly, all strains produced toxic volatile metabolites that having significant effects on the growth and development of the Foc. After seven days of exposure with antagonists, the mycelial growth of the test pathogen was inhibited by 24% until 44%. These results indicate that different strains showed variability in the level of antagonism.

Key words – antagonism – dual culture – Foc – VOC

Introduction

A definition of biocontrol proposed by O'Brien (2017) as "the control of disease by the application of biological agents to a host animal or plant that prevents the development of disease by a pathogen". In general, the application of biocontrol to overcome the threat of plant pathogen is considerably safer and more environmentally friendly than using pesticides. Since dawn of twentieth century, the searching of biocontrol agents has captured the interest of many researchers, but the main rise coming after 1960s (Campbell 1994). The fast progress of many crop diseases followed by the increasing food demand leads to invigorate the hunting of more effective and efficient biocontrol agents and technologies, both for curative or preventive purpose.

Banana is a major source of staple food and one of the main sources of income in many countries particularly in tropical countries such as in Asia, Africa, central and south America, and the Caribbean. For more than a century, Fusarium wilt disease has become a main limitation to optimize banana production. The disease is caused by the soil-borne fungus *Fusarium oxysporum* f.sp. *cubense* (commonly abbreviated as Foc). It is one of the most destructive diseases of banana

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worldwide causing the plant to wilt, turning yellow colour, exhibiting stunted growth and poor production. The pathogen enters via roots and colonize xylem vessel, hence blocking the flow of water and nutrients. Its new race Tropical Race 4 (Foc TR4) has been causing serious losses in Southeast Asia resulting in abandonment of thousands of hectares and recently spread to the Middle East, Africa (Mozambique) and South Asia raising concerns that it may spread further. (Ploetz 2015).

Trichoderma sp. have been widely used as biocontrol agent against soil borne pathogen. The proposed antagonistic mechanisms, either indirectly or directly, have been intensively studied. The indirect mechanism includes competition for nutrients and space, modifications of environmental conditions, antibiosis, mediated plant growth and induced plant immunity toward pathogens, while direct mechanisms leaded by mycoparasitism (Benitez et al. 2004). One of the species, *Trichoderma harzianum*, is a common soil, leaf litter, and wood fungus and presence in abundant amount in nature. The objective of this study is to evaluate and investigate the antagonism of ten isolates of *T. harzianum* strains against Foc, the well-known soil borne pathogen associated with banana wilt disease, through two in vitro antagonism approaches, dual culture test and volatile organic compound producing test.

Materials & Methods

Microorganisms and culture conditions

Ten isolates of *Trichoderma harzianum* strain and Foc (InaCC F822) were obtained from Indonesian Culture Collection (InaCC). The *Trichoderma* strains were isolated from different source of sample in various location in area of Java Indonesia (Table 1). The microorganisms were maintained on potato dextrose agar (PDA) plates at 30^{o}C prior to use in the experimental procedures.

Table 1 The *Trichoderma harzianum* strains for in vitro evaluation against Foc.

No. InaCC*	Locality	Source of Isolation
InaCC F86	Mt. Bromo, Ngadisari, Pasuruan Regency	Leaf litter
InaCC F87	Mt. Salak, Sukabumi Regency	Leaf litter
InaCC F88	Mt. Bromo, Ngadisari, Pasuruan Regency	Soil
InaCC F89	Mt. Salak, Sukabumi Regency	Leaf litter
InaCC F90	Mt. Salak, Sukabumi Regency	Leaf litter
InaCC F91	Mt. Salak, Sukabumi Regency	Leaf litter
InaCC F92	Mt. Salak, Sukabumi Regency	Leaf litter
InaCC F115	Mt. Bromo, Ngadisari, Pasuruan Regency	Soil
InaCC F116	Mt. Salak, Sukabumi Regency	Leaf litter
InaCC F144	Mt. Salak, Sukabumi Regency	Leaf litter

* Indonesian Culture Collection LIPI

Identification of Fungi

Fungal strains were identified using molecular approach based on sequence analysis of internal transcribed spacer (Hardoim et al. 2015) region. The total fungal genomic DNA was isolated using Nucleon PhytoPure, plant and fungal DNA extraction kits (GE Healthcare) according to the manufacturer`s instruction. PCR amplification was performed in 25-µl reaction mixtures with primer set ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG -3`) was used for amplified ITS1 and ITS 2 including 5.8S rDNA (White et al. 1990). Amplification was performed in a TAKaRa PCR Thermal Cycler P650 (TAKARA BIO Inc.,); programmed under following conditions: an initial denaturation at 95°C for 3 min, 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The product was purified

using PEG precipitation method (Hiraishi et al. 1995). Sequencing reactions were conducted using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer`s instruction. The reaction was performed in a TAKaRa PCR Thermal Cycler P650 (TAKARA BIO Inc.,); programmed for 25 cycles of 10s denaturation at 96° C followed by primer annealing 5s at 55°C and primer extension 4 min at 60°C for 1 min. Sequencing product were purified with ethanol purification method. The samples were analysed on an ABI PRISM 3130 Genetic Analyser (Applied Biosystems).

The raw ITS rDNA sequences were trimmed and assembled using BioEdit program and then applied for Basic Local Aligment Search Tool (BLAST) on National Centre for Biotechnology Information (NCBI). The assembled sequences were multiple aligned with those downloaded ITS rDNA on NCBI using ClustalW on BioEdit program. The phylogenetic analyses of sequence data were carried out using a Molecular Evolutionary Genetic Analysis (MEGA) programme version 5.2 (Kumar et al. 2008). A phylogenetic tree construction based on genetic distance with a neighbor joining (NJ) statistical method, maximum-composite likelihood algorithm, and complete deletion gaps. The strength of phylogenetic tree was tested using bootstrap method with 1000 replication (Felsenstein 1985).

Dual Culture Test

To examine the antagonism of *Trichoderma* strains against the Foc, discs of PDA medium (6 mm diam.) were taken from the edge of actively growing colonies of fresh fungal cultures and placed on the surface of a fresh nine-centimetre PDA plate 4 cm apart. The plates were incubated at 30ºC. The growth of *Trichoderma* against Foc was monitored every day for 7 days. At fifth day, the evaluation of antagonism was carried out according to the classification proposed by Bell's classification (Bell et al. 1982): grade 1–*Trichoderma* completely overgrew the pathogen and covered the entire medium surface; grade 2–*Trichoderma* occupies 75 % surface of the medium surface; grade 3–*Trichoderma* occupies 50 % of the medium surface; grade 4–*Trichoderma* occupies 25 % of the medium surface; grade 5–the pathogen completely overgrew the *Trichoderma*. The experiment was conducted with three repetitions for each *Trichoderma* strain.

Volatile metabolites

Separate nine-centimetre plates containing PDA medium were inoculated in the centre with a mycelial disc of 6 mm containing Foc or the different *Trichoderma* strains. The lids were removed and two plates were inverted and placed on top of another plate. The two plate bases were then sealed with a double layer of parafilm. The plates were kept at 30ºC. The pathogen was in the upper plate in order to avoid any interference by antagonistic spores in the plate inoculated with Foc. The moment of evaluation was determined after 3, 5, and 7 days post infection. The diameters of pathogens were measured and used to calculate the inhibition in relation to the control plate. The experiment was conducted with three repetitions for each *Trichoderma* strains.

Results

Identification of Fungi and Phylogenetic Tree Analyses

The phylogenetic analyses from ITS sequence data based on NJ method showed that all the strains have the same clade with *T. harzianum* or *Hypocrea lixii* species group with reliability of bootstrap value 100% (Fig. 1). Based on BLAST result and phylogenetic tree analyses all the strains were identified and reconfirmed as *T. harzianum*.

Dual Culture Test

The in vitro antagonistic activities of *T. harzianum* strains against Foc were studied in dual culture (Fig. 2). Every day, the growth profiles of each strains was monitored and analysed using ImageJ software (Fig. 3). Mostly for isolates, the occupation was significantly increase until third day, then subsequently showed plateau after fourth day. It shows that the percent of occupation of

T. harzianum in petri dish along with Foc are various between isolates. Observations after five days, the antagonists overgrew 63% until 88% of medium surface.

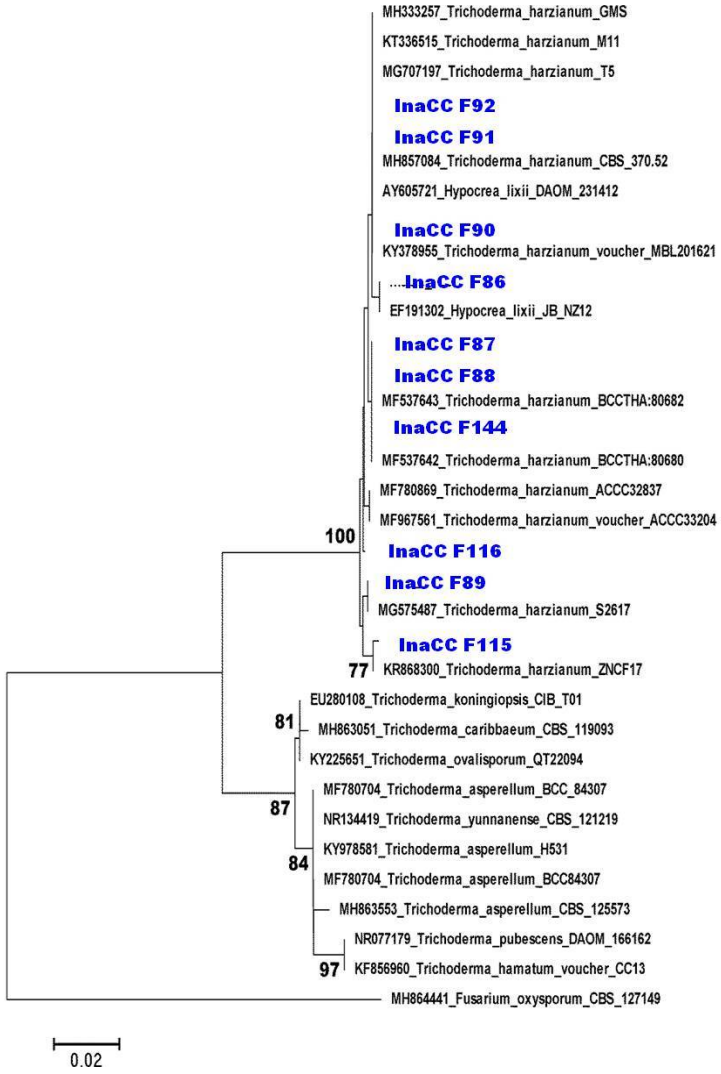

Fig. 1 – Phylogenetic tree of 10 strains of *Trichoderma harzianum* from InaCC based on ITS rDNA sequence using neighbor-joining method and *Fusarium oxysporum* as outgroup.

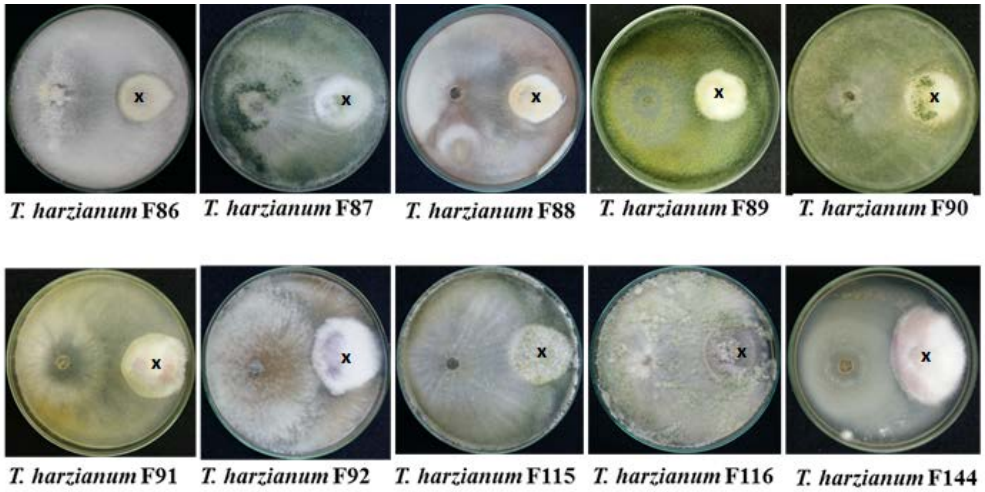

Fig. 2 – Dual culture test of ten *Trichoderma harzianum* strains against Foc (showed with "x" mark) after seven days.

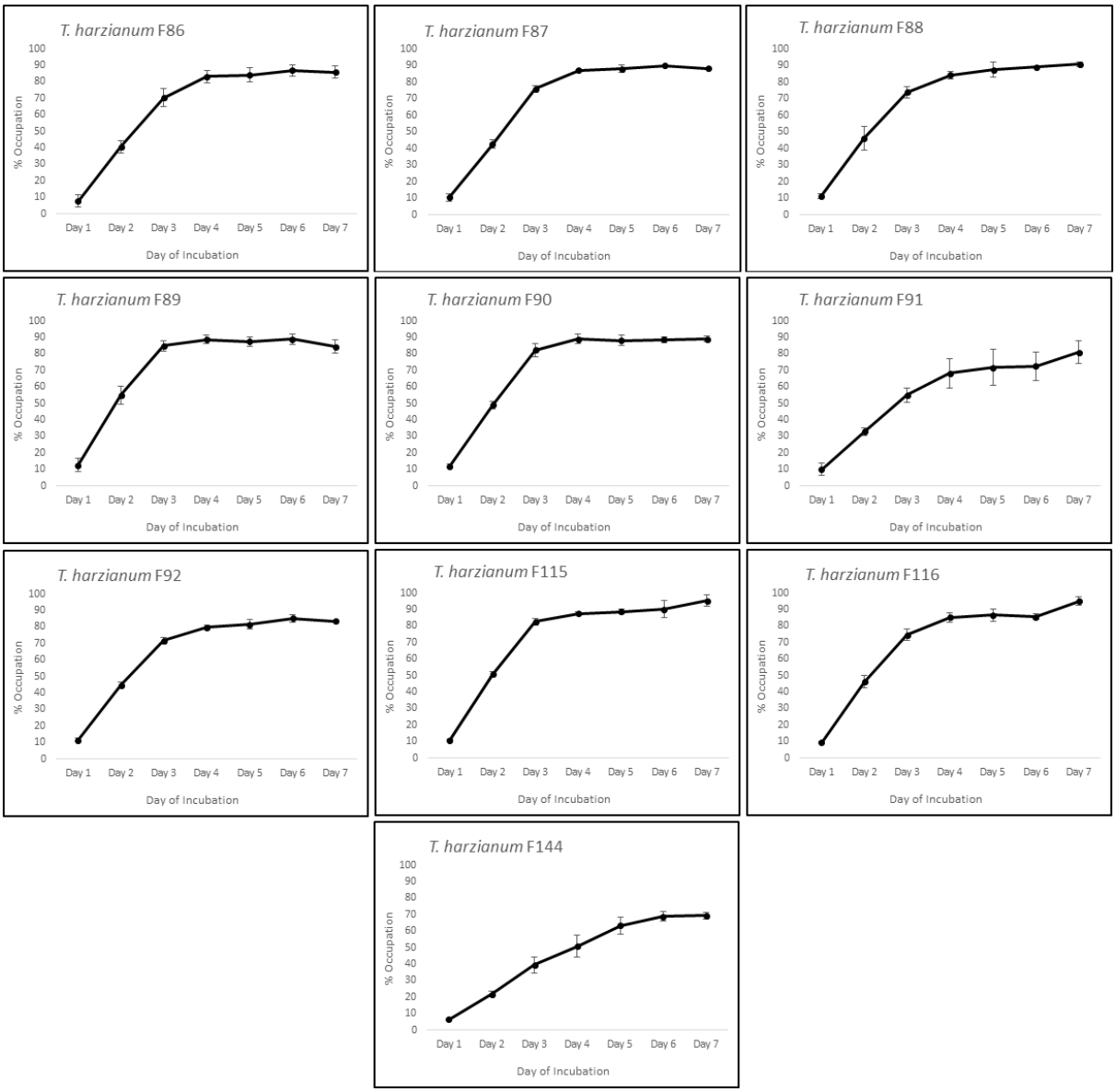

Fig. 3 – The growth profile of ten *T. harzianum* strains against Foc. The percent of occupation in petri dish was analysed every day for seven days using imageJ software.

The antagonism was evaluated by following the method described by Bell et al. (1982): a *Trichoderma* species was considered as antagonistic to the Foc if the mean grade score for a given comparison was ≤2, but not highly antagonistic if the number was ≥3. There were various in the average antagonistic abilities of the *Trichoderma* strains against Foc (Table 2). *Trichoderma harzianum* strains were efficiently antagonistic against Foc in that they received the best evaluations according to Bell's classification (Table 2).

Volatile Organic Compounds produced by *Trichoderma harzianum* strains against Foc

All of the *Trichoderma* species tested in this study produced toxic volatile metabolites having significant effects on the growth and development of the plant pathogens (Fig. 4). The volatile organic compounds produced by *T. harzianum* inhibited the growth of Foc by lowering the area of mycelial surface compared to control (Fig. 5). After seven days of exposure with antagonists, the mycelial growth of the test pathogen was inhibited by 24% until 44%. In this case *T. harzianum* F116 strain was the most efficacious in reducing mycelial growth with 44.63% inhibition measured by reducing the mycelial growth area compare to control.

Table 2 Dual Culture of ten isolates of *T. harzianum* against Foc after five days.

Isolates	Antagonism Class (grade scores) *	Interpretation
F86	2	Highly Efficient Antagonism
F87	2	Highly Efficient Antagonism
F88	2	Highly Efficient Antagonism
F89	2	Highly Efficient Antagonism
F90	2	Highly Efficient Antagonism
F91	2.3	Efficient Antagonism
F92	2	Highly Efficient Antagonism
F115	2	Highly Efficient Antagonism
F116	2	Highly Efficient Antagonism
F144	2.6	Efficient Antagonism

* Classification proposed by Bell et al. (1982)

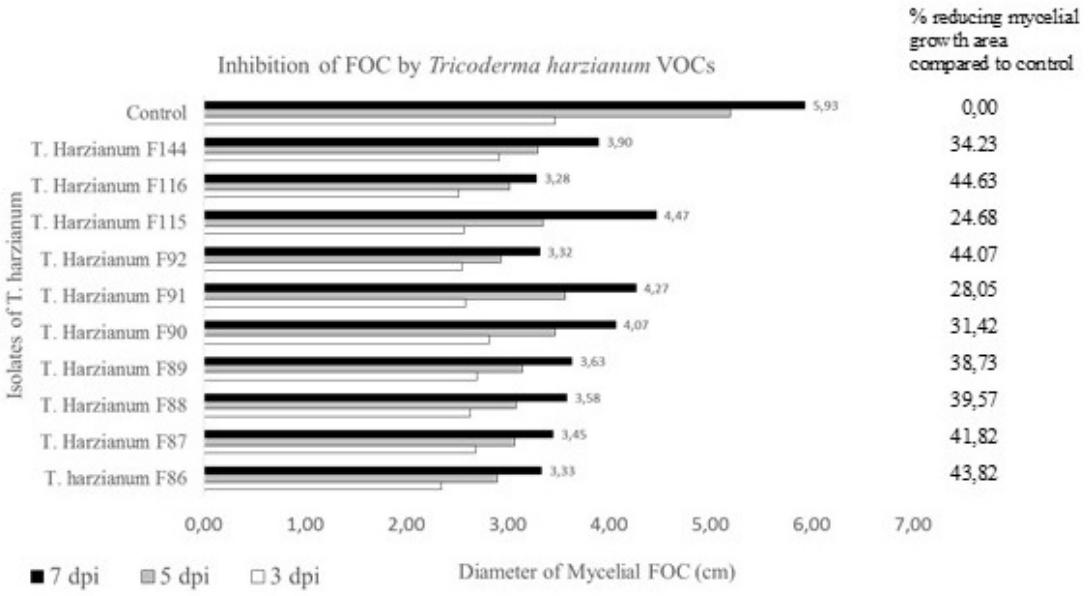

Fig. 4 – The inhibition profile of Foc by Volatile Organic Compounds of *Trichoderma harzianum* strains. The diameter of mycelial Foc was measured after 3, 5, and 7 days post inoculation. Percent reducing mycelial growth affected by VOCs *T. harzianum* strain area was calculated as 100% (diameter of mycelial Foc control - diameter of mycelial Foc affected by *T. harzianum* strain) / diameter of mycelial Foc control.

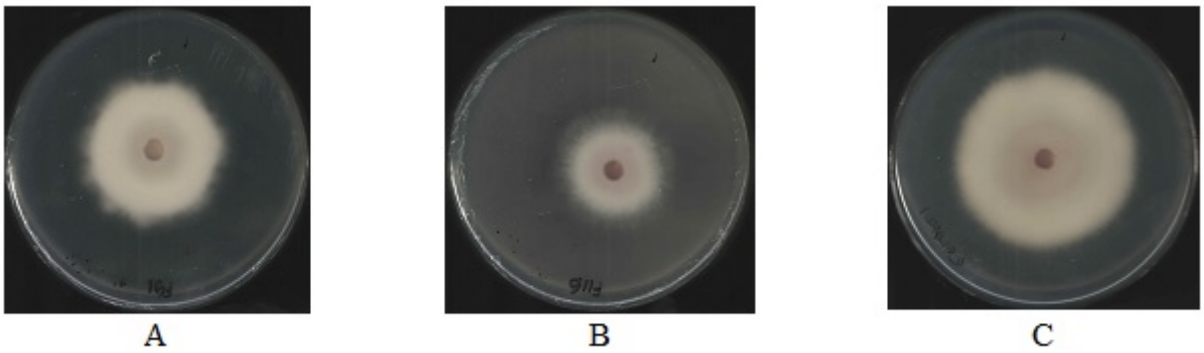

Fig. 5 – Mycelial area of Foc after seven days exposed by VOCs of *T. harzianum* strains. A by F91 strain, B by F116 strain, and C is control.

Discussion

Trichoderma sp. is considerably fast growth fungi and can be produced in high population in various substrates in relatively short time. In just three day, most of the strains has occupied three quarter of the petri dish that also containing Foc which also still growing but has lower growth. This feature is beneficial as biocontrol agent against plant pathogens, such as *Fusarium*. Moreover, the common proposed for mode of action of *Trichoderma* sp. against *Fusarium* is through mycoparasitism (Benitez et al. 2004) which is a direct mechanism that need a fast growth ability in order to overcome the pathogen. The search of fastest growing profile of *Trichoderma* sp. is significantly important to evaluate the ability of the agent as a promising biocontrol agent.

Trichoderma sp. produce and release small molecules gasses volatile organic compounds (VOCs) that able to diffuse through atmosphere and soil (Morath et al. 2012). These metabolites have biocontrol activity against various different pathogens such as *Alternaria* sp. and *Fusarium* sp. (Meena et al. 2017). *Fusarium* are reported to induce production of certain VOCs in *Trichoderma harzianum* (Zhang et al. 2014).

Our result confirmed the previous studies related to the effect of volatile organic compounds produced by *Trichoderma* sp. to control the plant pathogens. *Trichoderma viride* metabolites, both volatile and non-volatile, were reported to have biocontrol activity against ginger rhizome rot pathogens *Pythium myriotylum* and *Fusarium solani* (Rathore et al. 1992). Compounds such as dibutyl phthalate (DBP) along with many volatile substances had been identified in *Trichoderma virens* and reported to have antifungal activity (Tabarestani et al. 2016).

Similar with dual culture test and growth profile results (Fig. 3), the antagonism activity of *T. harzianum* strains against FOC via VOCs producing test also showed significant variability among strains. Genetic variability of *Trichoderma harzianum* isolates are reported against soil borne pathogens in dual culture test resulting differences in the capacity to produce extracellular metabolites (Choudary et al. 2007)

Conclusion

There was variability in the average antagonistic abilities of the *Trichoderma harzianum* strains against plant pathogen fungi *Fusarium oxysporum* f.sp. *cubense* (Foc) according to dual culture test and volatile organic compound (VOC) producing test toward 10 *T. harzianum* strains. All the strains produced volatile metabolites having inhibition effects on the growth and development of the Foc. *Trichoderma harzianum* F116 strain has the most effective antagonism activity according to the in vitro evaluations and has a potential for biological control.

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