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Phosphate Solubilizing and Antifungal Activity of Root Endophyte Isolated from *Shorea leprosula* Miq. and *Shoreal selanica* (DC) Blume

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Abstract

Fungal endophytes are fungi that lives within plant tissues without causing apparent disease. It is also suggested that these fungi have ability to enhance plant growth and plant resistancy against pest and disease. This research is a preliminary study about root fungal endophytes in dipterocarp since there are lack research concerning about this study focus. We examined root fungal endophyte isolated from seedling of Shorea leprosula and Shorea selanica taken from Dramaga Experimental Forest, Bogor. Furthermore, we also tried to find out the fungal potential ability to solubilize phosphate and suppres fungal pathogen by in vitro assay. Surface sterilization method was used to isolated fungal endophytes from root tissues. Trichoderma spirale, Velsalceae sp., Melanconiela ellisii, Chaetosphaeria callimorpha, and Trichoderma asperellum were isolated during this study. These fungi appear to have specific association between fungal species and host plant, but no evidence of fungal order-level specificiation in S. leprosula and S. selanica. In vitro test also suggested that root fungal endophyte Trichoderma spirale and Melanconiella elisii have potential ability to solubilize inorganic phosphate. In addition, this result also present that root fungal endophyte T. spirale and T. asperellum have the potential to inhibit pathogen fungi Fusarium sp.

Keywords: root endophyte, biofertilizer, bioinsectisicide, biocontrol, phosphate solubilizing fungi

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Introduction

Naturally, plants have interaction with soil organism (Das & Varma 2009). Studies have shown that interaction between plants and microorganism positively affect plant growth, plant health, increasing plant biomass, alter plant adaptation through stress condition, and etc (Bonkowski *et al.* 2000; Hrynkiewicz & Baum 2011). In forest tree, the interaction between trees and mycorrhiza is well studied. Currently, many forest plantations use mycorrhiza as biofertilizer in their plantation area. But unlike mycorrhiza, study about the interaction in forest trees and fungal endophyte in tropical region are less known.

Fungal endophyte are fungi that live within plant tissues without causing apparent disease and also suggested that fungal endophyte has functional effect on plant, producing various secondary metabolite, enhance plant resistance against pest and disease (Bayman 2007). Fungal endophyte are very ubiquitous in natural habitat (Schulz & Boyle 2005). Association between trees and fungal endophyte formed many benefits that still need to be explore further. Some research reveal fungal endophytes advantages such as: increase uptake of N from soil and plant biomass (Jumponnen *et al.* 2001), enhance plant resistance against plant pathogen (Miller *et al.* 2002), producing secondary metabolite which has potential ability as a new bioresources for pharmaceutical and agricultural needs (Surnayaran *et al.* 2002). In addition, microorganism have ability to dissolve insoluble form of phosphate into plant available form (Pradan & Suklana 2005). Therefore, microorganism could be potential bio-fertilizer due their ability to supply available phosphor for plant which are very limited in soil. Dipteorcarp tree is important trees in tropical forest due its function in ecology and economy. Some spesies of dipterocarp are now endangered due to overcutting, habitat degradation, and also illegal logging. Hence, many studied focused on establishment of dipterocarp tree including the interaction of dipterocarp tree with microorganisms. Based on many studies, dipterocarp trees are very well-known for their strong relationship with ectomycorrhiza (Lee 1998). Other research results also reveal that dipterocarp trees also have interaction with plant growth promoting bacteria (Sitepu *et al.* 2007). There is still inadequate information

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about interaction between dipterocarp trees and fungal endophytes. Study of endophyte in dipterocarp tree was done by Orachaipunlap *et al*. (2008) and Pragathi *et al.* (2013), but this study focuses on foliar endophyte.

Considering the lack study of endophyte in tropical forest especially in dipterocarp tree, this study was primarily aimed to observed the fungal biological biodiversity in tropical forest. In addition, this is also preliminary study for bioprospecting fungi in order to support the idea of environmental friendly and cost-effective approach forest eshtablishment.

Methods

Site location The study was carried out in September 2013 to July 2014 in Dramaga Experimental Forest of the Forest Research and Development Agency, Minsitry of Forestry (FORDA) in West Java Indonesia (S6º33'7" E106º45'11"). Dramaga Experimental Forest total area is about 60 ha and the elevation is 244 m asl. Annual rainfall is about 350 mm and the soil type is reddish latosol. Minimum temperature is at 20.1ºC and 30.1 ºC at maximum.

Sample collection and isolation Root sample from seedlings of *S. leprosula* and *S. selanica* (height 30−50 cm) were randomly collected from Dramaga Experimental Forest, Bogor, Indonesia and carefully excavated. Five seedling were collected for each species. The collected seedlings were placed in plastic bag and covered with watered tissues to keep the freshness; the seedlings were taken to the laboratory for the next process on the same day.

Isolation of root endophytic fungi Secondary root from seedling of *S. leprosula* and *S. selanica* were washed carefully with tap water to remove adhered soil in root surface and then cut into some pieces. Surface sterilization was done by modified immersion process refers to Achlich &Sieber (1996) as follows 96% ethanol for 1 min, 20% H_2O_2 for 3 min, 96% ethanol for 0.5 min, and rinse with sterile water for 5 min. Root pieces were cut into segment into 0.5−1 cm in length, then transferred into Petri dish containing 1% malt extract agar (MEA) that are very common medium used for isolating endophyte fungi (Arnold *et al.* 2001). In total, 120 root segments of *S. Leprosula* and 126 root segments of *S. Selanica* collected from the field Petri dishes were incubated at room temperature until fungal growth appear (2−30 days). To ensure the quality of sterilization process, 100µl water of rinsed-water on petri dish containing 1% MEA. Fungi that growing out from the root tips were transferred into petri dish contain 1% MEA to get the single culture. These activities were done in sterile condition under laminar air flow hood.

DNA Isolation, PCR and sequencing The edge of filamentous fungal colony were suspended in tube containing liquid 1% MEA and incubated for 3−7 days. Harvested mycelia were transferred into microcentrifuge-tube and ground the mycelia with steriled mini-pestle. Fungal DNA Isolation was done by using Wizard Genomic Kit from Promega, USA. PCR was perfomed using 50µL solutions consist of 5µL 5X Go Taq Flexi buffer, 1 primer ITS1 (5'-TCCGTAGGTGAACC TGCGG-3') (10µM), 1 primer ITS4 (5'-TCCTCCGCTT ATTGATATGC3') (10µM), and 5µL DNA template. PCR reaction was done by this following processes: initial denaturation for 5 min at 94 ºC, denaturation for 1 min at 94 ºC, annealing for 1 min at 55 ºC, elongation for 2 min at 72 ºC, elongation for 7 min at 72 ºC after 24 cycle, and final extension for 4 min at 72 ºC. DNA fragment visualized with 2% Agarose-electroporesis Gel in TAE buffer for 32 min at 100 V. Good Quality DNA from PCR demonstrated by DNA bands submitted for DNA Sequencing. Fungi were amplified using universal primer ITS 1 and ITS 4. The sequences were compared with available DNA sequences in GenBank database of NCBI using BLAST programme. Phylogenetic tree was performed with software Clustal X 1.83 and MEGA 5.2.2.

***In vitro* phosphate solubilizing ability test** Fungal plug of each isolate was placed in the center of PVK media which containing following ingredient (g L^{-1}) (Sharma *et al.* 2011): glucose, 10.0; yeast extract, 0.5; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.1; $Ca_3(PO_4)_2$, 5; NaCl, 0.2; KCl,0.2; $MnSO_4.H_2O$, 0.002; $FeSO4.7H_2O$, 0.002; agar, 15. For each isolates, the experiment was performed in triplicates. Observation was done after 72 hours. Fungal ability in phosphate solubilizing was indicated by clear zone solubilization index (SI) were calculated by the ratio of total diameter (colony and clear zone) and colony diameter.

Antagonistic test of fungal endophyte against pathogen *Fusarium* sp. Antagonistic test of fungal endophyte was carried out with dual inoculation method. Fungal root rot pathogen of *Anthocepalus cadamba, Fusarium* sp. was obtain from plant pathogen laboratory of Faculty of Forestry, Bogor Agricultural University. Petri dish (d = 9 cm) contain PDA were used for this antagonistic test with 5 × 5 mm fungal plug. The fungal plug was placed in 3 cm distance from the edge, opposed on the same diagonal line. Three replications were used for this antagonistic test. Single culture test of each isolates were uses as control. Percentage of growth inhibition were calculated using the formula: 100 × (C – T/C), where C = control and T = treatment. Monitoring were done in 3rd and 7th days after inoculation.

Results and Discussion

Isolation of root endophytic fungi surface sterilization is common method to isolate endophytic fungi from root (Schulz *et al*. 1993; Bayman 2007; Silvani *et al*. 2008). In this research, we refer to sterilization method used by Achlich and Sieber (1996) and we modified this method to obtain maximum result (Hakim *et al.* 2014). The presence of root endophytic fungi in *S. leprosula* and *S. selanica* was indicated by mycelia growth from the tip of sterilized root segment. A total 16 isolates were isolated from *S. leprosula* (7 isolates) and *S. selanica* (9 isolates). Colonization percentage is relatively low (Table 1).

Percentage of root endophytes colonization are varied depending on tree species. Achlich and Sieber (1996) reported the colonization percentage of dark septate endophytes in *Fagus sylvatica* is 5−14% and in common Pinacea trees was 43–67%. In addition, Achlich and Sieber (1996) also generalized that fungal isolated in root tree of temperate forest was between 70−100%. Colonization percentage of endophytic fungi in this study is relatively low

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Table 1 Result of fungal endophytes Isolation from root of *Shorea leprosula* and *Shorea selanica*

Parameter	Host	
	Shorea leprosula	*S. horea selanica*
Number of root segment	120	126
Number of colonized root	7	9
Colonization percentage	5.8%	7.1%

compare to other researcher which are 5.8% in *S. leprosula* and 7.1% in *S. selanica.* Thus, the theory is not applicable in this study. Low percentage of fungal colonization suggests that there are competition between endophyte fungi and ectomycorrhiza fungi that live in the root of *Shorea* sp. and assumed that both of fungi employed the same niche. The cooccurence of mychorrizal and endophyte in plant root were identified (Fuchs & Haselwandter K 2004; Wagg *et al.* 2008; Reinenger Sieber 2012; Soteras *et al*. 2013; Toju *et al.* 2013). According to Reinenger and Sieber (2012), there are correlation between ectomychorriza dan root endophytes in conifer species. This study reveal colonization of ectomycorrhiza form a phisical and physiological barrier to Dark Septeate Endophytes *Phialocepala fortinii*. In addition, the greater colonization of mycorrhiza could reduce the colonization of endophytes in the roots. Soteras *et al*. (2013) reported that higher colonization percentage of dark septate endophyte in root *Polyepis australis* decreasing the colonization of arbuscular mycorrhiza fungi. Furthermore, this result relevant to the assumtion that the colonization of root and foliar endophytes also correlated with colonization of arbuscular mycorrhiza (Novas *et al.* 2009; Eschen *et al.* 2010). Therefore, we can generated that the colonization of root endophytes influenced plant-mycorrhiza.

Identification Observation of fungal growth in 1% MEA, showed that there are 5 distinctly morphospecies of root fungal endophyte. Each morphospecies was observed with microscope (Figure 1) and subsequently samples were identified by molecular analysis.

ITS regions were amplified using universal primer ITS1 and ITS4 which are common primer to identify endophytic fungi (Hallman *et al.* 2006). Five distinct morphospesies was obtained in this study (Table 2 and Figure 1). As expected, all PCR product showed a single visible band about 450−550 bp on agarose gel 2% (Figure 2). Sequencing result then compared with sequence available from the gene bank. The result performed with BLAST showed that 5 isolated endophytic fungi identified as *Trichoderma spirale, Velsacea* sp*, Melanconiela elisii*, *Chaetosphaeria callimorpha*, and *Trichoderma asperellum*. All identified fungi are belongs to Ascomycota fungi. This result support study result of that majority of endophytic fungi belong to fungi group Ascomycota (Petrini 1991; Kageyama *et al.* 2008). Host specifity also appear in this study. Fungi isolated from *S. leprosula* were different with fungi isolated from *S. selanica.* According to Schulz & Boyle (2005), endophytic fungi in tropical area are specific to particular host. Fungal specificity in particular host was affected by secondary metabolic produced by host plant (Bayman 2007).

Phylogenetic analysis was conducted by comparing the sequences of isolated endophytic fungi with the 18 sequences available on gene bank. The phylogenetic tree result showed that five isolated fungi during this study belong to 3 groups of fungal order which are Diaporthales, Hypocreales, and Chaetosphariales (Figure 3).

Valsaceae sp and *M. elisii* which are isolated during this study belong to fungi order Diaphorthales. Diaphorthales fungi were reported as a saprobes, pathogen, and endophyte in woody plant (Rossman *et al.* 2007). Study result of Toju *et al.* (2013) and De Souza *et al.* (2013) succesfully isolated some Diaphorthales fungi from *Quercus serrate* and mangrove plants. According to Sieber (2007), plants which belong to Angiospermae are dominated by endophytic fungi that belong to order Diaporthales and Hypocreales. *T. spirale* and *T. asperellum* belongs to fungi order Hypocreales. Hypocreales fungi characterized by its green conidia and fungi in order Hypocreales divided into two genuses which are Hypocrea and Trichoderma (Chaverrri *et al.* 2003).

Trichoderma sp., commonly categorized as soil saprophytes, but some researches also describes *Trichoderma* species as a plant endophytes (Achlich & Sieber 1996; Bailey *et al.* 2011; de Souza Sebastianez *et al.* 2013). In Brazilian mangrove forest, *Trichoderma* species known as the one of the most frequent species isolated during the research (8.72%) (de Souza Sebastianez *et al.* 2013). In addition, Endophytic fungi isolated from *Trichoderma hamatum* enhances seedling growth and delays onset in *Theobroma cacao* (Bailey *et al.* 2011). Regarding to this condition, it is assumed that *Trichoderma* categorized as multifunctional fungi that has role as a endophytic fungus and saprobe as well. Term of multifunctional fungi, mentioned by Brundrett (2006). Brundrett (2006) reported that some fungus have multifunctional role. For example: *Fusarium spp*., known to have role as root endophyte, pathogen, orchid mycorrhizae, and saprophyte. Nowadays, there are many studies working on *Trichoderma* sp. including its application on forestry. According to Widyastuti (2007) *Trichoderma* sp. can be use for pathogen biocontrol in pine, acacia, and teak wood. *Trichoderma* sp. as a biocontrol also studied by Santos-Vilabos *et al.* (2013). In their study, *Trichoderma asperellum* has significant effect against pathogen in mango fruit.

Chaetosphaeria callimorpha is a fungal species that belongs to fungus order Chaetosphaeria and genus Chaetosphaeria. Fungi in thus genus characterized with black and septate hypha (Reblova & Winka 2000). Another study of Chaetosphaeria also done by Wright *et al.* (2009), and the study result showed that Chaetosphaeria was identified as ectomycorrhiza in tropical forest.

***In vitro* phosphate solubilizing ability test** In vitro assay by using Pikovskhaya Media (PVK) to screening phospate solulubilizing ability by microorganisms is a common methods used by many researchers (Nautiyal 1999). Based on observation on PVK Media, out of 5 isolates obtained during this study only 2 isolates showed ability to produce clear zone in PVK media which are *T. spirale* and *M. elisii* (Table 3) with solubilization index are 0.12 and 0.64, respectively.

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Table 2 Identification of endophytes isolates

Isolat Code	Species Name	Order	Identification (%)	Accesion No.
SLA	*Trichoderma spirale*	Hypocreales	100	KC581162.1
SLB	*Velsalceae sp.*	Diaporthales	89	AB334109.1
SSC	*Melanconiela ellisii*	Diap orthales	89	JQ926271.1
SSD	*Chaetosphaeria callimorpha*	Chaetosphaeriales	86	AF178555.1
SSE	*Trichoderma asperlum*	Hypocreales	100	KF815050.1

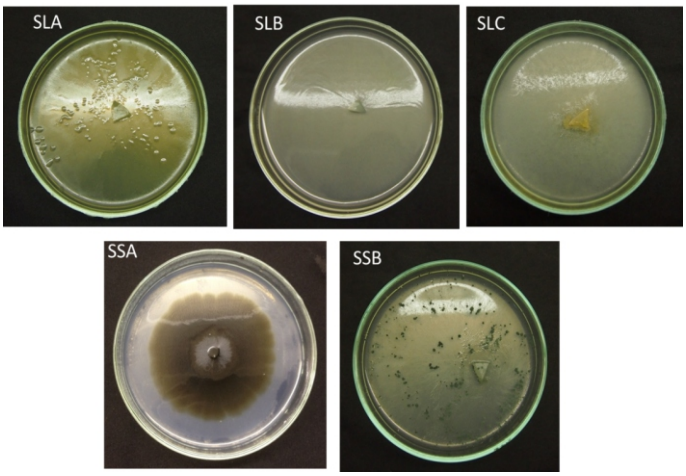

Figure 1 Fungal endophyte from root of *Shorea leprosula* and *Shorea selanica.*

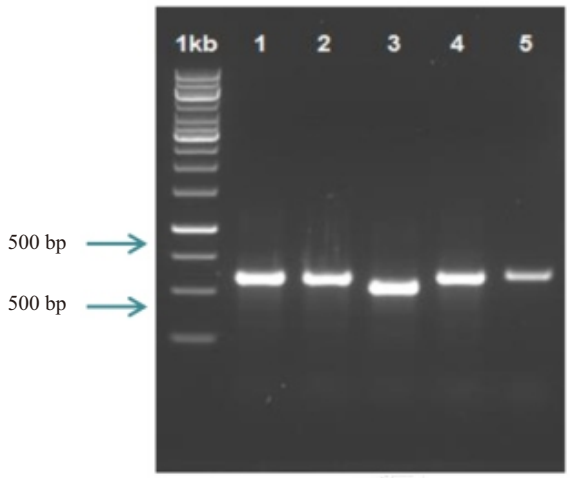

Figure 2 PCR amplification of sequence DNA with ITS1 and ITS 4 (Lane 1: Ladder; Lane 2,3,4,5,6 : SLA, SLB, SSC, SSD, and SSE).

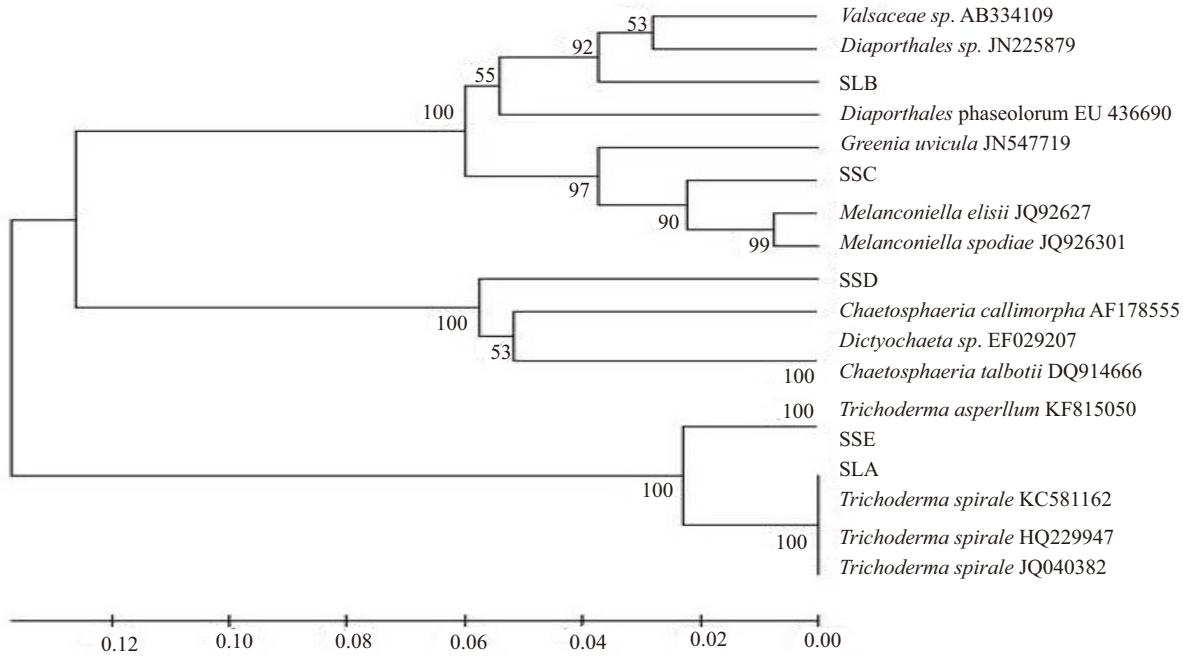

Figure 3 Phylogenetic tree of fungal root endophytes of *Shorea leprosula* and *Shorea selanica.*

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It is assumed that fungi with solubilize phosphate ability can produce clear zone has potential ability to support plant growth. This assumtion relevant with study result) that dark septate endophte isolated from *Atriplex canescens*, and *Aspergillus ustus* have ability to produce clear zone during *in vitro* test, can increase P content in inoculated plant compare with non-inoculated plant (Barrow & Osuna 2002). In some research, *Trichoderma sp*. has been recognize as a phospate solubilizing fungi (Altomare *et al.* 1999; Kapri *et al.* 2010; Rudesh *et al.* 2015). According to Rudesh *et al.* 2015, *Trichoderma sp*. has ability to produce organic acid which could solubilize insoluble phosphate. In addition, glasshouse experiment also reveal that the *Trichoderma* sp. also increase plant biomass.Therefore, it is assumed that fungal endophytes *T. spirale* also have potential to enhance plant growth. Unlike the *Trichoderma* sp., there are lack report about the ability of *Melanconiella elissii* to solubilize phosphate. However, it is assumed that the fungal with producing clearzone ability in vitro have similar mechanism.

Antifungal activity of fungal endophyte Naturally, there are many biological active compounds which have antifungal activity against fungal pathogens. These active biological compounds could be isolated from living organ such as plant leaves, bark, stem (Darma *et al.* 2006), and from fungal endophytes. *Trichoderma* sp. are the most frequently studied fungi as biocontrol agents for disease control and also studied for its secondary metabolites production (Verma *et al.* 2007; Widyastuti 2007; Hoyos-Carvajal *et al.* 2009). Therefore, antagonistic test of fungal endophyte and pathogen *Fusarium sp*. focused only on *Trichoderma* sp. which are isolated in this research. There are two *Trichoderma* species isolated during this research which are *T. spirale* and *T. asperellum*. Antagonistic test was carried out by dual inoculation method.

Dual culture test showed that endophytic fungi *Trichoderma* sp has growth inhibition effect against pathogen *Fusarium* sp. (Table 4), ilustrated that *Trichoderma* sp in 3 day after inoculation, radial growth inhibitionover

Table 3 Mycelia growth and clear zone of fungal endophyte from root of *Shorea leprosula* and *Shorea selanica* on PVK media after 3 days

Incubation time (days)	Antagonistic test (*Fusarium* sp.) species	Radial growth*	% Radial growth inhibition*
3	*Trichoderma spirale*	2.33	8.63%
	Trichoderma asperellum	1.97	22.75%
	Control		
7	*Trichoderma spirale*	2.63	45.58%
	Trichoderma asperellum	3.8	21.37%
	Control	4.83	

*Each value is the means of three replicates ± standard error

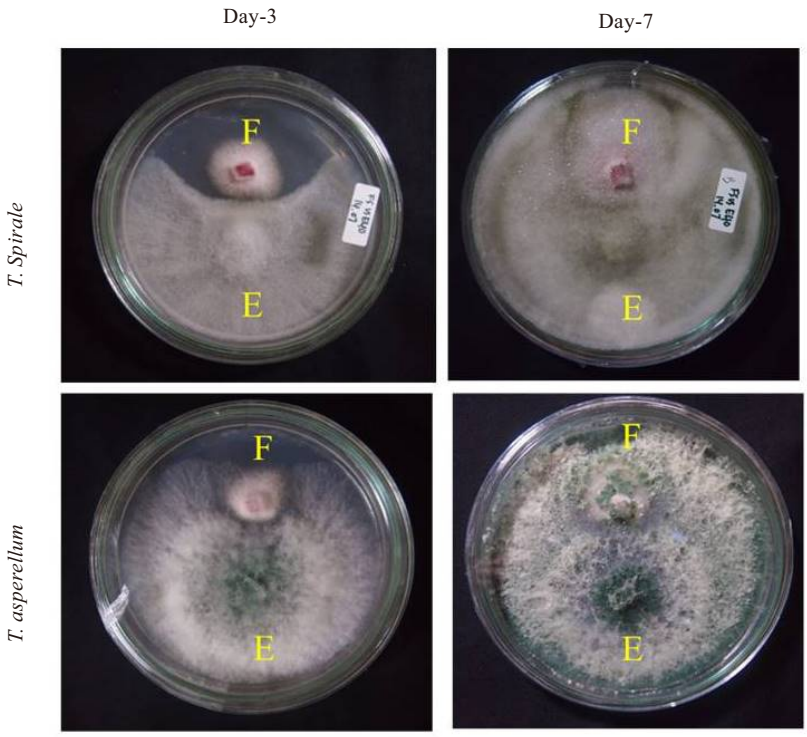

Figure 4 Antagonism test of endophyic fungi *Trichoderma spirale* and *Trichoderma asperellum* to pathogenic fungi *Fusarium* sp in 3rd and 7th days after inoculation (E = endophyte fungi; F = pathogen).

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Table 4 Evaluation of root endophyte *Trichoderma spirale* and *Trichoderma asperellum* against pathogen *Fusarium* sp. in 3rd and 7th days after inoculation

Species	Mycelia growth* (cm)	Clear zone* (cm)	Solubilization Index (SI)
Trichoderma spirale	8±0	0.98±0.13	0.12
Velsalceae sp.	4.62±3	0	0
Melanconiela ellisii	7.93±0.13	5.1±1.05	0.64
Chaetosphaeria callimorpha	0.65±0	0	0
Trichoderma asperellum	8±0	0	0

* Each value is the means of 3 replicates

control are 8.63% (*T. spirale*) and 22.75% (*T. asperellum*). In addition, seventh days after inoculation *T. spirale* and *T. asperellum* inhibited pathogen growth by 45.58% and 21.37% over control, respectively. The ability of Trichoderma species supress growth of pathogen *Fusarium* sp has been widely demonstrated (Cotxarrera *et al.* 2002; Srivastava *et al.* 2010; Bhale *et al.* 2013).

According to Verma *et al.* (2007), there are 3 mechanism modes of *Trichoderma sp.* fungi to inhibit pathogen fungi which are mycoparasitsm, antibiosis, and competition. Antagonism mechanism of *Trichoderma* sp. fungi against pathogen are involving many complex processes. In mycoparasitsm mechanism, there are several step which are host recognition process, penetration and killing the pathogen fungi. In this mechanism, fungi *Trichoderma* sp. release cell wall degrading enzym (CDWE) (Vinale *et al.* 2008). Unlike the mycoparasitism process that resulting the dead of the antagonist, in antibiosis mechanism, *Trichoderma* sp. only supressing fungal growth by space competition which resulting growth inhibition of antagonist fungi (Verma *et al.* 2007). In the near future, the ability of endophyte fungi to surpress plant pathogen growth area should include *in vivo* studies.

Conclusion

Isolation and *in vitro* assay of root fungal endophytes from *S. selanica* and *S. leprosula* suggested that fungal endophyte has potential ability as a biofertilizer and biofungicide. Based on in vitro test, fungal endophytes capable to solubilize phosphate. Furthermore, the dual inoculation between fungal endophytes and pathogen *Fusarium* sp. play a potential role of fungal endophytes to control the growth of plant fungal pathogen. This result suggest that fungal endophytes have a many undiscovered potential roles to support plant growth.

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