DETEKSI CENDAWAN ENDOFIT PADA TEMBAKAU (*Nicotiana tabacum* L.) DAN PENGARUHNYA TERHADAP KESEHATAN TANAMAN

THE DETECTION OF ENDOPHYTIC FUNGI ON TOBACCO (Nicotiana tabacum L.) AND THEIR EFFECT ON PLANT HEALTH

SKRIPSI

Oleh
AMIRUDIN AKHMAD FAUZI
NIM. 111510501141

PROGRAM STUDI AGROTEKNOLOGI
FAKULTAS PERTANIAN
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Diajukan guna memenuhi salah satu persyaratan untuk menyelesaikan
program sarjana pada program studi Agroteknologi
Fakultas Pertanian Universitas jember

Oleh
AMIRUDIN AKHMAD FAUZI
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PERSEMBAHAN

Dengan memanjatkan puji syukur kehadirat Allah SWT Yang Maha Pengasih lagi Maha Penyayang, Saya persembahkan Skripsi ini kepada :

1. Keluarga tercinta, ibunda Bawon Damayanti, kakakku Zulfikar Vanni Hanjaya dan istrinya Ratih Ekamaysla, Adikku Figo Muhaimin Yahya, Nenekku, dan Alm. Kakekku yang senantiasa memberikan dukungan dan do'a yang tiada henti kepada saya.
2. Semua Guru dan Dosen yang telah memberi bimbingan yang besar sepanjang hidup saya.
3. Almamater yang saya banggakan Program Studi Agroteknologi Fakultas Pertanian Universitas Jember.

MOTTO

"Barangsiapa sungguh-sungguh, sesungguhnya kesungguhannya itu adalah untuk dirinya sendiri" (*QS Al-Ankabut : 6*)

"Musuh yang paling berbahaya di atas dunia ini adalah penakut dan bimbang. Teman yang paling setia, hanyalah keberanian dan keyakinan yang teguh."
(*Andrew Jackson*)

"Kita melihat kebahagiaan itu seperti pelangi, tidak pernah berada di atas kepala kita sendiri, tetapi selalu berada di atas kepala orang lain."
(*Thomas Hardy*)

PERNYATAAN

Saya yang bertanda tangan di bawah ini:

Nama : Amirudin Akhmad Fauzi

NIM : 111510501041

Menyatakan dengan sesungguhnya bahwa karya ilmiah yang berjudul **Deteksi Cendawan Endofit pada Tembakau (*Nicotiana tabacum* L.) dan Pengaruhnya terhadap Kesehatan Tanaman**, adalah benar-benar hasil karya saya sendiri, kecuali jika dalam pengutipan substansi disebutkan sumbernya, dan belum pernah diajukan pada institusi manapun, serta bukan karya jiplakan. Saya bertanggung jawab atas keabsahan dan kebenaran isinya sesuai dengan sikap dan etika ilmiah yang harus dijunjung tinggi.

Demikian pernyataan ini saya buat dengan sebenarnya, tanpa adanya tekanan dan paksaan dari pihak manapun serta bersedia mendapat sanksi akademik jika ternyata dikemudian hari pernyataan ini tidak benar.

Jember, 25 Juni 2015

Yang menyatakan,

Amirudin Akhmad Fauzi
NIM. 111510501141

SKRIPSI

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Oleh:

AMIRUDIN AKHMAD FAUZI

NIM. 111510501141

Pembimbing:

Pembimbing Utama	: Ir. Hari Purnomo, M.Si., Ph.D., DIC.
NIP	: 19660630 199003 1 002
Pembimbing Anggota	: Ir. Sigit Prastowo, M.P.
NIP	: 19650801 199002 1 001

PENGESAHAN

Skripsi berjudul "**Deteksi Cendawan Endofit pada Tembakau (*Nicotiana tabacum* L.) dan Pengaruhnya terhadap Kesehatan Tanaman**" telah diuji dan disahkan pada :

Hari : Kamis

Tanggal : 01 Juni 2015

Tempat : Fakultas Pertanian Universitas Jember

DPU,	DPA,
Ir. Hari Purnomo, M.Si., Ph.D., DIC.	**Ir. Sigit Prastowo, M.P.**
NIP. 19660630 199003 1 002	**NIP. 19650801 199002 1 001**

Penguji 1,	Penguji 2,
Ir. Kacung Haryono, M.S., Ph.D.	**Ir. Sundahri, M.P.**
NIP. 19640814 199512 1 001	**NIP. 19670412 199303 1 007**

Mengesahkan
Dekan,

Dr. Ir. Jani Januar, MT
NIP. 19590102 198803 1 002

RINGKASAN

Deteksi Cendawan Endofit pada Tembakau (*Nicotiana* tabacum L.) dan Pengaruhnya Terhadap Kesehatan Tanaman; Amirudin Akhmad Fauzi; 111510501141; 2015; 51 halaman; Program Studi Agroteknologi Fakultas Pertanian, Universitas Jember.

Tembakau merupakan salah satu komoditas penting di Indonesia. Kualitas tembakau seringkali tidak tercapai akibat adanya serangan OPT sebagai faktornya. Kesehatan tanaman tembakau *on farm* banyak dipengaruhi oleh teknik budidaya yang masih konvensional dan rendahnya pengetahuan petani. Penyakit tanaman tembakau antara lain mozaik, lanas, dan busuk batang dapat menurunkan kualitas dan produksi tembakau. Cendawan endofit merupakan cendawan yang hidup didalam jaringan tanaman dan tidak bersifat parasit terhadap tanaman. Cendawan endofit dapat berperan sebagai bio-proteksi yang dapat melindungi tanaman dari serangan OPT terutama patogen penyebab penyakit. Penelitian ini bertujuan untuk mendeteksi cendawan endofit yang ada pada tanaman tembakau yang berkaitan langsung dengan kesehatan tanaman tembakau.

Penelitian ini memiliki 2 eksperimen yang berbeda. Eksperimen pertama menggunakan rancangan acak lengkap sederhana dengan 3 taraf dan 5 replikasi. 3 taraf tersebut antara lain adalah kontrol, media+Chaetomium dan media+Trichoderma. Sementara itu, eksperimen kedua menggunakan rancangan acak lengkap sederhana dengan 2 taraf ddan 5 replikasi. 2 taraf tersebut antara lain adalah media+Chaetomium dan media+Trichoderma yang masing-masing diinokulasikan cairan zoospora dari *Phytophtora* spp.

Hasil penelitian menunjukkan bahwa *Trichoderma* spp. dan *Chaetomium* sp. merupakan cendawan endofit yang berada di jaringan tanaman tembakau. Aplikasi kedua cendawan endofit ini tidak memberikan pengaruh nyata terhadap parameter pertumbuhan, akan tetapi memberikan perlindungan terhadap serangan *Phytophtora* spp. Cendawan *Trichoderma* spp. yang ditemukan merupakan cendawan *Trichoderma* spp. yang diaplikasikan pada media sesuai dengan hasil PCR yang dilakukan pada yang terdeteksi pada 1.5 kB.

SUMMARY

The Detection of Endophytic Fungi on Tobacco (*Nicotiana Tabacum* L.) and Their Effect on Plant Health; Amirudin Akhmad Fauzi; Study Program of Agrotechnology, Faculty of Agriculture, University of Jember.

Tobacco is an important commodity in Indonesia. Tobacco quality is oftenly unfulfilled because of biotic interference as the factor. On-farm plant health is much affected by conventional practice and unskilled farmer. Tobacco disease such as mosaic, black shank, and rotten stem are able to decrease tobacco quantity and quality. Endophytic fungi is a microorganism which stays or colonizes in the plants without incurring negative effect to the host. Endophytic fungi play role as bio-protection that protect the plant from biotic interference especially from plant pathogen. This research objective is to detect and determine the present of endophytic fungi on tobacco that relate with plant health.

This research contained 2 different experiments. The first experiment used complete randomized design with 3 levels and 5 replications. The levels include control, *Chaetomium*+media, and *Trichoderma*+media. Meanwhile, the second experiment used complete randomized design with 2 levels and 5 replications. The levels include *Chaetomium*+media, and *Trichoderma*+media which both of them were inoculated with zoospore of *Phytophtora* spp.

The result shown that *Trichoderma* spp. and *Chetomium* spp. were found as endophytes in the tobacco tissue. Application of these endophytes was not show significant different on tobacco plant growth, but both of them protected the plants from *Phytophtora* spp. infection. *Trichoderma* spp. which found in the tissue was declared as the same type with the inoculum that applied to the media by the result from PCR technique in 1.5 kB.

PRAKATA

Puji syukur kepada Allah SWT karena hanya dengan limpahan rahmat dan hidayah-Nya penulis dapat menyelesaikan skripsi yang berjudul **"Deteksi Cendawan Endofit pada Tembakau (*Nicotiana tabacum* L.) dan Pengaruhnya terhadap Kesahatan Tanaman"**. Skripsi ini disusun sebagai salah satu syarat untuk menyelesaikan Pendidikan Program Sarjana (S1) pada Program Studi Agroteknologi Fakultas Pertanian Universitas Jember. Penyusunan tugas akhir ini tidak terlepas dari bantuan berbagai pihak, oleh karena itu penulis ingin menyampaikan ucapan terima kasih kepada:

1. Dr. Ir. Jani Januar, M.T. selaku Dekan Fakultas PertanianUniversitas Jember.
2. Ir. Hari Purnomo, M. Si., Ph. D, DIC, selaku Dosen Pembimbing Utama dan Ir. Sigit Prastowo, M.P., selaku Dosen Pembimbing Anggota, yang memberikan perhatian, bimbingan, meluangkan waktu, dan pikiran sehingga skripsi ini dapat diselesaikan.
3. Ir. Kacung Haryono, M.S., Ph.D., selaku Dosen Penguji satu dan Ir. Sundahri, M.P., selaku Dosen Penguji dua yang memberikan arahan dan pikiran sehingga skripsi ini dapat diselesaikan.
4. Nanang Tri Haryadi, S.P., M.Sc., selaku Dosen Pembimbing Akademik yang telah membimbing selama menjadi mahasiswa.
5. Ibunda Bawon Damayanti, Kakak Zulfikar Vanni Hanjaya beserta istrinya Ratih Ekamayslah, Adik Figo Muhaimin Yahya, Nenek dan Alm. Kakek yang senantiasa menjadi cermin semangat penulis dalam menjalani kehidupan.
6. Segenap Dosen dan Tim *Student Exchange* di Fakultas Pertanian yang telah memberikan dukungan dan kerja kerasnya dalam membimbing penulis selama menjalani pertukaran pelajar di Kasetsart University, Thailand.
7. Dr. Chainarong Rattanakreetakul selaku Supervisor dan segenap civitas akademika di Kasetsart University selama penulis menjalani pertukaran pelajar di Kasetsart University, Thailand.

8. Kerabat-kerabatku Mbak Nungky, Dina Ajeng, Mas Ian, Mas Rahmat, Pisut Keawmanee, Ratchanok Piwtamai, Dewi Masitoh, dan teman-teman lainnya yang tidak dapat disebutkan satu per satu, terima kasih atas dukungan, motivasi, pengalaman, dan saran yang bermanfaat bagi kehidupan penulis.
9. Teman-teman IMAGRO (Ikatan Mahasiswa Agroteknologi), UKM CR (Chorus Rusticarum), dan UKM Plantarum yang telah bersedia membagi ilmu dan pengalaman berorganisasi selama menjadi mahasiswa.
10. Teman-teman Program Studi Agroteknologi angkatan 2011 dan semua pihak yang tidak dapat disebutkan satu persatu, terima kasih telah meluangkan pikiran dan memberikan semangat serta pengalaman hidup yang tidak terlupakan.

Penulis menyadari bahwa skripsi ini sangat jauh dari kesempurnaan. Oleh karena itu, dengan segala kerendahan hati penulis senantiasa mengharapkan saran dan kritik yang membangun demi kesempurnaan skripsi ini. Akhirnya penulis berharap, semoga skripsi ini dapat bermanfaat bagi semua pihak, terutama bagi perkembangan ilmu di bidang pertanian.

Jember, Juni 2015 Penulis

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CHAPTER I. PREFACE

1.1 Background

Tobacco (*Nicotiana tabacum* L.) is one of the most important commodities in Indonesia. Main product of tobacco is the leaves which become the main material of cigarettes. The demand of tobacco in the world is rising, for that reason farmers should increase the productivity of tobacco yield. Farmers have to manage their tobacco production technique till postharvest stage. Indonesia is one of the biggest tobacco exporters in the world. Since 1970 until 2007 Indonesian tobacco yield was under China (38.87%), Brazil (14.73%), India (8.43), USA (4.73%), and Argentina (2.86%) (FAO, 2009). Therefore, the productivity of tobacco yield is very important for Indonesia. Because it's yield give huge contribution for Indonesian economic.

Tobacco is an annual plant which grows on tropical country such as Indonesia with elevation between 0 until 900 ASL (Deptan, 2012). Indonesian tobacco is categorized on *Na-Oogst* and *Voor-Oogst* (Rachman, 2013). *Voor-Oogst* (VO) tobacco is the main material for the filler on the cigarettes, but *Na-Oogst* (NO) tobacco is the material for covering the cigar (*dekblad*). VO tobacco is usually planted on dry season. It is waterless, but requirement of water must be provided. NO tobacco is usually planted on rainy season, because it needs more water than VO tobacco. Perfect yield of NO tobacco have to be far from deformedless which commonly caused by the pests.

Farmers in Indonesia basically used their local wisdom to manage their field. Moreover, regeneration of the new farmers is very low. In 2013, the growth rate decreased about 6% from 31.17 million to 26.13 million farmers (Tempo, 2013). This condition was severed by the unstable climate. Climate became one of nature aspect that can determine the plant yield in which pest and disease emergence was related with. On farm management of tobacco pay so much attention from other sector. This condition relate with how the farmers overcome major pest problem. Uncontrolled application of pesticide causes many environmental issues. According to USEPA (United State Environment Protection Agency), more than

2,600 pesticide chemical ingredients are published in the market and more than 3,500 formulation of pesticide are marketed around the world. This enhancement happened especially on developed country (Yuliani *et al.*, 2011). Most of Indonesian farmers still depend on chemical pesticide to overcome pest invation which against the concept of sustainable agricultural.

Tobacco diseases is caused by various microorganism (include fungi and bacteria). They have their specific symptom which allowed us to diagnose and find the best way to control them. Plant protection is the most reliable activity to safe the product from disease disorder. Preventive and curative methods are the best way to protect the plants from biotic disturbance. The decreasing of chemical prevention is the purpose of sustainable agriculture and mostly supported by using endophytic fungi. Endophytic fungi are located on plant tissue such as leaves, branch, or on plant roots (Purwanto, 2008). Endophytic fungi produced some antibiotic compound to against the pathogenic bacteria or fungi (Worang, 2003).

In modern agriculture, the endophytic fungi can be used as a biological control which is the best way to protect plants from disease disorder. In this study, the detection of endophytic fungi on tobacco is very useful method to realize the sustainable agriculture.

1.2 Problem identification

There are several problems which identified based on the background described before.

1. What kind(s) of the endophytic fungi that can be used in tobacco (*Nicotiana tabacum* L.)?
2. What is the role of endophytic fungi to tobacco?

1.3 Objective

The main research objective was to detect and determine the effectifity of endophytic fungi in tobacco (*Nicotiana tabacum* L.) and relate with plant growth and health.

1.4 Benefit

1. To promote the used of biological control to reduce the disease disorder.
2. Implementation of the research will reduce the application of chemical pesticide, so tobacco plant will get better growth and health.

CHAPTER II. LITERATURE REVIEW

2.1 Tobacco in Indonesia

Tobacco (*Nicotiana tabacum* L.) is an annual plant which it is one of *solanaceae* family from genus *Nicotiana*. In this genus, *N. tabacum* is the famous one than another *Nicotiana's* plants such as *N. rustica*, *N. silvestris*, *N. glutinosa*, and *N. petunoides* which they are less valueable (Santoso, 2001 ; Deptan, 2012). They can adapt and develop well in a few region of Indonesia and it makes various common name of tobacco in Indonesia, such as Selopuro Tobacco, Temanggung Tobacco, Kendal Tobacco, Madurese Tobacco, and Paiton Tobacco. Tobacco is divided according to the harvested season as, *Na-Oogst* Tobacco (Harvested on the end of rainy season) and *Voor-Oogst* tobacco (Harvested on the end of dry season). But, according to the use of tobacco, it is grouped in to filler tobacco, cover of *cigarillos* (*dek-omblad*), filler of filter cigerettes (Rachman, 2013).

Indonesian tobacco is characterized as ascociated tobacco, as they have much cultivated by small farmer in Indonesia. Ascociate tobacco is produced to fulfil the market cigarettes needs (Santoso, 2001). Production of tobacco, especially for NO tobacco in Indonesia is focused on 3 region, respectively in Deli (Sumatra Island), Klaten (Center Java), and Jember (East Java). The region is divided based on the ecological system that very suitable to get the best production of NO tobacco. But, historically that area was managed to product VO tobacco which used as the filler of cigarettes (Djajadi, 2008).

2.2 Diseases on Tobacco

Tobacco production is affected by several factors and one of them is diseases disorder that can reduce up to 80% of tobacco production. Here is several diseases which commonly found in Tobacco cultivation.

a) Mosaic (Tobacco Mosaic Virus)

The infection of this disease is very easy, and commonly infected another host plant by the material of tobacco cultivation or by the friction between the

infected host plants to the healthy one. TMV is a soil borne virus which able to penetrate from injured roots. TMV live well on the debris in the soil and will infect the host plant whenever the injury happened on roots (Akin and Nurdin, 2003). TMV is considered as the most heat resistant plant pathogen, stable *in vitro,* and able to survive over 10 years in dried leaves and cigarettes or cigars (Lucas 1975). Therefore, the infected dried leaves, cigar or cigarettes can be the source of inoculums of TMV. In the field, the most common source of virus inoculums is the debris of infected plants which play role as reservoirs for virus transmission to the halthy plants. Inproper disposal of these plant residues contribute as the recycle of virus as pathogens (Conway, 1996).

b) Black Shank (*Phytophtora nicotianae*)

Black shank which it is caused by *P. nicotianae* is a dangerous tobacco disease especially for NO tobacco. The infection occurs from seeding till mature tobacco which ready to be harvested (Csinos and Bertrand, 1994). This disease can be detected by the symptom such as withered plant, chlorosis, and brown rotten stem and if it's splitted, heartwood sectional will appear. *P. nicotianae* is a soil borne fungi which live well in the soil and become the vector of Black Shank disease. Zoospore is flagellate and able to move well on water. The irrigation system and rainflow are the best way to spread the infection to the whole field. (Jaarsveld *et al*., 2002). Wet and moist condition is the suitable condition for *P. nicotianae* to infect the host plant. Black shank disease reported able to decrease about 25% from total yield product (Suripno and Yulianti, 2009).

c) Black Stalk (*Erwinia carotovora*)

E. carotovora infects the stem and make it rotten and also defective on the leaves. In the field, farmers are haunted by this disease because they can't solve and prevent the infection of this disease. *E. carotovora* infects the plants via the hole or the injury which it is caused by mechanical practice in the field. This bacteria move to the heartwood and caused necrose which later make a hole in it. Preventive and curative control of this disease must be applied as soon as possible to prevent the infection. Control can be implemented by using biological control to reduce the application of chemical pesticide (Yulianti and Suhara, 2009).

2.3 Endophytic Fungi as Biological Control

Endophytic is a microorganism which stays or colonizes in the plants without incurring negative effect to the host. It can help to protecting the host from biotic or abiotic interference. Every plant reported as the host of various endophytic fungi. Endophytic fungi have an important role to increasing the tolerance of drought and hold the expansion of herbivores insect, pathogenic fungi, virus, and roots nematode. As a biological control, endophytic fungi have a mechanism as parasitism, antibiotic, nutrient competition, and resistance induction. Endophytic fungi produce fungtional metabolism which include on terpenoids, steroids, xanthones, chinones, phenol, iso-coumarins, benzopyranones, tetralones, cytochalacins and enniatines which play role as antibacterial, antirival, and antifungal (Suryanarayanan *et al*, 2009).

Several endophytic fungi are able to decrease the infection of some pathogen treat. Endophytic fungi as *Chaetomium* sp. and *Phoma* sp. species have been successful to reduced number of pustuls and leaves widespreak attacks on grain caused by *Puccinia recondita* f.sp. *tritici*. Moreover, leaching media *Chaetomium* sp.and *Phoma* sp. isolates have been activated active defence reaction from the plant, that limit the multiplication of the pathogen (Dingle and Mcgee, 2003). Emergence of endophytic fungi has good chance as a biological agents, because fungi included on host plant system and suitable as promotor to introducing foreign gene on the plants tissue. Host of endophytic fungi can be manipulated genetically to produce the active compound which prospected for the host plants via genetic engineering, such as biopesticide (Petrini, 1992).

2.3.1 *Trichoderma* spp. as Endophytic Fungi

Species of *Trichoderma* Pers. (teleomorph Hypocrea Fr.; Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreales, Hypocreaceae) may occur mostly as soil inhabitants, saprophytes and parasites of other fungi (Samuels *et al*., 2002). Genus *Trichoderma* species have been described as opportunistic avirulent plant symbionts due to their abilities to benefit from and provide benefit in direct interactions with plants. The biocontrol capabilities of several *Trichoderma*

species have been extensively documented (Harman *et al.*, 2004). Some species of Trichoderma that also are found as endophytes in stems of woody plants include *T. aggressivum* Samuels & W. Gams, *T. caribbaeum* Samuels & Schroers, *T. erinaceus* Bissett, C. P. Kubicek & Szakacs, *T. evansii* Samuels, *T. hamatum* (Bonord.) Bainier, *T. harzianum* Rifai, *T. koningiopsis* Samuels, C. Sua´rez & H. C. Evans, *T. martiale* Samuels, *T. ovalisporum* Samuels & Schroers, *T. pubescens* Bissett, *T. spirale Bissett*, *T. stilbohypoxyli* Samuels & Schroers, *T. strigosum* Bissett, *T. stromaticum* Samuels & Pardo-Schulth, *T. theobromicola* Samuels & H. C. Evans, *T. viridescens* (A. S. Horne & H. S. Will.) Jaklitsch & Samuels and *T. virens* J. H. Mill., Giddens & A. A. Foster) Arx (Evans *et al.*, 2003, Crozier *et al.*, 2006, Samuels *et al.*, 2006, Hanada *et al.*, 2008, Samuels and Ismaiel 2009).

Fungal antagonistic *Trichoderma* species are considered as promising biological control agents against numerous phytopathogenic fungi include *F.oxysporum* (Sarhan *et al*., 1999). These filamentous fungi are very common in nature, with high population densities in soil and plant litters (Samuels, 1996). They are saprophytic, promptly growing and easy to culture, in addition to producing huge quantities of conidia of long lifetime (Mohamed and Haggag, 2006). *Trichoderma* species have shown efficiency on biocontrol of plant pathogens. According to Dolatabadi *et al*., (2012), *Trichoderma harzianum* can effectively avoid Lentil (*Lens culinaris*) from *Fusarium oxysporum* infection. In addition, *Trichoderma* has more report on the plant induced resistant to *Fusarium* species.

The ability of Trichoderma isolates to enhance plant growth has been characterized in other cropping systems, although the mechanisms involved have not been fully explained (Harman *et al.*, 2004). Plant growth promotion is often observed in response to Trichoderma colonization (Bae *et al*., 2009). Enhanced nutrient availability through solubilization and chelation of minerals and increased nutrient uptake efficiency, among others, are proposed mechanisms involved in Trichoderma-induced plant growth promotion (Altomare *et al*., 1999; Yedidia *et al*., 2001; Harman *et al*., 2004).

2.3.2 *Chaetomium* spp. as Endophytic Fungi

Chaetomium species are normally found in soil and organic compost. *Chaetomium* is one of the largest genera of saprobic ascomycetes with more than 300 species worldwide. *Chaetomium* species are potential degraders of cellulose and other organic material and can be antagonistic against various soil microorganisms. *Chaetomium globosum* and *C. cochlioides* are antagonistic to species of *Fusarium* and *Helminthosporium* (Soytong *et al.*, 2001). The mechanism of disease control is competition, antibiosis/lysis, antagonism, induced immunity in plants and hyphal interference. *C. cupreum* found to produce rotiorinol (Kanokmedhakul *et al.*, 2006) and *C. globosum* produces chaetoglobosin-c (Sibounnavong *et al*., 2011).

It has been found that by using specific strains of *C. globosum*, it is possible to obtain promising control over many plant pathogens. By coating seeds of corn with spores of *C. globosum* it was possible to prevent seedling blight caused by *Fusarium roseum* f sp. *cerealis* 'graminearum'. Such seed coating treatments were also found to reduce disease incidence of apple scab caused by *Venturia inequalis* (Soytong *et al*., 2001). It has also been reported that some isolates of *C. globosum* produce antibiotics that can suppress damping-off of sugar beet caused by *Pythium ultimum* (Di Pietro *et al.*, 1991). A further isolate of *C. globosum* was found to be antagonist against *Rhizoctonia solani* and *Alternaria brassicicola* (Soytong *et al*., 2001) and also reduced the quantity of sporulation of *Botrytis cinerea* on dead lily leaves exposed in the field (Kohl *et al.*, 1995).

Zhang *et al*., (2013) reported that *Chaetomium* have antifungal substance as *chaetoglobosin* and it can inhibit the growth of *Rizopus stolonifer*. The mycelia growths of 24 h cultivation of *R. stolonifer* and 72 h cultivation of *C. diplodiella* were nearly completely inhibited as *Chaetoglobosin A* concentration 40 and 20 g/mL, respectively. *Chaetoglobosin A* had acute antifungal activity against phytopathogenic fungi, which indicated that the studied propolis had the potential to be a natural preservative that can be applied to control plant diseases.

Endophytic mutualism can extend beneficial growth regulatory effects on host-plant under normal as well as extreme environmental conditions. In current

study, the *C.globosum* has significantly increased the shoot growth and allied growth characteristics of the host pepper plants. The plant had higher chlorophyll content, shoot biomass and leaf area compared to both the controls, indicating growth ameliorative impacts on plants. In endophyte-host symbioses, secondary metabolites may be a contribution of the endophytic partner for such mutualistic relationship. Plants treated with endophytes are often healthier than those lacking such interaction (Schulz and Boyle, 2005), which may be attributed to the endophyte secretion of phytohormones such as IAA (Khan *et al*., 2011) and GAs. Endophyte *C. globosum* produces various physiologically active and in-active GAs in its culture medium. It was observed that the culture biltrate of *C. globosum* significantly promotes the growth attributes of mutant rice *Waito*-C (Khan *et al*., 2012).

CHAPTER III. METHOD OF RESEARCH

3.1 Time and Place

This research was implemented during December 2013 till June 2014 in the Greenhouse and Laboratory of Physiology of Plant Disease, Department of Plant Pathology, Faculty of agriculture at Kamphaeng Saen Campus, Kasetsart University.

3.2 Research Method

3.2.1. Preparation

1. Preparation of Endophytic Fungi

Trichoderma culture was taken from Biological Control Laboratory, Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen campus, Kasetsart University. The culture was multiplied via steamed rice as carrier. Two hundred grams of fresh steamed rice was put into the plastic bag (1 kg size) with aseptic technique, sealed the plastic bag after the steamed rice filled in. After the temperature reduced, *Trichoderma* culture was poured to the rice about 0,5 g respectively. The plastic bag was stabbed with the needle to make aeration in to the plastic bag ecactly on the upside part. Number of the holes was about 15 holes to each bag and the bag was placed in the incubation room with room temperature and with lying position to get equal surface on *Trichoderma* growth. The incubation took about 3-4 days to be harvested. After *Trichoderma* completely growth in the steamed rice, then it was stored in the refrigerator.

Chaetomium spp. was collected from laboratory culture and the whole method for make *Chaetomium* inoculum was the same as explained in *Trichoderma* inoculum, except steamed corn subtituted steamed rice as the carrier.

2. Preparation of *Phytophtora* spp. Zoospore Suspension.

Phyophthora spp. was grown on V8 agar plates at 25 °C under dark condition. For zoospore production, mycelial plugs were transferred to a flask containing 25 mL of V8 vegetable juice and incubated in the dark at 25 ± 2 °C.

After 1-2 weeks, mycelial plugs were removed from the medium and macerated with sterile distilled water in a sterile blender for 30 s. Drops of mycelial suspension were placed onto the surface of water-agar plates using a sterile syringe. Sporangial formation in *Phytophthora spp.* was induced by removing uncolonized agar from around the mycelium and incubating the culture for an additional 3 days at 25 °C; plates were placed under fluorescent lights (40 W, daylight) at a distance of 12 cm. Zoospores were induced to release by incubating the culture plates in sterile water at 4°C at room temperature for 1 hour. The zoospores were collected and filtered through Whatman No. 54 to remove sporangial cases and mycelial. The concentration was then adjusted to 10^2, 10^3, and 10^4 zoospores per milliliter using a hemocytometer (Ward and Stoessl, 1974).

3. Tobacco Seedling Preparation

Tobacco was seedled using tobacco seed and organic matter as the media. Media was moisted with water addition. Tobacco seed was poured in to the media and then covered with double-layer filter paper to keep the moisture. After 20-25 days filter paper was removed from the media and then tobacco seedling grew untill reach the vigorous stage (about 40-45 days) and ready to be transplanted to the new media.

4. Media Preparation

Media was composed as sterilized soil and sterilized peatmoss. Soil was sterilized in mass media sterilization. Peatmoss was sterilized manually in autoclave with 121^{o}C and 72 psi/1atm for 15 minutes. Media was mixed with proportion 3:1 of soil:peatmoss (v/v). Media was added with 20 g of inoculum of endophytic fungi (*Trichoderma* spp. and *Chaetomium* sp.) as treated media, the rest which was not added with inoculum of endophytic fungi as the control media.

5. Detection of Endophytic Fungi Preparation

Detection of endophytic fungi used 2 different methods, tissue transplanting and mollecular method (PCR). Tissue transplanting method used PDA (Potato

Dextrose Agar) and RBA (Rose Bengal Agar) as the culture media. PDA contain of potato as carbon source, dextrose as sugar source and agar. RBA was a selective media contain of KH_2PO_4, $MgSO_4$, Rose Bengal, Agar, Peptone, and Glucose. PCR method was prefaced by DNA extraction using extraction kit (Thermo.ltd) for pure *Trichoderma* culture extraction. Trichoderma in tobacco plant parts were extracted by modified extraction which used lysis buffer (250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS), PCI, 100% ethanol, and TE buffer.

3.2.2. Experimental Design

This research contained 2 major experiments which separately runned. The first experiment which focused on efficacy of endophytic fungi on tobacco growth used simple complete ramdomized design with 3 treatments and 5 replications respectively. Treatments was responsible to Ch (*Chaetomium*+media), Tri (*Trichoderma*+media), and Co (Control). The second experiment which focused on effect of endophytic fungi on controlling *Phytophtora* spp used simple complete randomized design with 2 treatments responsible to Ch (*Chaetomium*+media), Tri (*Trichoderma*+media) and 5 replications.

Ch	Co	Tri
Ch	Tri	Co
Tri	Ch	Co
Tri	Co	Ch
Co	Ch	Tri

a

Ch	Tri
Tri	Ch
Tri	Ch
Ch	Tri
Tri	Ch

b

Figure 3.1. Experimental design (a: first ex. design ; b: second ex. design)

3.3.3. Research Procedure

1. Efficacy of Endophytic Fungi on Tobacco (*Nicotiana tabacum* L.) Growth.

The efficacy of endophytic fungi on plant growth was tested on tobacco seedling. The endophytic fungi were mixed with mixed sterile media. 41 days age

tobacco seedling which have already prepared in the nursery was planted to each treatments. The growth variable measurements included height, leaf number, stem size, and root length was concerned.

Three and five months after tobacco was transplanted, five grams of media in each treatment were taken for checking the availability of the both endophytic fungus. Five grams of the treated media was diluted upto 10^{-5} using sterilized deionizing water. Dilution 10^{-3} and 10^{-5} were used as the sample of colony availibility measurement. One hundred microliter (100µl) of the designed dilution was poured into RBA media with 3 replication respectively, so that each fungi will be responsible to 6 RBA plates. After 5 and 7 days of incubation, colony was counted manually according to the characteristic of each endophytic fungus.

In the end of plantation, some parts of tobacco, especially for the root zone and the lower stem was isolated using tissue transplanting method to detect the presence of the endophytic fungi.

2. Effect of Endophytic Fungi to Control *Phytophtora* spp.

Vigorous tobacco seedling was transplanted to peatmoss that have been inoculated with twenty gram of tested endophytic fungi. After seven days transplanted in the media, plants root were injured by pinning knife vertically surrounding the plants. Then, media were soaked with fifty milliliter of each fungus zoospore suspension. To accomplish the best distribution of the inoculum within the root system, each pot was watered immediately after inoculation with no water outflow (Mitchell and Rayside, 1986). Favorable ecology for disease development was created by watered the plants in 2-3 days intervals to keep the soil moisture stay high.

3. Detection of Endophytic Fungi Using Morphological Characteristic

Tobacco plant was removed from the media and washed using flown water. Tobacco plants are cut on 3 different parts, 2 parts on root zone (upper and lower root) and last part on stock near root zone. Tissue transplanting was applied to all sample and cultured on RBA and PDA then incubated on room temperature. Each

fungal mycelium will be identified morphologically (Legiastuti and Aminingsih., 2012).

4. Detection of Endophytic Fungi with PCR Method

a). Trichoderma DNA Extraction

Endophytic fungi which isolated from tobacco plant part were cultured on PDA for 4 days. After the fungus fully covered the PDA, the culture was chopped into small agar plug, about 0,5 cm. The agar plug was soaked into flasks contained 100 mL of Czapek Dox Broth and incubated the cultures at 25^{o}C for 3-5 days with an orbital shaker (120 rpm). Mycelia were harvested, washed with sterilized distilled water, frozen, and lyophilized. Total DNA was extracted from 50 mg of freeze-dried mycelia. DNA extracted was obtained using the DNA extraction kit according to manufacturer's instructions. The DNA extracted from each sample was resuspended in 100 ml of TE buffer (Rubio *et al.*, 2005).

b). Tobacco Parts Extraction

Tobacco parts were separated into small piece and grinded with lysis buffer. Plant solution was incubated at 55-65^{o}C for 1 hr followed by centrifugation at 12,000 rpm for 15 min. The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min. The supernatant was further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 15 min; the supernatant was then transferred in a new tube and the DNA was precipitated with chilled ethanol (100%). DNA was pelleted by centrifuged at 12000 rpm for 15 min and washed with 70% ethanol by centrifugation. The pellets were air dried and suspended with TE buffer (pH 8.0) (Chakraborty *et al.*, 2010).

c). DNA Amplification

Amplification of fungus DNA using a pair of primer SCAR a1 (5'-GGAAGCTTGGCGTTTATTGTACAA-3') and SCAR a1c (5'-GGAAGCTTGGGTATTGAGCTGGGC-3') (WardMedic, Inc). Each PCR

reaction contained 5.0 µl of fungal DNA, 1.0 µl of Taq polymerase, 2,5 µl of DNA polymerase buffer, 2,5 µl of each deoxynucleoside triphosphate, 2,5 µl of $MgCl_2$, 10 µl of H_2O, and 2,0 of soluted primer in a total volume of 25,5 μl. PCR conditions included an initial denaturation of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min 30 s at 65°C, and 2 min at 72°C, with a final extension step of 72°C for 7 min, with a thermal cycler. PCR products were analysed by electrophoresis in a 0.8% agarose gel in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8]), stained with Gel Star. Lambda pstl 24 (Fermentas, Inc) is used to be the marker (Rubio *et al.,* 2005).

3.2.4. Research Variable

Some parameters were measured on this research include:

1. Plant Growth Development
   a. Plant height (cm): Measured from the surface of the media till the last growing point. Measurement was implemented once in a month.
   b. Leaf number (unit): Measured by counting the vigourous leaves manually. Measurement was implemented once in a month.
   c. Stem diameter (cm): Measured using vernier caliper in the 2 cm above the surface. Measurement was implemented once in a month.
   d. Root length: Measured from the root stock to the end of the root parts below. Measurement was implemented in the end of plantation.
2. Endophytic fungi colony (cfu/g): measured by counting the colony manually and converted the number into cfu/g.
3. Mortality

Plant mortality was responsible to the respon of tobacco plant on *Phytophtora* spp. inoculation. Data was recorded by counting the dead plant and presentaged the number of missed plant using formula:

$$M = \frac{a - b}{b} \times 100\%$$

Note :

a : number of healthy plant(s) b : number of total plants

3.2.5. Data Analysis

Data recorded was analyzed using ANOVA (Analysis of Variance) and then continued with mean square test (post hoc) using DMRT (Duncan Multiple Range Test) with 95% significant level used R-Stat (Freeware) to found out the difference of each treatment applied.

CHAPTER IV. RESULT AND DISCUSSION

4.1 Result

4.1.1 Effect of Endophytic Fungi on Tobacco Growth

The effect of *Trichoderma* and *Chaetomium* treated media was evaluated on tobacco growth. Tobacco was grown in the growing media (3:1 soil:peatmoss v/v) added with Trichoderma and Chaetomium inoculum separately. The variable of plant height, leaf number, stem size, and root length was determined. Growth comparasion of each treatments based on ANOVA result was described below.

Table 4.1. Analysis of varians (ANOVA) on effect of endophytic fungi on tobacco growth variable.

Source of Variation	df	F-value	Pr>F
Height	2	$2{,}545^{ns}$	0,12
Leaf number	2	$2{,}668^{ns}$	0,11
Stem size	2	6,536*	0,012
Root length	2	938,9**	0,000000655

*) significant in α=0.05 ; **) significant in α=0.01

Endophytic fungi were observed on tobacco plant growth for 5 months observation. Analysis of varians was tested using Duncan Multiple Range Test (DMRT) with alpha (α) = 0,05. Result on tobacco plant height varian as the effect of endophytic fungi addition on the media was shown on figure 4.1. Tobacco plant height average was not significantly different between control treatment and endophytic added media treatments both on *Chaetomium*+media and *Trichoderma*+media on the whole months. This result claimed that endophytic fungi addition to the media didn't play role on tobacco plant height variable. Based on the tren of the figure 1, shown that *Trichoderma*+media have potential effect compared than both control and *Chaetomium*+media treatment. Result on leaf number varian shown that there was no significant differ between leaf number on control treatment with *Trichoderma*+media treatment in the last month (Figure 4.2). The difference was shown at control treatment versus *Chaetomium*+media treatment. Stem size varian result shown that both treatments didn't give any

contribution on tobacco stem development untill the last month (Figure 4.3). The last growth variable, root length, shown significant difference between treatments (Figure 4.5). *Trichoderma*+media treatment gave the best result with 21,6 cm average and significantly different compared with *Chaetomium*+media treatment with 16,3 cm average and control treatment with 14,2 cm average.

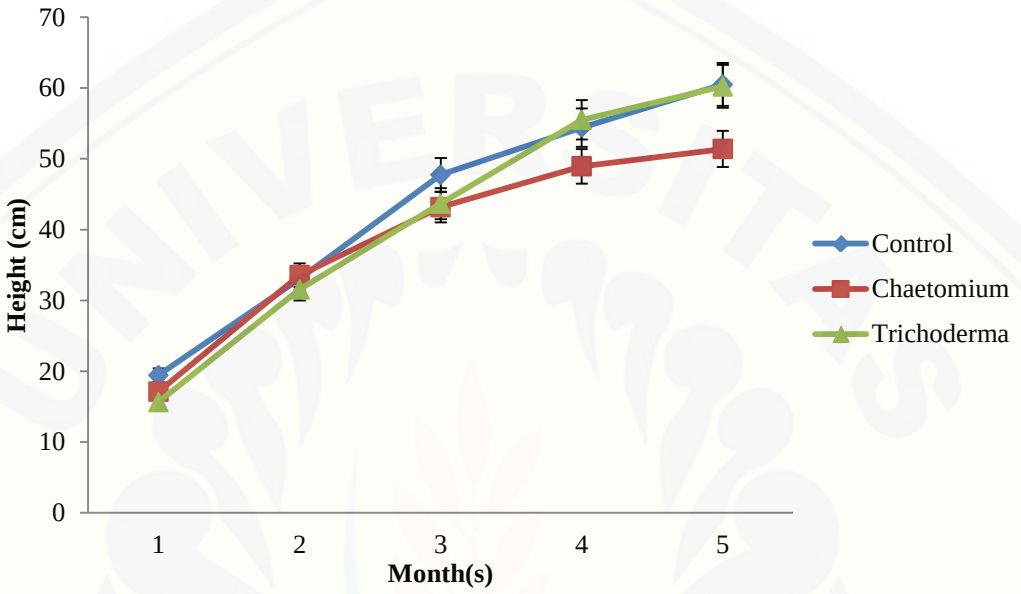


Figure 4.1. Tobacco plant height (each number on each month which followed with the same letter do not differ significantly at α=0.05 according to Duncan test)

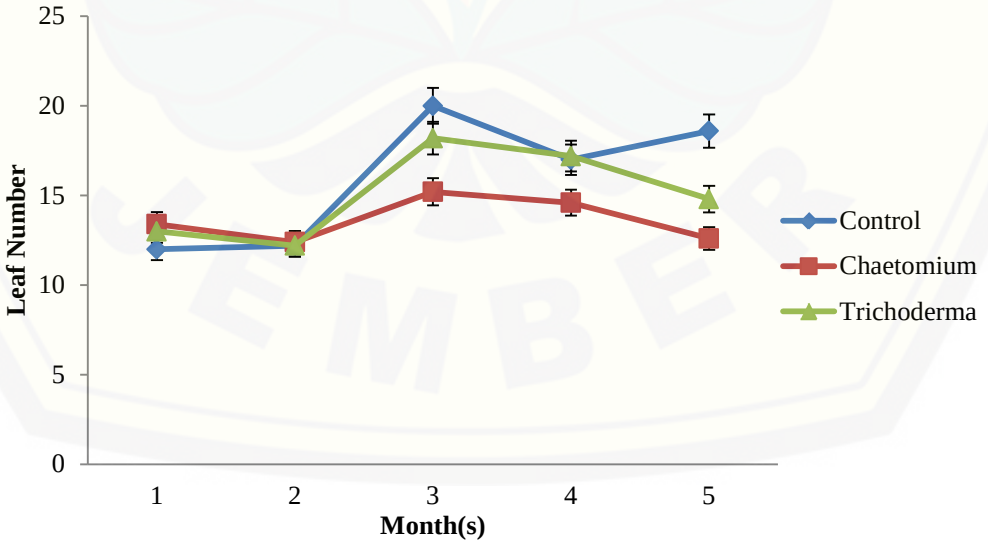


Figure 4.2. Tobacco leaf number (each number on each month which followed with the same letter do not differ significantly at α=0.05 according to Duncan test)

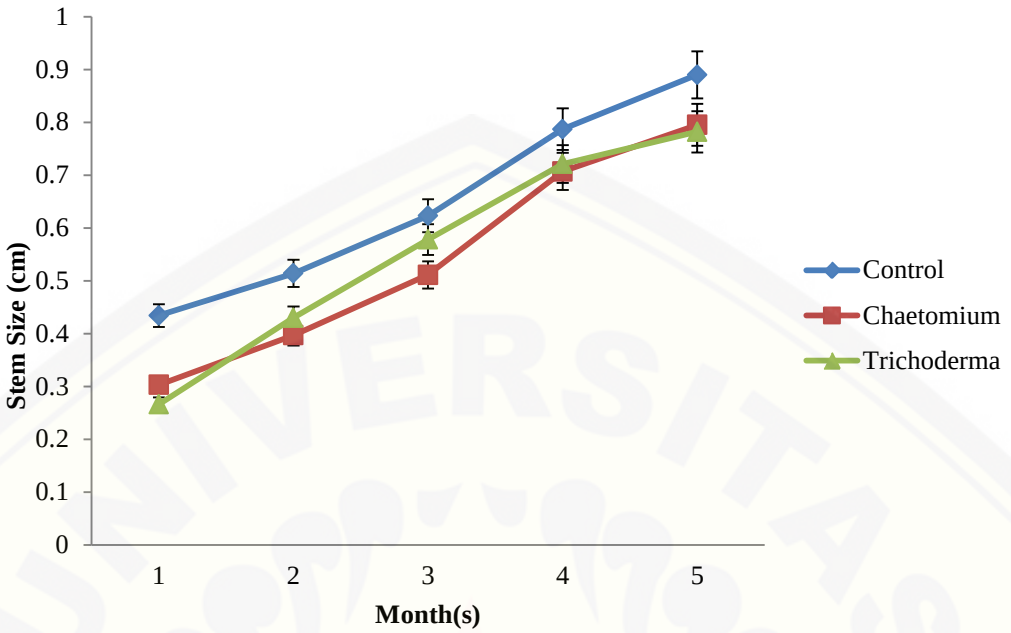
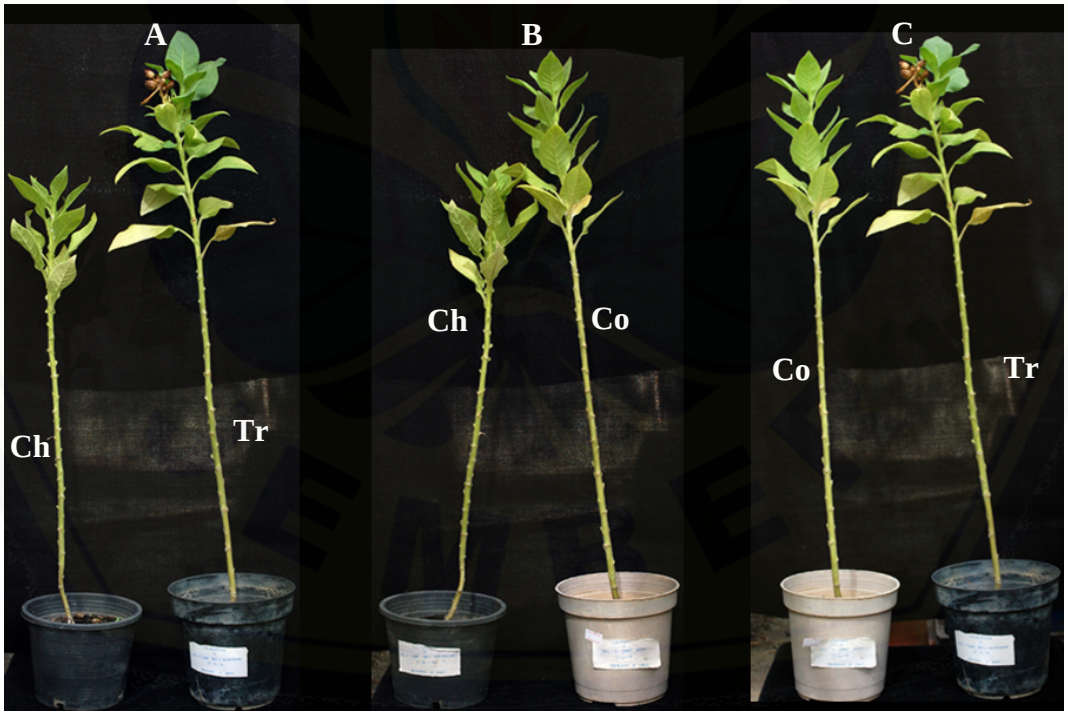


Figure 4.3. Tobacco stem size (each number on each month which followed with the same letter do not differ significantly at α=0.05 according to Duncan test)



(Co: Control ; Ch: Peatmoss+*Chaetomium* ; Tr: Peatmoss+*Trichoderma*)

Figure 4.4. Growth comparasion between treatments after 5 months plantation.

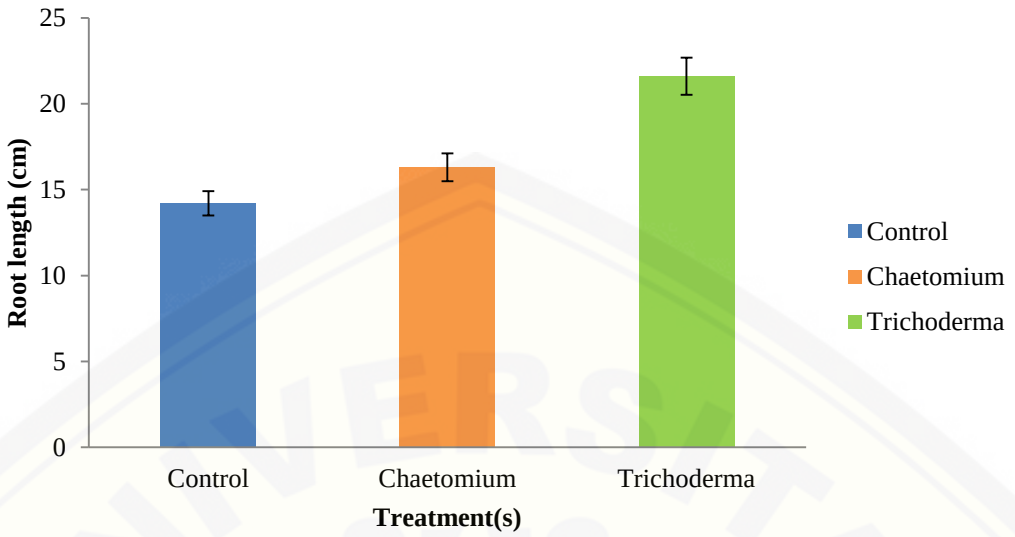


Figure 4.5. Tobacco root length (each number on each treatment which followed with the same letter do not differ significantly at α=0.05 according to Duncan test)

(Co = Control; Ch = *Chaetomium*+media; Tri = *Trichoderma*+media)

Figure 4.6. Root length comparision between treatments after 5 months plantation

Persistancy of applied endophytic fungi to the media was determined on 3 month and 5 month after planting [MAP] (Table 2). The result shown that applied *Chaetomium* sp. was decrease from 2,53 x 10^6 in the 3 MAP to 8,5 x 10^5 in the 5

MAP. Otherwise, *Trichoderma* spp. persistancy was increase from 1,3 x 10^3 in the 3 MAP to 6,6 x 10^5 in the 5 MAP.

Table 4.2. Population of Endophytic fungi on media

Time[1]	Endophytic Fungi	
	Chaetomium (cfu/g)	Trichoderma (cfu/g)
3 MAP	2.53 x 10^6	1.3 x 10^3
5 MAP	8.5 x 10^5	6.6 x 10^5

[1]Months After Planting.

Several endophytic fungi were isolated from tobacco plant parts include root-low part, root-basepart, stem-base part. Kind(s) of endophytic fungi observed was described below.

Table 4.3. Number of identified potential endophytic fungi (according to the number of plant parts transplanted).

No	Fungi	Numbers founded			
		Co[1]	Cha[2]	Tri[3]	Total
1	*Fungi 1*	3	1	6	10
2	*Fungi 2*	0	0	1	1
3	*Fungi 3*	0	0	1	1
4	*Fungi 4*	6	0	0	6
5	*Fungi 5*	3	0	0	3
6	*Fungi 6*	17	47	4	68
7	*Fungi 7*	0	0	11	11

[1]Control; [2]Chaetomium treated media; [3]Trichoderma treated media. Total of tissue transplanted was 144.

Identification was implemented based on morphology and microscopic character using Barnett and Hunter (1972) imperfecti fungi taxonomy.

1. Fungi 1

Colony color was grey and growth was looks circular and mycelia was fibrous and thick. From bottom plate, looks clearly the circular margin and have dark gradient color (Figure 4.7). The color was brighter on young mycelia than the old one. The growth of this fungus was fast.

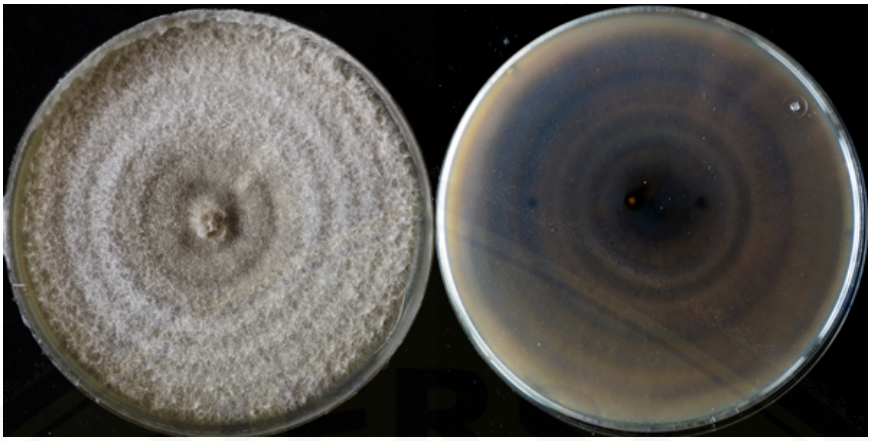
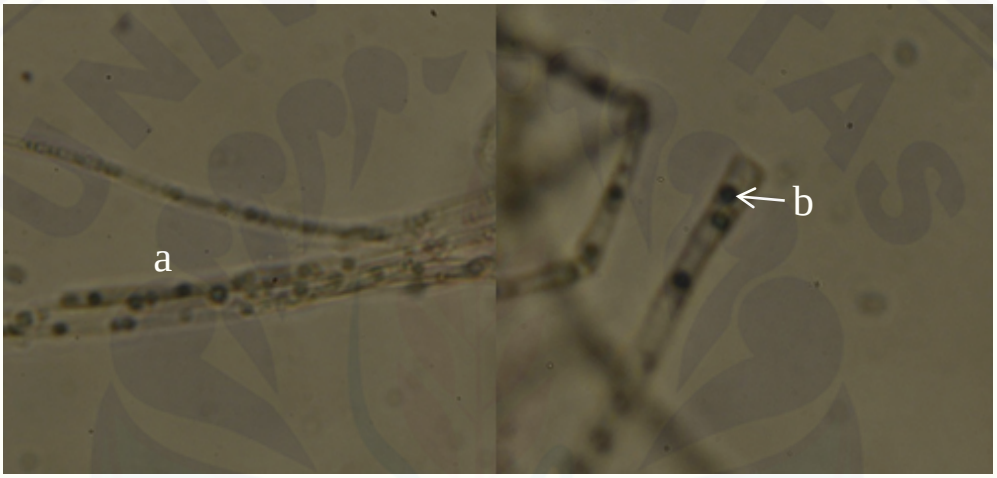


Figure 4.7. Fungi 1 growth on PDA from top and bottom view



(a. Hiphae, b. Nucleus)

Figure 4.8. Microscopic view of fungi 1

Microscopic view shown that this fungi didn't have spore. Hypha was nonseptate and has nuclei along the hypha. Hypha is in single form, rather not have branching (Figure 4.8). This fungi can not be identified because of no evidence of conidia shown under microscope observation.

2. Fungi 2

Colony on fungi 2 was looks white with fibrous mycelia and thick (Figure 4.9). Growth was looks as irregular circular and has orange color from bottom view.

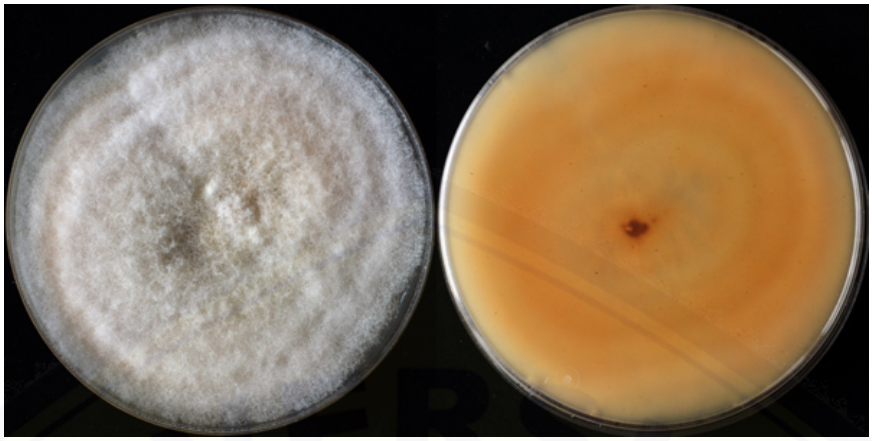
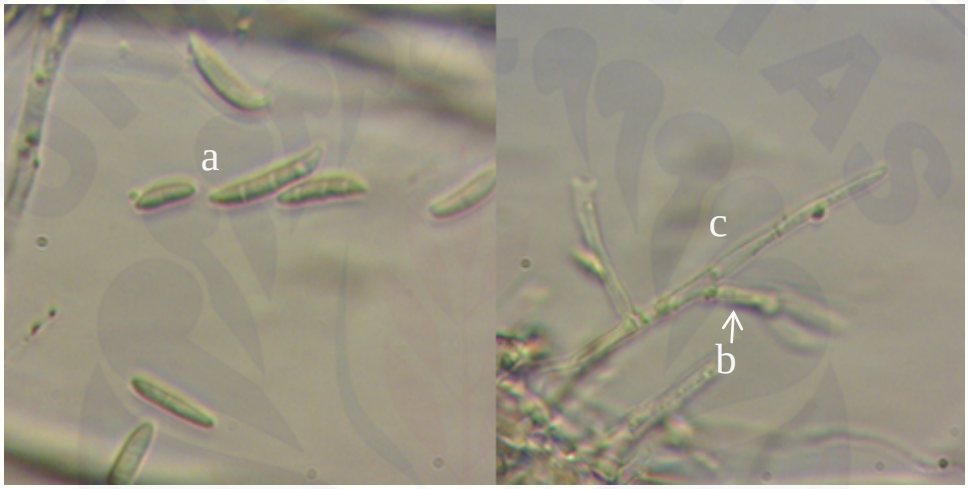


Figure 4.9. Fungi 2 growth from top and bottom view



(a. Conidia, b. Conidiophore, c. Hypha)
Figure 4.10. Microscopic view of fungi 2

Microscopic view shown that Fungi 2 have long elipsed conidia and hyalined by 3. Conidium was looks like rice grain and curved. Conidiophore was long and septated hyphae with rare nucleus (Figure 4.10). According to Barnett and Hunter (1972) taxonomy, this fungus was identified as *Fusarium sp* from Tubercularia family and mostly became plant pathogen.

3. Fungi 3

Colony of this identified fungus was white and has thin mycelia. Growth looks circular and mycelium was spread out fast. In this picture, looks the fungi 3 was inhibted by chaetomium which being the contaminant in the culture (Figure 4.11).

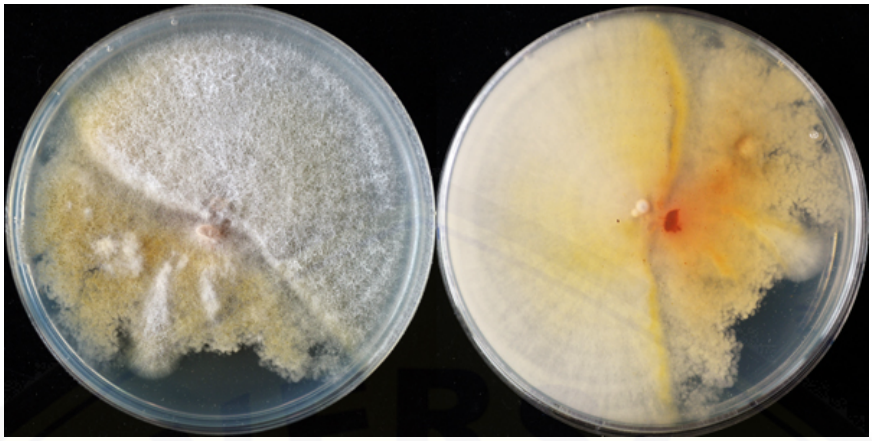
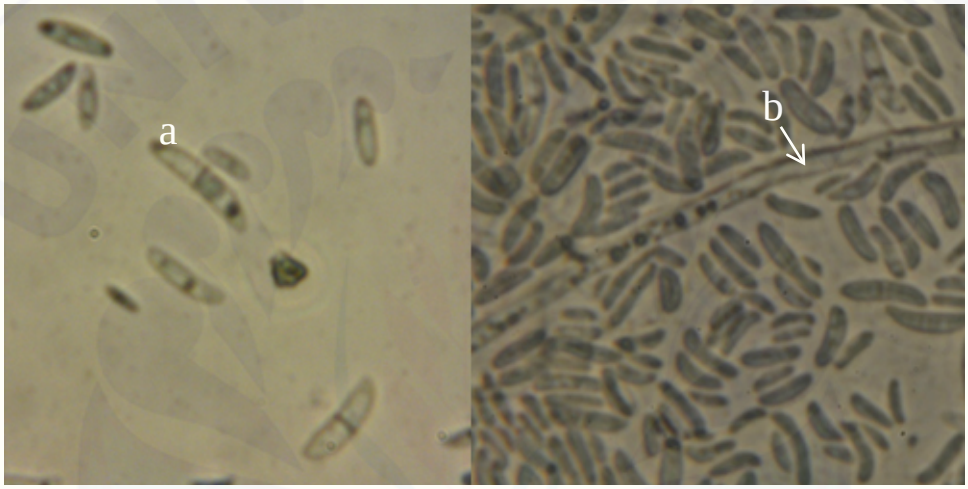


Figure 4.11. Fungi 3 growth from top and bottom view



(a. Conidia, b. Hyphae)
Figure 4.12. Microscopic view of fungi 3

Fungi 3 had elipsed and curved conidia and hylined by 1. Nucleus also appeared inside the conidia. Conidium was dispersal and nearly covering the hyphae. Hypha was nonseptated with rare nucleus inside (Figure 4.12). From Barnett and Hunter taxonomy, this fungus was known as *Colletrotichum sp* from Glomerellaceae family and mostly play role as plant pathogen.

4. Fungi 4

Colony was looks dark greeny with circular growth. Mycelium was fibrous and thick. Colony margin was shown clearly circular from bottom view. Darker color was shown on the center of culture (Figure 4.13).

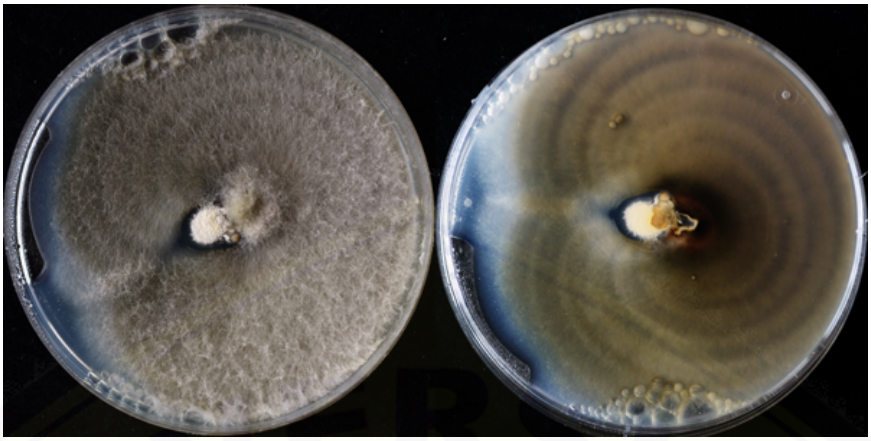
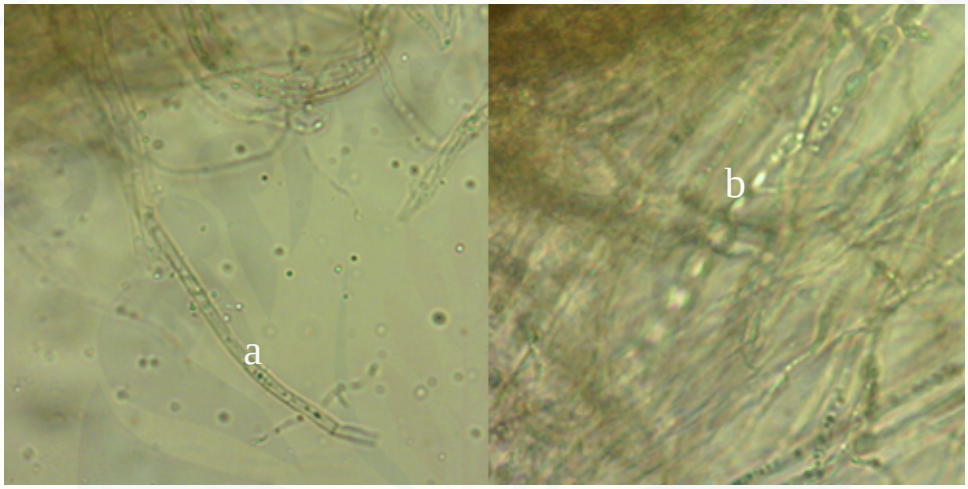


Figure 4.13. Fungi 4 growth from top and bottom view



(a. Hyphae, b. Looks like Clamydiophore)
Figure 4.14 Microscopic growth of fungi 4

Microscopic view of fungi 4 shown that hypha was septated with nucleus inside. From the overview, did not show both conidiophore and conidia or even spore also (Figure 4.14). This fungus was difficult to be identified because the fungi didn't have conidia produced.

5. Fungi 5

Colony color was dark in the center of culture and gradiented into chalk white in the edge of culture. Culture was flat and growth with circular pattern. The colony did not have any different between top and bottom view (Figure 4.15).

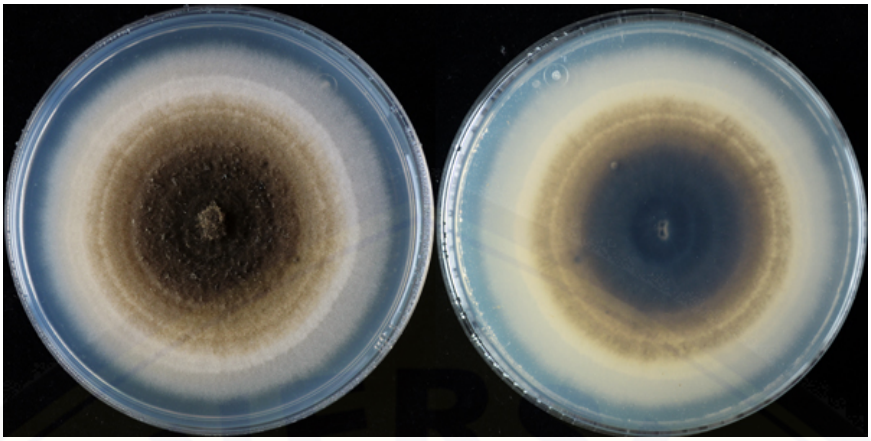
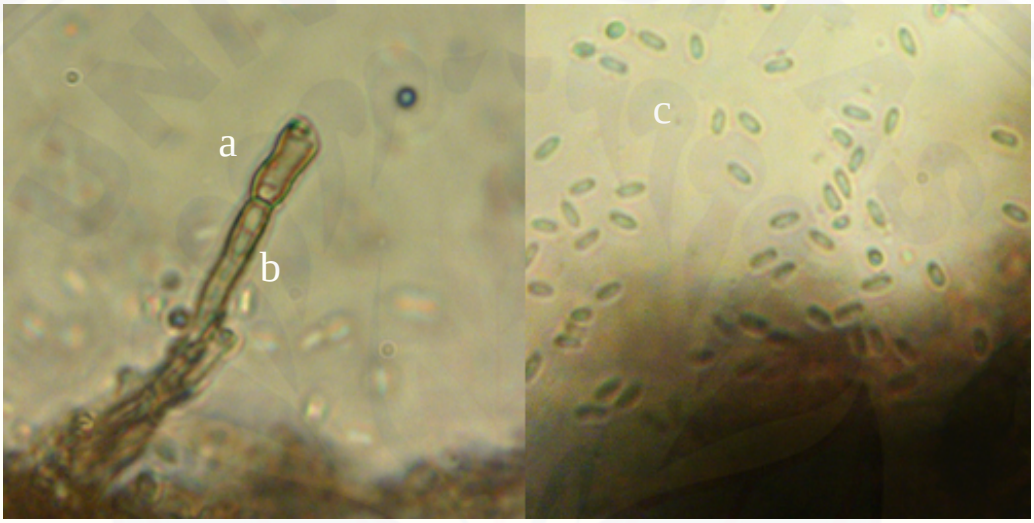


Figure 4.15. Fungi 5 growth from top and bottom view



(a. Conidiophore, b. Hyphae, c. Conidia)
Figure 4.16. Microscopic view of Fungi 5.

Microscopic view on fungi 5 was shown that conidia looks dispersal and have nucleus on both edge. Conidium was small oval and short. Hyphae was nonseptate and nucleus inside the hypha. Conidiophore on small form and bordered with septate (Figure 4.16). From the characteristic and matched with Barnet and Hunter taxonomy, this endophytic fungus was identified as *Cladosporium sp.* from Davidiellaceae family and mostly as plant pathogen.

6. Fungi 6

Culture was fibrous white on the center and greenish on the edge irregularly surrounding the center. From bottom view, the center of culture shown

have yellow colour. The culture was identified as *Chaetomium* which applied on the *Chaetomium* treatment (Figure 4.17).

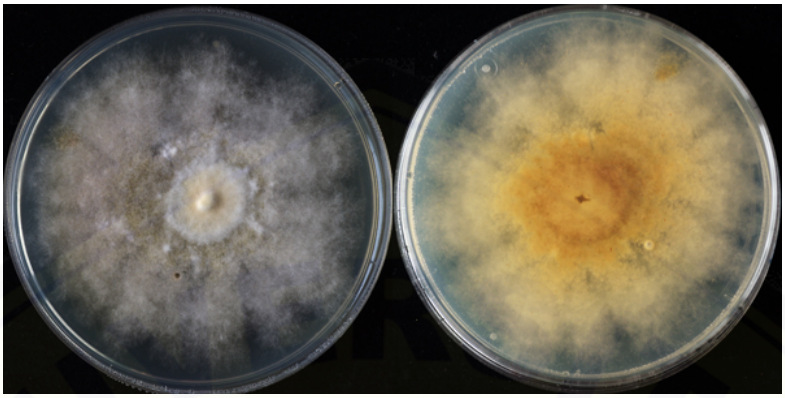
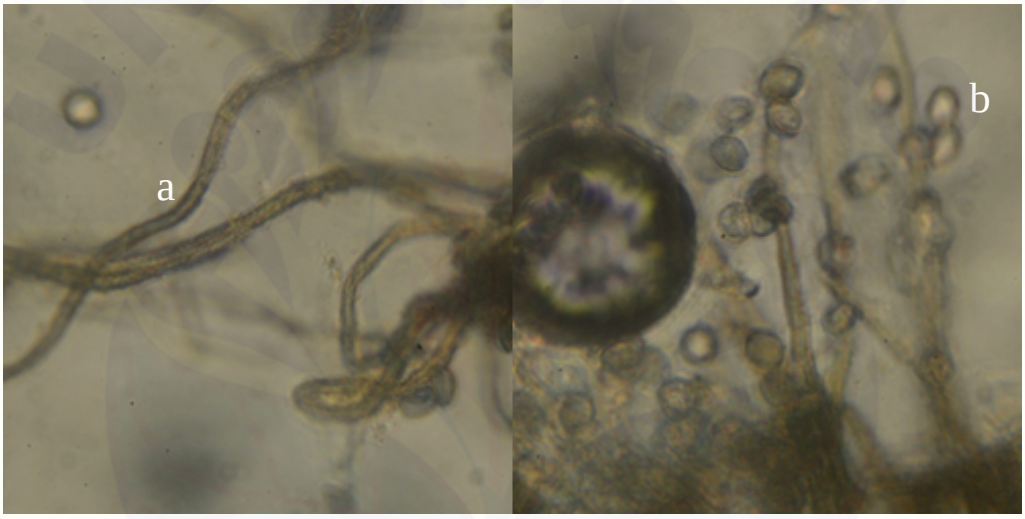


Figure 4.17. Fungi 6 growth from top and bottom view



(a. Hyphae, b. Ascospores)
Figure 4.18. Microscopic view of fungi 6

This fungi was known as *Chaetomium spp* according to the characteristic which shown both in the microscopic view and macroscopic view (Figure 4.18.). Septate hyphae, perithecia, asci and ascospores are visualized. Perithecia are large, dark brown to black in color, fragile, globose to flask shaped and have filamentous, hair-like, brown to black appendages (setae) on their surface. Perithecia have ostioles (small rounded openings) and contain asci and ascospores inside. Asci are clavate to cylindrical in shape and rapidly dissolve to release their ascospores (4 to 8 in number). Ascospores are one-celled, olive brown in color,

and lemon shaped (de Hoog *et al.*, 2000). This fungus was identified as potential endophytic fungi according to the role based on the literature reviewed.

7. Fungi 7

Colony was greenish and white in the surface of colony. Colony was fibrous with thick mycelia. Colony was glaucous on the bottom view with grey majority on the center of culture (Figure 4.19). This fungus was identified as *Trichoderma* which founded on *Trichoderma* treatment media. The last fungi which isolated from tobacco plant was identified as *Trichoderma* spp. (Figure 4.20). Septate hyaline hyphae, conidiophores, phialides, and conidia are observed. Conidiophores are hyaline, branched, and may occasionally display a pyramidal arrangement. Phialides are hyaline, flask-shaped, and inflated at the base. They are attached to the conidiophores at right angles. The phialides may be solitary or arranged in clusters. Conidia are one-celled and round or ellipsoidal in shape. They are smooth or rough-walled and grouped in sticky heads at the tips of the phialides. These clusters frequently get disrupted during routine slide preparation procedure for microscopic examination. The color of the conidia is mostly green (de Hoog *et al*., 2000,). This fungus was identified as potential endophytic fungi according to the role based on the literature reviewed.

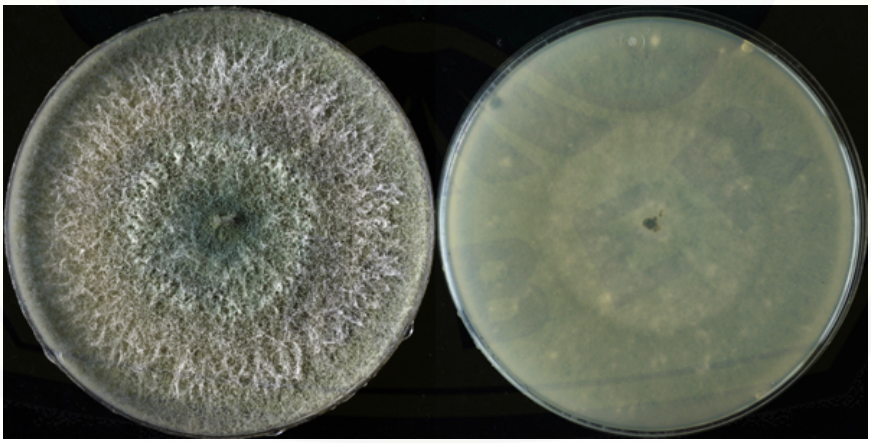
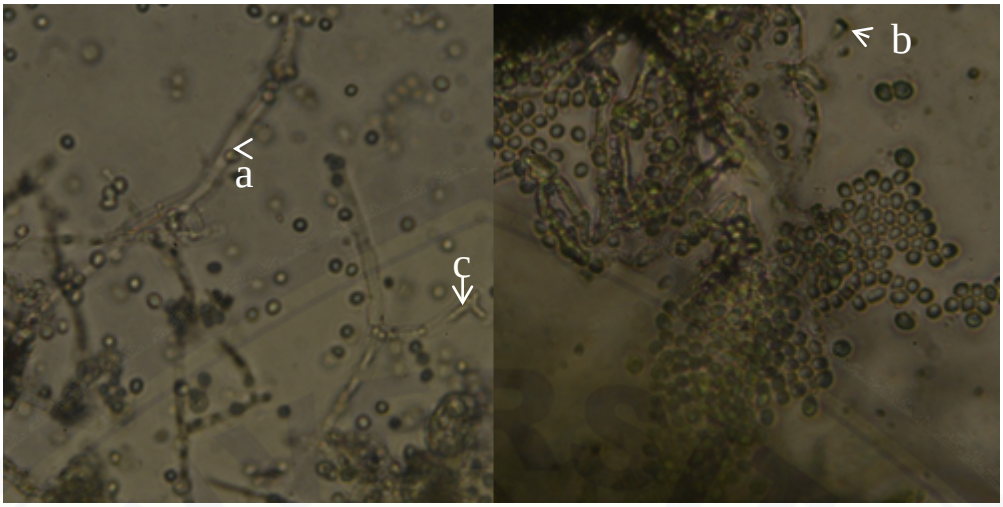


Figure 4.19. Fungi 7 growth from top and bottom view



(a. Hyphae, b. Spores, c. Phialide)
Figure 4.20. Microscopic view of fungi 7

4.1.2 Effect of Endophytic Fungi Aginst *Phytophtora* spp. Infection

Two endophytes, *Chaetomium* sp. and *Trichoderma* spp., were studied further against plant pathogen infection. *Phytophtora* spp. was known as the major disease which commonly appeared at seedling period even also in mature tobacco. Several variables include plant growth variable such as plant height and leaf number was measured and with addition of mortality variable as plant health defence variable. Significant difference was shown on tobacco plant height between *Trichoderma*+media and *Chaetomium*+media treatments. The difference was shown from week 4th untill the last data gained. On the last record, *Trichoderma*+media induced tobacco plant height untill 49.15 cm average and *Chaetomium*+media induced tobacco plant height reached 26,62 cm average. According to DMRT with α=0.05 shown that *Trichoderma*+media treatment was differ signicanly compared than *Chaetomium*+media treatment (F-value = 8.513, Pr>F = 0.0194). This result claimed that *Trichoderma*+media treatment induced tobacco seedling better than *Chaetomium*+media treatment.

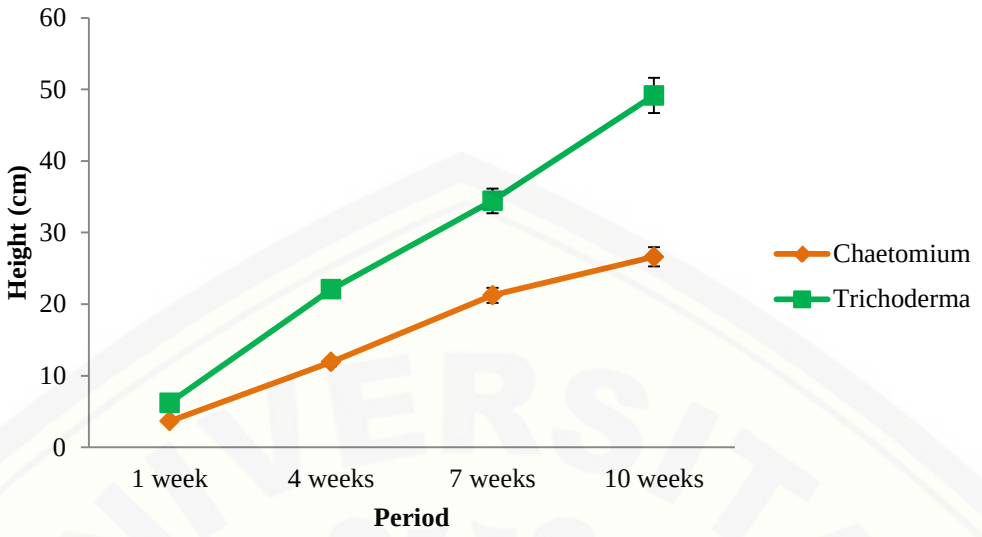


Figure 4.21. Tobacco seedling height (each number on each period which followed with the same letter do not differ significantly at α=0.05 according to Duncan test)

Leaf number variable didn't give significant difference between both treatments (Figure 4.22). On the last observation, leaf number on *Trichoderma*+media treatment gained 9.8 leaves average and *Chaetomium*+media treatment with 7.8 leaves average (F-table = 1,923 ; Pr>F = 0.203).

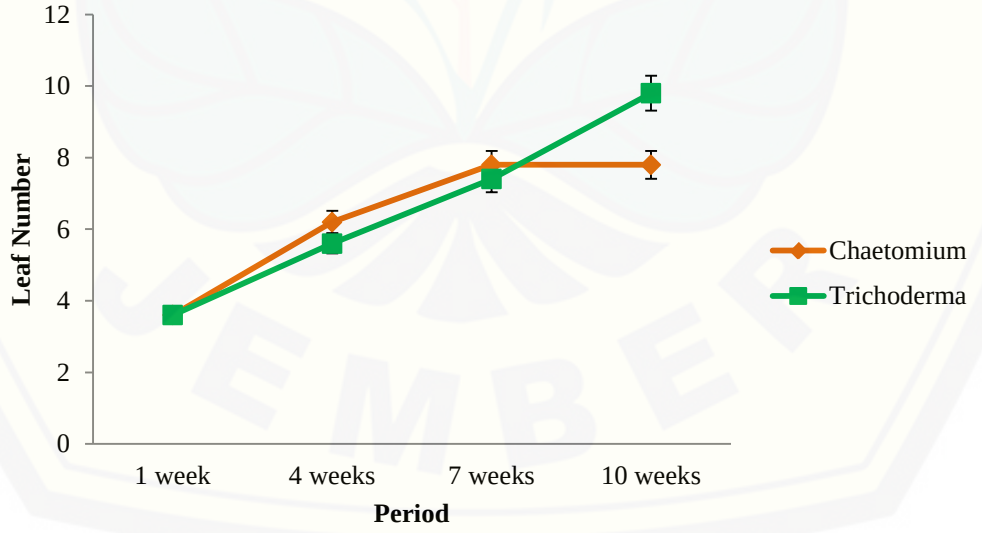


Figure 4.22. Tobacco seedling leaf number (each number on each period which followed with the same letter do not differ significantly at α=0.05 according to Duncan test)

Mortality variable shown to be concerned as the plant health parameters on this research. Both treatments successfully contributed to the plant defence induced system against *Phytophtora* spp. infection. No mortality recorded or even plant suffered as the effect of applied pathogen infection on both treatments. It means that *Phytophtora* did not work as literature marked. Dual-culture was implemented to proof the effectifity of endophytes (*Trichoderma* spp) against *Phytophtora* spp (Figure 4.23).

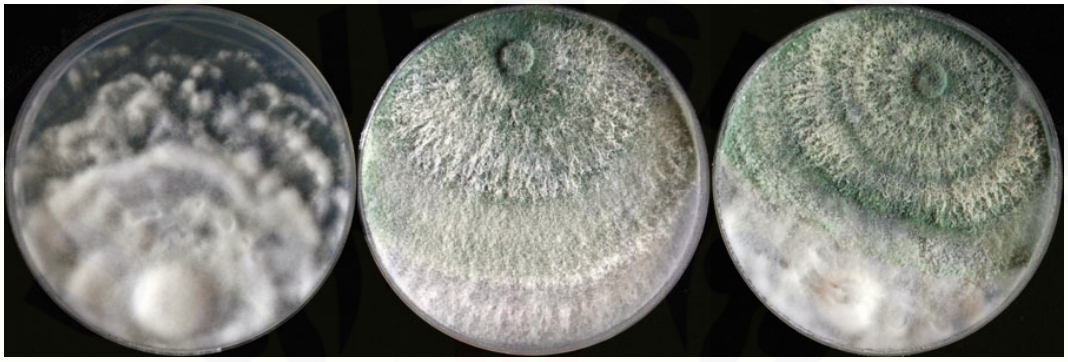


Figure 4.23. Dual-culture of *Trichoderma* spp. against *Phytophtora* spp. for pathogencity test of *Trichoderma* spp. (left: Pure *Phytophtora* spp. culture ; middle: Pure *Trichoderma* spp. culture ; right: *Trichoderma* spp. versus *Phytophtora* spp. culture)

Persistancy of applied endophytic fungi to the media was determined on the last observation (Table 4.5). The result shown that applied *Chaetomium* sp. population reached up to 3.2 x 10^4 cfu/g and *Trichoderma* spp. population was higher with 3,3 x 10^5 cfu/g.

Table 4.5. Population of applied endophytic fungi in peatmoss media after 2 months.

Fungi	Population (cfu/g)
Trichoderma	3.3 x 10^5
Chaetomium	3.2 x 10^4

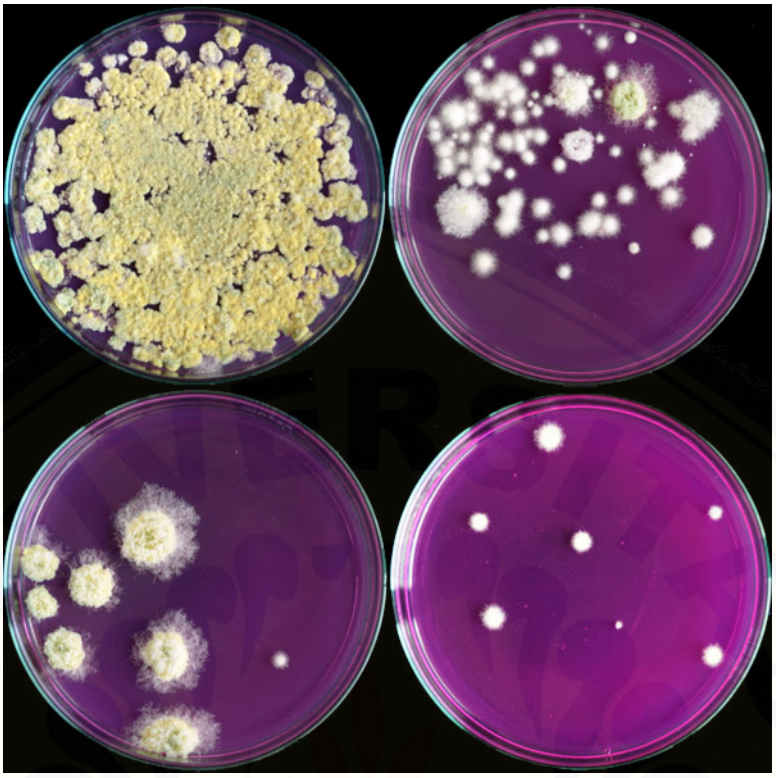
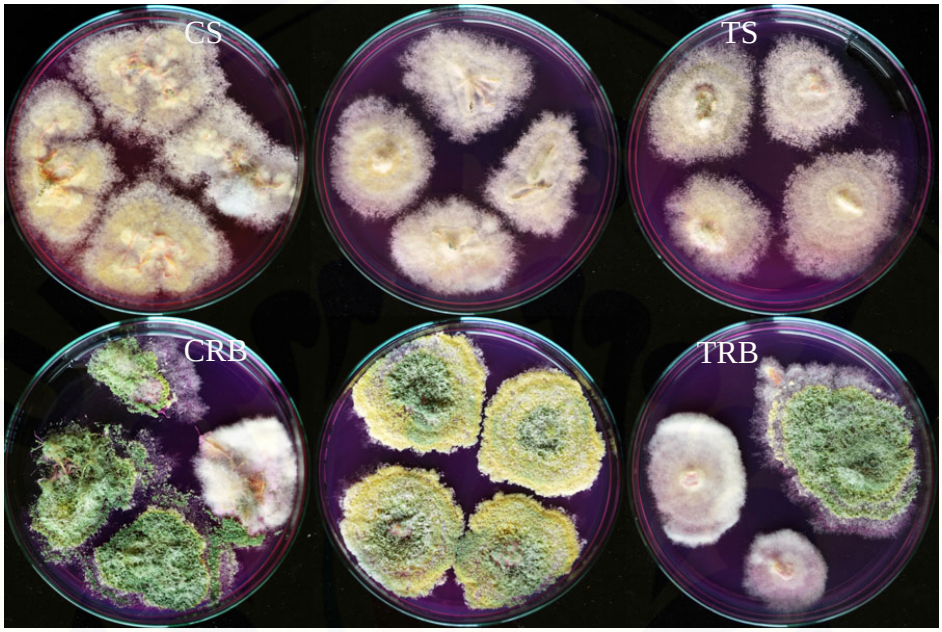


Figure 4.24. Population of applied endophytic fungi on RBA media. (T1= Trichoderma on 10^{-3} dilution ; T2= Trichoderma on 10^{-5} dilution ; C1= Chaetomium on 10^{-3} dilution ; C2= Chaetomium on 10^{-5} dilution)

Trichoderma spp. and *Chaetomium* sp. persistancy was observed in the tobacco plant parts include rootzone parts and stem-base part, morphologically by tissue transplanting method (Figure 4.25) and mollecular method (Figure 4.26) used PCR technique. The result on tissue transplanting method described that both applied endophytes were present inside the tobacco plant parts with the same character as the pure culture. It means that both applied endophytes penetrated well to plant tissue. The truth of this detection was supported by PCR technique result which implemented on *Trichoderma* spp. isolate.

Four replications in each tobacco plant parts on Trichoderma+media treatment was resulted as PCR product and fragmented on 1.5 kb. This result (R1, R2, R3, R4 and S1, S2, S3, S4) shown similar with the comparasion PCR product of pure *Trichoderma* culture result (T) (Figure 4.26). SCAR a1 and a1c primers were used for screening *Trichoderma* species in the previous study (Hermosa *et al.*, 2001). The similarity between *Trichoderma* PCR band and extracted tobacco

parts band was shown that *Trichoderma* detected on each plants parts. About 1.5 kb band was detected in related to the previous study by Rubio *et al*., 2005 which detecting *Trichoderma harzianum* on the same size with SCAR a1 and a1c with 68^{o}C annealing temperature. It was meant that applied *Trichoderma* applied and identified as *T. harzianum*.



(CS= +Chaetomium Stem ; CRB = +Chaetomium Root Base ;
CRL= +Chaetomium Root Low ; TS= +Trichoderma Stem ;
TRB= +Trichoderma Root Base ; TRL= +Trichoderma Root Low)
Figure 4.25. Detection of applied endophytes on tobacco plant parts.

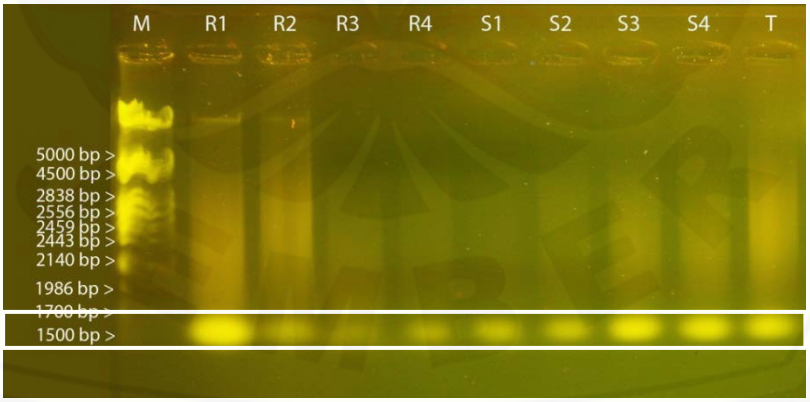


Figure 4.26. Agarose gel showing PCR product of tobacco tobacco plant parts. Root (R1, R2, R3, R4) and tobacco stem (S1, S2, S3, S4) inoculated with Trichoderma (T). Amplified using SCAR a1 and a1c at annealing temperature 62^{o}C for 1.5 minutes. Marker used lambda pstl 24 (Fermentas.ltd)

4.2 Discussion

4.2.1 Effect of Endophytic Fungi on Plant Growth

Endophytic fungi have been widely studied have role as plant growth promotion. Especially for *Trichoderma* spp. that has been known as plant growth regulator and biocontrol agents. *Chaetomium* spp. also have secondary metabolism that induce plant growth such as high chloropyl content, higher biomass, and increase shoot growth (Schulz and Boyle, 2005). Tobacco growth on after 5 month shown that there were no differences between each treatments on plant height in the end of observation. There were no differences on leaf number and stem size in all treatments. Looking back from each month growth, there were no difference growths between treated tobaccos with control. Growth rate between treated tobacco and control also did not have any different at all.

Role of endophytic fungi on plant growth variables were shown on tobacco seedling together with plant pathogen infection. *Trichoderma*+media treatment induced plant height and the result was significantly different compared than *Chaetomium*+media treatment. Both treatments were not significantly different on plant leaf number. This result shown that applied *Trichoderma* spp. gave better result in case of plant growth induced system. Better result on Trichoderma treatments shown that *Trichoderma* application is more effective than *Chaetomium* treatments. Peatmoss, as the growing media of tobacco plant seem to be the factor affecting of *Trichoderma* success result. Trichoderma can survive well on peatmoss media because of high organic matter inside the peatmoss. Zaidi and Singh (2004) and Gangadharan (1989) reported that Trichoderma was on high population in several organic substrate like cow dung, FYM (farm yard manure), and paddy husk. The existence of Trichoderma inside organic matter will highly multiplicate, but they will reduced slightly under longer incubation.

Stimulation of *Trichoderma* on rhizosphere was help plants to get defense from soil borne pathogen which lives immidiately can infected plants root zone. Result on plants root length determined that *Trichoderma* play more role in the root stimulation. *Trichoderma* treatment was significantly induced plants root activity compared with *Chaetomium* treatment and control. This result showed

that Trichoderma were going inside and contributed as plant growth regulator. Another reason was based on the result of persistancy of *Trichoderma* spp. inside the media was increased. Both endophytic fungi was added only once in the beginning of the plantation. Trichoderma had their ability to grow in wide range substrates like pigeonpea, farmyard manure, wheat bran, neem cake, mustard cake, saw dust, coffee husk, vermicompost, sorghum grains etc but their use in mass multiplication and formulation remained little explored (Kumar *et al*., 2013).

In this experiment, there is no replacement of endophytic fungi to media after tobacco planted. Long period of plantation and also different weather between the plantation period also affecting the shelf life of both endophytic fungi in the media. According to the data, the existence of *Trichoderma* was very low on the third month and increased up to 2 times amount. The result overtook the population of *Chaetomium* which otherwisely decreased. According to Agosin *et al* (1997) Trichoderma shelf life was affected by pH of media, C/N ratio media, harvesting time of *Trichoderma spp.*, and also duration spores were left in the culture. This result shown that *Trichoderma* spp. was slowly adapt to the media and become more convenience for *Trichoderma* spp. multiplication after then. It can be proven by the growth tren of *Trichoderma*+media treatment was better than other treatments.

Trichoderma was well known as great biocontrol agents that widely control many pathogens, especially soil borne pathogens like *Pythium* sp., *Phytophtora* sp., *Rhizoctonia* sp., etc. Trichoderma interact with root, soil and leaf surroundings. They produce and release many components, which induce local or systemic plant resistance to abiotic stress. According to Rosado *et al.*, (2007), the main factor for ecological success of this genus is a combination of very active mycoparasitism mechanisms and an effective defensive strategy, induced in the plants. Mycoparasitism involve physical contact and synthesis of hydrolytic enzymes, toxic compounds and/or antibiotics that act synergistically with the enzymes. *Trichoderma* as biology control agents can even exert positive effects on plants with an increase in plant growth (biofertilization) and the stimulation of plant-defense mechanisms (Benitez *et al*., 2004).

Chaetomium sp. population also recorded according to 3 MAP and 5 MAP. This amount was indicated that existence of *Chaetomium* sp. and it was not affected by the environment or any factors. *Chaetomium* sp. as biocontrol agents are known have long shelf life in the favorable environments such as soil pH, content of organic matter, soil aeration, moisture and chemical residue in the soil. As a biological control agent, ecology and climate might be affected on poor result of biological control. *Chaetomium* sp. can survive at biopellets formulation about 77% on 1 year storage and can survival about 59% at biopowder formulation on 1 year storage (Soytong *et al.*, 2001). According to the result, this amount of *Chaetomium* sp. seems not to be enough to give effect on tobacco plant growth.

The role of endophytic fungi was also detected by tissue transplanting method. Both applied endophytic fungi were observed as the main endophytes inside tobacco tissue. Several fungi include the inoculated endophytic fungi were identified in 3 parts from the 5 months old tobacco atroot low (RL), root base (RB), and stem base (SB). Tobacco plant parts were transplanted into PDA media (Radji, 2005) and incubated for 7 days. Several fungi were successfully identified according to the macroscopic and microscopic morphology using Barnett and Hunter taxonomy (1972). Most of identified fungi were identified as Ascomycota (*Chaetomium* spp., *Trichoderma* spp., *Fusarium* spp., and *Colletrotichum* sp.) and Deutromycota division (Petrini, 1992). Only *Trichoderma* spp. and *Chaetomium* sp. which described as endophytic fungi, and the rest (*Fusarium* spp., and *Colletrotichum* sp. and *Cladosporium* sp.) were identified as plant pathogen.

The result also determined the effectivity of *Chaetomium* and *Trichoderma* addition. From the detection result, *Chaetomium* was penetrated into the plants tissue. *Chaetomium* penetrated to the plant tissue through the stem base (near the rootzone).The same result also shown on *Trichoderma* spp. *Trichoderma* spp. also penetrated well into the plant tissue. According to the general literature, these both endophytic fungi will help the host plants as plant growth regulator by penetrating to plant tissue. But according to the result, both of this endophytic fungi didn’t

give any effect on plant growth, even *Chaetomium* treatment was similar to be the worst treatment between Control and *Trichoderma* treatment.

4.2.2 Effect of Endophytic Fungi against *Phytophtora spp.* Infection.

Tobacco seedling was transplanted into single pot and was inoculated by *Phytophtora spp* zoospores suspension which already prepared as previously. Inoculation was implemented after the seedling vigourous. The inoculation of *Phytophtora spp* to tobacco seedling did not affect on the tobacco growth and development. Tobacco plant lived well without any interference from *Phytophtora* spp infection. *Phytophtora* spp. has been known well as plant pathogen especially on tobacco seedling. More than 43 species of *Phytophthora* have now been described. Members of this genus cause a wide variety of diseases on major food crops, forest, fruit, and nut trees, and many ornamental plants. The species *Phytophthora parasitica* causes root, stem, and fruit rot on more than 90 plant species, including tobacco (Ribeiro, 1978). *Phytophtora* is one of pathogen which causing of dumping-off on tobacco and any economic plants seedling together with *Pythium* spp., *Rhizoctonia solani, Sclerotium rolfsii,* and *Fusarium oxysporum* (Loliam *et al*., 2012). *Phytophtora* which infected the plants will lived and produced mainly diploid hyphae, oospores and chlamydospores within plant tissue. Zoospores are biflagellate and able to swim in the water. Oospores of this pathogen also have ability to be long lasting in the organic part of media (Lilja *et al*., 2006).

Inoculation of *Phytophtora spp* to tobacco seedling did not successfuly infect to tobacco. Percentage of tobacco seedling which infected by *Phytophtora* spp. were nothing. It means that *Phytophtora* did not work as literature marked. From the result above, applied endophytic fungi seem to be the reason why was the *Phytophtora* did not give any effect on tobacco plant growth. *Chaetomium* and *Trichoderma* was applied to the media which composed by 100% peatmost. Both of endophytic fungi play role as biocontrol agents. The ability of these both endophytic fungi was known well especially for Trichoderma. *Trichoderma spp.* has been reported inhibit *Phytophtora* spp. growth by Moayedi *et al*., (2009) in

various test. In this experiment, dual culture also implemented to measure the inhibition ability of *Trichoderma* spp. over *Phytophtora* spp.

After 7 days culture, the effect of *Trichoderma* spp. was shown with inhibition over *Phytophtora* spp. culture. Culture of *Trichoderma* spp. looks overdominant inhibit the growth of *Phytophtora* spp. by growing over the mycelia of *Phytophtora* spp. culture. It means that when *Trichoderma* spp. mycelia started to contact with *Phytophtora* spp., the inhibition by *Trichoderma* spp. was begun. Inhibitions of Trichoderma spp. make the hyphae lysed, parasitizides, and disorganited over the *Phytophtora* spp. cell host (Moayedi and Mustowfizadeh-ghalamfarsa., 2010). The lysed mycelium was caused by enzyme activity of *Trichoderma* spp. isolates at the contact points (Elad *et al*., 1983).

Antagonism role of *Trichoderma* causing the significant inhibition to *Phytophtora* spp. even it also destroy the hyphae by lysis effect. Application of endophytic fungi, especially for Trichoderma has given impact to *Phytophtora* spp. shelf life. *Phytophtora* spp. was failed to penetrate too far because of the inhibition of endophytic fungi which protected the plants tissue from the infection. Uneffective inoculation of *Phytophtora* spp. also gave chance the plants to grown without any disturbance. Endophytic fungi also play role as plant growth regulator Shown that *Trichoderma* application was looks better than *Chaetomium* Population was detected by using Rose Bengal Agar and continued with spread plate method. The result has shown that existence of *Trichoderma* in the peatmoss as major media shown greater than *Chaetomium* existence. Population of *Trichoderma* reached up to 3,3 x 10^5 cfu/g. The colony counting result shown on *Chaetomium* population just only 3,2 x 10^4cfu/g, and it meant that *Chaetomium* needed to adapt longer in peatmoss media. Otherwise, Trichoderma was able to survive and multiplicate well in peatmoss media. Smaller amounts of *Chaetomium* found shown that pure peatmoss give unfavorable condition to *Chaetomium* compared with to previous experiment. This result also affected tobacco growth which unconstantly promoted by *Chaetomium* as occured on *Trichoderma* treatment.

The effectifity of both applied endophytic fungi on *Phytophtora* spp. infection were supported by detecting both endophytic fungi inside plant tissue used tissue transplanting method and PCR technique. Morphological identification has been used widely as endophytes isolation. Some researcher may need to use specific media to gain more success on endophytes isolation. Selective medium like Rose Bengal also used by Frohlich *et al*., 2000 was used to inhibit the fast growing endophytes. Specific technique may also needed reflecting on the objectives of each researcher want to do with. The result of tissue transplanting method detection shown, that both *Chetomium* and *Trichoderma* lived inside of the plant tissue. *Chaetomium* looks penetrated better than Trichoderma in all tobacco tissue parts. *Trichoderma* did not penetrate well on stem compared with *Chaetomium* which have more penetration to this area. Otherwise, *Trichoderma* similar to be suitable to stay at root base (RB) and much detected in this plant part. From the result can be determined that both of applied endophytic fungi penetrated well to the plant tissue to gain and were symbiosis with the host plants. Interaction between endophytic fungi and host plants was widely study. *Trichoderma* which found in each part of treated tobacco claimed that *Trichoderma* did not just only stay and play role in the rhizosphere, but they are also penetrated into the plant tissue and give contribution by promoting the growth and also protect from the pathogens. Endophytes also play role as environment tolerance like drought (Frohlich *et al*., 2000 ; Sieber, 2007).

The result on tissue transplanting method was confirmed by mollecular detection method by PCR technique. Detection with molecular technique was also applied due to gain the accuracy of the specific endophytes applied. Endophytic fungi isolation usually resulted in a considerable number of sterile mycelia and recent studies have used molecular analysis to provide taxonomic placement of these sterile fungi (Promputtha *et al*., 2005; Wang *et al*., 2005). The identification of organisms on the basis of DNA investigation requires the characterization of discriminating DNA targets (Rubio*et al*., 2005). Trichoderma which is applied as endophytes in the present study was detected using SCAR a1 (5'-GGAAGCTTGGCGTTTATTGTACAA-3') and SCAR a1c (5'-

GGAAGCTTGGGTATTGAGCTGGGC-3') (WardMedic, Inc) as the primers with annealing temperature on 62^{o}C for 1.5 minutes. Amplification was cycled for 34 times. Plant DNA extraction was done before using the written method according to Chakraborty *et al*., 2010. Two plant parts was concerned to be extracted according to the result of morphology detection. Tobacco root and stem were amplified. Trichoderma also amplified as the comparasion. Electrophoresis with 0.8% of agar was runned flown the TAE buffer. Marker was also runned using lambda pstl 24 (Fermentas, Inc).

The result on this PCR technique claimed that all of tested plant parts were positifly containt *Trichoderma* spp. which applied in the growing media. The similarity between *Trichoderma* PCR band and extracted tobacco parts band was shown that Trichoderma detected on each plants parts. About 1.5 kb band was detected and related to the previous study by Rubio *et al*., 2005 which detecting *Trichoderma harzianum* on the same size with SCAR a1 and a1c with 68^{o}C annealing temperature. It was meant that applied *Trichoderma* identified as *T. harzianum*. Bands were detected on each sample on the same fragment. The result also proved that *Trichoderma* penetrated up to tobacco stem as endophytes.

PCR result from tobacco tissue which detected with *Trichoderma* inside proved accurately that *Trichoderma* penetrated to the plants tissue, contributed as plant endophytes and helped the host plants as biocontrol and plant growth promoters. It also proved that *Trichoderma* was more effective became the endophytes inside tobacco plant tissue and symbiozed well with the host plants. *Trichoderma* seems to be mobile beneficial endophytes which penetrate through the plant tissue. Combination of morphology and PCR result seems to be a great conclusion that *Trichoderma* was good endophytic fungi that induced the host plant health. PCR technique also can be the way to gain accuracy of endophytic fungi identification with the specific primers. It is very usefull and be choosen as identification method due to the ability of PCR which can identificate the unknown microorganism to the species level. It is also was used as the solver when morphological identification was hardly finished.

CHAPTER V. CONCLUSION

5.1 Conclusion

From the result above, this research was concluded as:

1. Several fungi detected lived inside tobacco tissue such as *Trichoderma* spp., *Chaetomium* sp., *Fusarium* sp., *Colletroticum* sp. and *Cladosporium* sp. Only *Trichoderma* spp. and *Chaetomium* sp. which added to the media that played role as endophytic fungi, the rest was identified as plant pathogens.
2. *Trichoderma* has been proven to be effective endophytes which also induce resistance on the host plant from the inoculated pathogens. *Trichoderma* with their metabolites activity also showed inducing the plant health due their contribution on plant protection and plant growth promoters. Application of endophytic fungi, especially *Trichoderma*, was successful to be an plant defence inducer which contributed against *Phytophtora* spp infection and also promoted the growth of tobacco. Activity of applied endophytes was found in the tobaco plant tissue. The result of morphologic detection also supported by PCR result which successfully detect Trichoderma using SCAR a1 and SCAR a1c primers on 1,5 kb. Detected Trichoderma in the tobacco tissue showed that applied endophytes contribute on plant caring system with play role as biocontrol and also plant growth promoters. Combination of both detection method was accurately identified the activity of *Trichoderma* applied in the plants tissue. Furthermore, it also identified *Trichoderma harzianum* as the species of Trichoderma applied.

5.2 Recommendation

The existences of each endophyte inside of each plant tissue need to be identified and studied further. Moreover, it also need to be confirmed the mechanism of plant-microbe interaction between endophyte and host plant itself.

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APPENDIX

1. Potato Dextrose Agar PDA Media (1 Liter)
   - 200 g Potato
   - 15 g Dextrose
   - 20 g Agar
   - 1 L Deionized water

2. Rose Bengal Agar (RBA) Media (1 Liter)
   - 1 L Deinozed water
   - 0,05 g Rose bengal
   - 5 g Peptone
   - 0,5 g Magnesium sulphate ($MgSO_4$)
   - 1 g Monopotassium phospate (K_2PO_4)
   - 10 g Agar
   - 15 g Dextrose

3. V8 Juice Broth Media
   - 250 ml V8 Juice
   - 750 ml Deionized water
   - 2 g Calcium carbonate ($CaCO_3$)

4. Surface Sterilization of Plant Part Material
   - 20% of Sterilized chlorox
   - Sterilized water
   - Timer
   - Sterilized plate

5. Raw data of tobacco height (cm) and ANOVA table in the effect of endophytic fungi on tobacco growth at 1st observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	21	14.9	21.7	23.4	16.2	19.44
Chaetomium	14.8	18.6	14.5	23.5	14.2	17.12
Trichoderma	16.2	13.6	11	18.6	18.8	15.64

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	36.69	18.34	1.353	0.295
Residuals	12	162.75	13.56		

6. Raw data of tobacco height (cm) ANOVA table in the effect of endophytic fungi on tobacco growth at 2nd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	27.5	35	32.9	38	32	33.08
Chaetomium	31.25	32	28	45	31.6	33.57
Trichoderma	27.1	28	30.5	34.2	38	31.56

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	10.98	5.49	0.209	0.815
Residuals	12	315.76	26.313		

7. Raw data of tobacco height (cm) ANOVA table in the effect of endophytic fungi on tobacco growth at 3rd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	38	54	43.5	59.2	44	47.74
Chaetomium	42.2	33.6	38.9	60.8	40.4	43.18
Trichoderma	40.6	38	40.1	47	52.7	43.68

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	62.5	31.27	0.43	0.66
Residuals	12	873.5	72.80		

8. Raw data of tobacco height (cm) ANOVA table in the effect of endophytic fungi on tobacco growth at 4th observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	46	60	52.7	60.8	52.6	54.42
Chaetomium	43.7	40	52.7	61.6	46.7	48.94
Trichoderma	51.2	48.3	48	65.5	64.5	55.5

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	123.7	61.86	0.999	0.397
Residuals	12	743.4	61.95		

9. Raw data of tobacco height (cm) ANOVA table in the effect of endophytic fungi on tobacco growth at 5th observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	55.2	59.8	62.8	60.6	64.1	60.5
Chaetomium	44.7	43.4	56.8	63.9	48.2	51.4
Trichoderma	54.1	55.2	53.2	68.2	70.5	60.24

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	268.4	134.19	2.545	0.12
Residuals	12	632.7	52.72		

10. Raw data of tobacco leaf number ANOVA table in the effect of endophytic fungi on tobacco growth at 1st observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	8	10	9	15	18	12
Chaetomium	11	11	20	15	10	13.4
Trichoderma	12	13	16	12	12	13

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	5.2	2.60	0.201	0.821
Residuals	12	155.2	12.93		

11. Raw data of tobacco leaf number ANOVA table in the effect of endophytic fungi on tobacco growth at 2nd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	9	16	7	16	13	12.2
Chaetomium	10	12	10	19	11	12.4
Trichoderma	12	10	11	15	13	12.2

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	0.13	0.067	0.006	0.994
Residuals	12	138.80	11.567		

12. Raw data of tobacco leaf number ANOVA table in the effect of endophytic fungi on tobacco growth at 3rd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	13	30	11	25	21	20
Chaetomium	16	9	14	20	17	15.2
Trichoderma	18	16	14	22	21	18.2

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	58.8	29.40	0.96	0.411
Residuals	12	367.6	30.63		

13. Raw data of tobacco leaf number ANOVA table in the effect of endophytic fungi on tobacco growth at 4th observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	14	26	12	16	17	17
Chaetomium	13	15	18	15	12	14.6
Trichoderma	14	18	14	22	18	17.2

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	20.93	10.47	0.69	0.52
Residuals	12	182.00	15.17		

14. Raw data of tobacco leaf number ANOVA table in the effect of endophytic fungi on tobacco growth at 5th month.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	20	28	13	15	17	18.6
Chaetomium	11	14	12	16	10	12.6
Trichoderma	12	13	12	19	18	14.8

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	92.13	46.07	2.668	0.11
Residuals	12	207.20	17.27		

15. Raw data of tobacco stem size (cm) ANOVA table in the effect of endophytic fungi on tobacco growth at 1st observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	0.58	0.38	0.5	0.375	0.335	0.434
Chaetomium	0.175	0.3	0.235	0.44	0.365	0.303
Trichoderma	0.275	0.145	0.18	0.4	0.33	0.266

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)	
Trt	2	0.07792	0.03896	3.606	0.0594	.
Residuals	12	0.12963	0.01081			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

16. Raw data of tobacco stem size (cm) ANOVA table in the effect of endophytic fungi on tobacco growth at 2nd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	0.68	0.515	0.615	0.48	0.435	0.514
Chaetomium	0.36	0.355	0.34	0.605	0.45	0.397
Trichoderma	0.53	0.45	0.365	0.61	0.455	0.43

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	0.03783	0.01892	1.833	0.202
Residuals	12	0.12381	0.01032		

17. Raw data of tobacco stem size (cm) ANOVA table in the effect of endophytic fungi on tobacco growth at 3rd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	0.735	0.67	0.72	0.69	0.475	0.623
Chaetomium	0.36	0.39	0.52	0.78	0.62	0.511
Trichoderma	0.685	0.58	0.55	0.67	0.57	0.578

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	0.03919	0.01960	1.315	0.305
Residuals	12	0.17887	0.01491		

18. Raw data of tobacco stem size (cm) ANOVA table in the effect of endophytic fungi on tobacco growth at 4th observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	0.87	0.81	0.815	0.8	0.64	0.787
Chaetomium	0.705	0.73	0.63	0.79	0.68	0.707
Trichoderma	0.74	0.66	0.72	0.755	0.73	0.721

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	2	0.01825	0.009127	2.218	0.151
Residuals	12	0.04938	0.004115		

19. Raw data of tobacco stem size (cm) ANOVA table in the effect of endophytic fungi on tobacco growth at 5th observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Control	0.945	0.87	0.89	0.94	0.84	0.89
Chaetomium	0.775	0.81	0.7	0.87	0.82	0.795
Trichoderma	0.8	0.765	0.695	0.81	0.84	0.782

[1]Plants die because of geminiviruses infection

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)	
Trt	2	0.04813	0.024065	6.538	0.012	*
Residuals	12	0.03641	0.003034			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

20. Raw data of tobacco seedling height (cm) ANOVA table in the effect of endophytic fungi against *Phytophtora spp.* at 1st observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Chaetomium	3.3	2.6	1.9	7.9	2.5	3.64
Trichoderma	12.5	6.5	4.1	2.5	5.5	6.22

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	1	16.64	16.641	1.623	0.238
Residuals	8	82.04	10.26		

21. Raw data of tobacco seedling height (cm) ANOVA table in the effect of endophytic fungi against *Phytophtora spp.* at 2nd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Chaetomium	17.1	4	4.2	24.3	10	11.92
Trichoderma	25.4	24.3	19.3	21.4	20	22.08

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)	
Trt	1	258.1	258.1	6.171	0.0379	*
Residuals	8	334.6	41.82			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

22. Raw data of tobacco seedling height (cm) ANOVA table in the effect of endophytic fungi against *Phytophtora spp.* at 3rd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Chaetomium	26.1	10.5	10.3	38.7	20.7	21.24
Trichoderma	39.1	38.9	30.2	32.6	31.4	34.44

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)	
Trt	1	434.3	434.3	5.463	0.0476	*
Residuals	8	636	79.5			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

23. Raw data of tobacco seedling height (cm) ANOVA table in the effect of endophytic fungi against *Phytophtora spp.* at 4th observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Chaetomium	37.8	11.7	11.6	48.5	23.5	26.62
Trichoderma	55.3	54	42	45	49.4	49.15

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)	
Trt	1	1269	1269	8.513	0.0194	*
Residuals	8	1191	148.9			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

24. Raw data of tobacco seedling leaf number ANOVA table in the effect of endophytic fungi against *Phytophtora spp.* at 1st observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Chaetomium	4	3	4	4	3	3.6
Trichoderma	4	4	3	3	4	3.6

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	1	0.0	0.0	0.0	1.000
Residuals	8	2.4	0.3		

25. Raw data of tobacco seedling leaf number ANOVA table in the effect of endophytic fungi against *Phytophtora spp.* at 2nd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Chaetomium	5	6	5	7	8	6.2
Trichoderma	6	5	5	7	5	5.6

[1]Plant die because of environmental interference (plant lost)

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	1	0.9	0.9	0.72	0.421
Residuals	8	10	1.25		

26. Raw data of tobacco seedling leaf number ANOVA table in the effect of endophytic fungi against *Phytophtora spp.* at 3rd observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Chaetomium	8	9	7	8	7	7.8
Trichoderma	9	8	7	6	7	7.4

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	1	0.4	0.4	0.4	0.545
Residuals	8	8	1		

27. Raw data of tobacco seedling leaf number ANOVA table in the effect of endophytic fungi against *Phytophtora spp.* at 4th observation.

Treatment	Replication					Avg
	1	2	3	4	5	
Chaetomium	10	5	7	11	6	7.8
Trichoderma	10	12	7	9	11	9.8

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Trt	1	10	10	1.923	0.203
Residuals	8	41.6	5.2		

28. Raw data of endophytic fungi population ANOVA table in the effect of endophytic fungi against *Phytophtora spp*.

Treatment	Replication			Avg
	1	2	3	
Chaetomium	382	354	258	331.333
Trichoderma	16	80	1	32.333

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)	
Rep	2	8540	4270	2.483	0.2871	
Trt	1	134102	134102	77.989	0.0126	*
Residuals	2	3439	1719			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1