Characterization of Soybean Stem Diseases in Myanmar and Their Biological Control Using Beneficial Fungus, Trichoderma virens

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CONTENTS

List of Tables

List of Figures

Abstract

Soybean (*Glycine max*) is one of the major pulses in Myanmar. In 2017 and 2018, pre-matured falling leaves and dry rot with discoloration on the epidermis of the stem were observed in field conditions at the reproductive growth stage of the soybean plants in the Southern Shan State and Nay Pyi Taw region. Based on the morphological characteristics, the causal microorganisms were identified into three fungal genera, namely – *Colletotrichum*, *Diaporthe* and *Macrophomina.* In the pathogenicity test, isolates of *Macrophomina* spp. were less pathogenic to the soybean cultivar (Yezin-10) and further molecular identifications were performed for other two genera. *ACT, CHS1, GAPDH* and ITS genes were sequenced for *Colletotrichum* spp. and *EF1-α*, ITS and *TUB* genes were used for *Diaporthe* spp. According to the multi-locus phylogenetic analysis, the collected *Colletotrichum* isolates from Nay Pyi Taw region and Shan State were *C. truncatum* and *C. plurivorum*, respectively, and the *Diaporthe* spp. isolated from soybean of Myanmar during 2017 and 2018 were identified as *D. endophytica*, *D. melonis*, *D. tectonendopytica* and *D. ueckerae*. All isolates showed virulent reaction in inoculation tests. This is the first report of stem fungal diseases on soybean caused by *Colletotrichum* and *Diaporthe* species complex in Myanmar.

Another main finding in the present study was discovery of fungal biocontrol agent. Soil saprophytic fungi isolated from the waste paper sludge were identified by partial sequencing of ITS gene. Their antagonistic effects on mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOLy) were tested *in vitro*, and *Trichoderma virens*, 911 (Tv911) inhibited 64.27 % of the growth compare to the control. Moreover, Tv911 was selected for investigating plant growth promoting abilities on Japanese mustard spinach, radish and tomato in manufacturer recommended soils; farmer use nursery soil (FNS) and home garden soil (HGS). Plant heights of one-month old Japanese mustard spinach and

tomato increased by 16.22% and 50.26% in treated FNS and by 9.84% and 7.00% in treated HGS in comparison with those in non-treated control, respectively. Fresh shoot and root weight of radish also increased by 23.83% and 58.86% in treated FNS and by 12.77% and 64.45% in treated HGS. Disease suppression ability of Tv911 against fusarium wilt diseases of lettuce and tomato was also examined, and disease severity was significantly reduced in both diseases. Additionally, the Tv911 colonization was observed as a stable in soil and increased in root tissue over growing period for one month.

The beneficial effects of Tv911 against anthracnose of soybean caused by *Colletotrichum truncatum* were also evaluated in the germination and early seedling stages of plant growth under greenhouse conditions. Tv911 reduced the pre- and post-emergence seedling mortality of soybean and enhanced the germination and seedling vigour. Fungal colonization of the two fungi in plant tissue and rhizospheric soil was estimated via quantitative real-time PCR (qPCR) with respective species specific primers which were newly designed in this study, and Tv911 supressed the colonization and infection of *C. truncatum* in the root tissue of soybean and rhizospheric soil. Therefore, this strain has the potential for plant growth promotion and disease suppression against the tested soil borne fungal diseases, and it would be useful as a biological agent in crop production.

CHAPTER 1

General Introduction

Myanmar agriculture and soybean cultivation

Myanmar is a developing country, and 70% of the population is living in rural area and their livelihoods depend on the agricultural production systems (CSO 2017). The ratio of agriculture sector (excluding livestock, fishery and forestry) in national gross domestic product (GDP) in 2018 was 15.4% and it is one of the most important sectors of Myanmar economy (MOALI 2018). A variety of cereals, oil seeds, food legumes, fibers, perineal plantations, fruits, and vegetables crops were grown through the country. Total cultivated area was approximately 20.5 million ha, and rice is a staple food and major cultivated crop (7.3 million ha) (MOALI 2018). Soybean, *Glycine max* (L.) Merrill, is also one of the most important pulses in Myanmar and sown area was 1.4 million ha in 2017 (MOALI 2018), and it is mainly sown in central plain area and Shan state. The yield was 1.07 tons per ha and was also only approximately one third of world average yield (2.68 tons/ha) (USDA 2018).

Importance of diseases occurrence on soybean

Plant diseases are one of the major limitations in soybean cultivation, and soybean diseases were mainly caused by more than 300 species of parasitic microorganisms, such as bacteria, fungi, nematodes and virus (Hartman and Hill 2010). However, few species cause economic damage. The estimated yield losses caused by 24 major plant diseases and other minor diseases in top eight soybean-producing countries was 59.9 million metric tons in 2006, and among these diseases, 20 diseases were caused by different species of fungal causal plant pathogen (Wrather et al. 2010). Among the soybean diseases, stem diseases

including anthracnose, stem blight, stem rot, and stem canker are caused by several fungal pathogens (Backman et al. 1985; Kim and Diers 2000; Mengistu et al. 2007; Hartman and Hill 2010; Wrather et al. 2010).

Anthracnose of Soybean

Anthracnose of soybean is caused by *Colletotrichum* species complex, including *C. truncatum* (Schwein.) Andrus & Moore (syn. *C. dematium* var. *truncate*) and other species, namely *C. cliviae* Yang, Liu, Hyde & Cai (now *C. plurivorum*), *C. coccodes* (Wallr.) Hughes, *C. destructivum* O'Gara, *Glomerella glycines* Lehm. & Wolf, *C. graminicola* (Ces.) Wils., and *C. gloeosporioides* (Penz.) Penz. & Sacc. [sexual stage *G. cingulata* (Stonem.)] (Kale B.G. 2016; Rogério et al. 2017; Dias et al. 2018).

The causal organisms of soybean anthracnose attack all stages of growth, but the symptoms commonly develop only during the germination and seedling stages, and during pod filling and senescence stages (Bowers and Russin 1999). However, the typical anthracnose symptoms of irregular shaped brown areas appear on stems and pods especially in late growth season (Hartman and Hill 2010; Ramos et al. 2013).

The anthracnose diseases caused by *Colletotrichum* were distributed throughout the soybean cultivating countries on the world including neighbouring countries of Myanmar, such as Bangladesh, China, India and Thailand (CABI and EPPO 2001). The estimated yield losses caused by anthracnose disease in soybean was 1.7 million metric tons in China and 2.6 million metric tons in worldwide in 2006 (Wrather et al. 2010). The disease development largely depends upon the weather condition, and prolong period of rainfall and high relative humidity would favour the severe disease occurrence and cause heavy damage to yield and seed quality (Zhang et al. 1998; Singh et al. 2001).

Diaporthe species complex in soybean disease

Soybean seed decay, seed rot, pod and stem blight and stem canker diseases are caused by *Diaporthe* (anamorph = *Phomopsis*) species complex that includes *Diaporthe longicolla* Hobbs., and *D. sojae* Lehman (Bowers and Russin 1999). Additionally, *D. melonis* (Beraha & O'Brien) is able to infect soybean and causes stem disease symptoms, and *D. ueckerae* (Udayanga & Castl. Sp. nov.) was also reported as a soybean endophytes (Udayanga et al. 2015). *D. longicolla* (syn. *Phomopsis longicolla*) is a common seedborne pathogen, which can also be isolated from other parts of the plant and severely reduce the grain quality and cause Phomopsis seed decay of soybean (Li et al. 2010; Udayanga et al. 2015). This fungal pathogen has already been reported in most of the major soybean cultivating countries including China and India, which are neighbouring countries of Myanmar (CABI and EPPO 2005). *D. sojae* (syn. *D. phaseolorum* (Cke. & Ell.) Sacc. f. sp. *sojae* (Lehman) Wehm., *D. glycines* Petr., *Phomopsis camptothecae* C.Q. Chang, Z.D. Jiang & A.J. Young., and *D. melonis* var *brevistylospora* Tak. Kobayashi et Tak. also attacked soybean and these species are distributed throughout several soybean producing countries including China and India, (Udayanga et al. 2015).

The symptoms of Phomopsis seed decay range from symptomless to shriveled, or cracked and often chalky-white appearance, and the pathogens were frequently isolated from all vegetative plant parts (Li 2011). The most obvious symptoms of pod and stem blight caused by *Diaporthe* species complex are the row of black, speck-sized fruiting bodies of pycnidia on the pods, petioles and stems. The black pycnidia appear on the main stem and upper branches after death of the plant in the maturity stage, and the death stems may be covered with the small patches of pycnidia especially near the nodes (Bowers and Russin 1999).

The estimated yield losses due to seed decays, pod and stem blight, and stem canker caused by *Diaporthe* species complex was 2.4 million metric tons in 10 soybean producing countries in 1994 (Wrather et al. 1997). The main aspect of this disease complex is affecting on the seed quality under high relative humidity, and yield reduction is as a consequence of a shortened period of seed-filling due to premature plant death (Bowers and Russin 1999).

Management of soybean fungal diseases

In field conditions, there are many biotic problems (e.g. fungi, bacteria, virus, nematodes, insects, and etc.) and abiotic constraints (e.g. nutrient deficiency, chemical toxicity, extreme weather, physiological disorder, and etc.) in crop production (Agrios 2005). Therefore, correct disease diagnosis is very important for plant disease management in agricultural production.

Fungal plant diseases are major components of biological constraints in soybean production and several control strategies have been applied to reduce the impact of the diseases. The general disease management practices are crop rotation, destruction of crop residue, field and seed sanitation, fungicide application, and usage of resistant cultivars (Hartman and Hill 2010). The integrated disease management strategies such as combination of destruction of previous crop residues and sowing of resistant cultivars can significantly reduce the stem canker damage (Damicone et al. 1990). Additionally, Usage of disease free seeds or fungicide application (common name = captan) seed treatment is recommended to control anthracnose and Phomopsis pod and stem blight at the seedling stage, and a curative foliar fungicide (such as Triazole (tebuconazole)) spraying at the reproductive stage can reduce the disease damage and significantly increase the yield (Wrather and Sweets 1998; Swoboda and Pedersen 2009).

However, the disease management practices using chemical fungicide would cause harmful impact on biodiversity, environment and human health (Debenest et al. 2010; Geiger et al. 2010). Organic agriculture and using biological resources in integrated disease management strategies have attracted an increasing attention and are interest from the human's health and environmental concerns.

Biological control of plant disease means all measures of controlling disease or reducing the damage caused by plant pathogens using biological mechanisms or organisms. It includes (1) cultural practices such as crop rotation, tillage system and fertilizer application which affect microbes, (2) using antagonistic microbes to attack the pathogens or enhance plant growth, (3) the chemicals to alter the microflora and (4) plant breeding and genetic engineering to transfer the disease resistance genes. However, the term of biological control is usually used as narrow approach that is artificial introduction of antagonistic microorganism into the environment to control the pathogen (Campbell 1989). Currently, many commercial microbial products of bacteria and fungi are available for plant diseases management and plant growth promotion as a biofertilizer (Rodríguez and Fraga 1999; Vessey 2003; Berg 2009).

Objectives of the thesis research

This PhD study was conducted with the following objectives.

i) To identify the causal organisms of soybean stem diseases in Myanmar using multilocus phylogenetic analysis
ii) To discover the effective beneficial biological agents for plant growth promotion and disease suppression
iii) To investigate the plant-microbe interaction of antagonistic biocontrol agent, *Trichoderma virens,* Tv911 and plant pathogenic fungi, *F. oxysporum* f. sp.

lycopersici, *F. oxysporum* f. sp. *lactucae* and *Colletotrichum truncatum* on tomato, lettuce and soybean, respectively, using quantitative real-time PCR (qPCR)

CHAPTER 2

Soybean Stem Fungal Diseases and Their Characteristics in Myanmar

2.1 Introduction

Soybean (*Glycine max*) is one of the major crops in Myanmar, where it is cultivated on 0.15 million hectares and its yield averaged 1.07 tons per ha in 2016 (CSO 2017). The yield in Myanmar is approximately one-third of the world average yield (USDA 2018) because of low agricultural inputs and problems concerning pests and diseases. The yield and quality of the product are critical to soybean farmers. Unfortunately, plant diseases are a significant constraint on soybean production and caused losses of approximately 59.9 million metric tons in the top eight producing countries in 2006 (Wrather et al. 2010). Commonly, Burmese soybean farmers do not adopt any control measures for plant diseases, and they would face losses when weather conditions favour disease development.

Fungal pathogens were the causal agents of the majority of the 24 reported soybean diseases in the top 10 soybean-producing countries in 1994 (Wrather et al. 1997). Among these diseases, stem blight, pod rot and seed decay are caused by *Diaporthe* spp. (and their *Phomopsis* anamorph) and resulted in yield and quality losses (Santos et al. 2011). Moreover, a *Diaporthe* species complex (including *D. endophytica*, *D. longicolla*, *D. phaseoli*, *D. phaseolorum* and *D. sojae*) is associated with soybean (Gomes et al. 2013; Udayanga et al. 2015; Zhang et al. 1998).

Soybean anthracnose is mainly caused by *Colletotrichum truncatum* (Schwein.) Andrus & W.D.Moore and is also an economically important disease in soybean production. The estimated yield reductions caused by anthracnose in China and India were 1.66 million tons and 0.18 million tons, respectively, in 2006 (Wrather et al. 2010). Other species such as *C. gloeosporioides* (Penz) Penz & Sacc. (teleomorph *Glomerella cingulata*), *C. coccodes* (Wallr.) Hughes, and *C. destructivum* O'Gara (teleomorph *G. glycine* F.Lehm. & F.A.Wolf)

are also related to anthracnose diseases in soybean (Chen et al. 2006; Manandhar 1986; Riccioni et al. 1998). However, some of the strains that were previously identified as *C. gloeosporioides* and *C. truncatum* in fact could be any of the newly described species in the orchidacearum species complex (Damm et al. 2019). One species that belongs to this species complex, *C. plurivorum* (formerly *C. cliviae*), was recently reported in Brazil as a causal agent of soybean disease (Barbieri et al. 2017).

As described above, soybean pod and stem blight are caused by several *Diaporthe* spp., and soybean anthracnose is caused by the *Colletotrichum* species complex. Identification of causal pathogens is important for disease management. Traditional techniques of identification based on morphological characteristics present limitations because of phenotypic variation in the different geographical locations and environmental conditions in which *Colletotrichum* spp. occur (Bailey and Jeger 1992). Moreover, the identification of *Diaporthe* spp. is complicated due to inter- and intraspecific variability and a wide host range (Mostert et al. 2001; Rehner and Uecker 1994). Therefore, identification at the species level based on morphological characteristics alone is impossible for these species. Currently, species delimitation via molecular identification based on internal transcribed spacer (ITS) region sequences of ribosomal DNA (rDNA) and other loci such as actin (*ACT*), beta-tubulin (*TUB*), chitin synthase (*CHS-1*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and translation elongation factor 1-alpha (*EF1-α*) has become to the standard (Santos et al. 2011; Udayanga et al. 2015; Weir et al. 2012).

In the case of Myanmar, scientific information on soybean fungal diseases is scant, and it is very difficult to precisely diagnosis the species, which is essential for disease management. During a field survey in the rainy season of 2017, we found that soybean plants in farmers' fields were affected by fungal diseases causing premature leaf fall and unfilled pods. Therefore, the objective of this study was to characterize the causal fungal

species community in soybeans from two major soybean production areas to develop disease control strategies in Myanmar.

2.2 Materials and Methods

2.2.1 Sample collection and isolation

Samples were collected in September 2017 and 2018 in a survey of two major crop production locations in Myanmar. The first location, the Nay Pyi Taw area (Pyinmana and Tetkone) in Central Myanmar, has a tropical climate with intermittent rainfall, and other locations of Shan State (Heho, Kyaukme, Lawksawk, Moemeik and Taunggyi), are mountainous areas and has a mild climate with high humidity in the growing season. During field collections, premature leaf fall, dry rot, discoloration on the epidermis of the stem and pods with shrivelled seeds were observed (Fig. 2.1). These typical disease symptoms were widespread and easily observed throughout the fields of both locations during the field survey. Twenty diseased stem samples exhibiting black specks and blotching were collected from each location. The epidermis of the collected stem samples was cut into 2- to 3-mm pieces and surface-sterilized with 2% (v/v) NaOCl solution, then incubated on water agar at 25 ± 1°C for 24 h. The emerging mycelial tips were transferred to potato dextrose agar (PDA) (Merck-EMB, Germany) plates, and single-spore cultures and single mycelial tips from the cultures for non-spore-forming isolates were prepared to obtain pure cultures for further studies.

2.2.2 Morphological characterization

Agar discs (5 mm diameter) of each fungal isolate were placed on PDA and incubated at 25 ± 1°C with four replicates. Colony diameter was measured at 24-h intervals for 7 days, and colony morphology was observed. The isolates were placed on sterilized soybean stems on water agar media to observe the morphology of the fruiting bodies. Images were obtained using a light microscope mounted with an Olympus DP70 camera (Olympus,

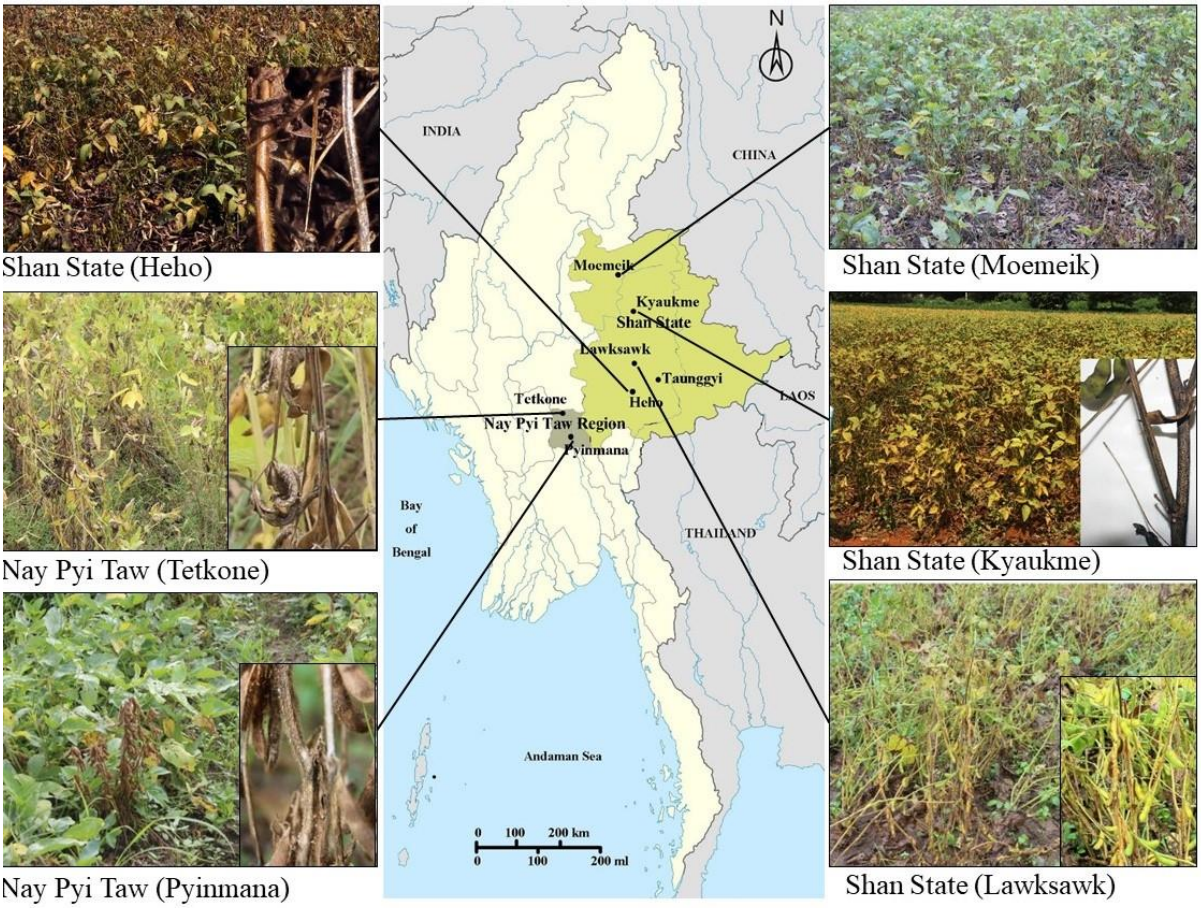

Fig. 2.1 Sampling locations and field symptoms of soybean stem diseases in Myanmar

Tokyo, Japan). The microscopic images were viewed and measured with WinRoof 2015, Lite Ver 3.6.0 software. The range measurements of the conidia are presented as mean ± standard deviation, and 50 conidia were measured. The representative isolates were selected from each morphologically similar group for the experiment.

2.2.3 Pathogenicity on soybean

The pathogenicity of the collected isolates was examined using a stem cutting inoculation technique (Li et al. 2010) with slight modifications. Briefly, soybean (Yezin-10 cultivar) seeds were surface-sterilized with a 2% (v/v) NaOCl solution and grown in autoclaved potted soil in the phytotron (Biotron Application Center, Kyushu University) at 25 ± 2°C. Two-week-old seedling stems were cut into equal lengths at 15 cm above the soil line but below the first trifoliate leaf. Then, small mycelial discs were collected from the margins of 10-day-old cultures on PDA plates using the large end of 200 µl micropipette tips and immediately placed onto fresh-cut stems (Fig. 2.2). Inoculation with PDA-only discs served as the control. A completely randomized design (CRD) was used with five replications, and each replicate included three plants. The micropipette tips were removed 2 days after inoculation, and stem and diseased lesion lengths were measured at 10 days after inoculation. Lesion length was measured and calculated as a percentage of total stem length using the following formula: Lesion length (as % of total stem length) = (Lesion length/Stem length) × 100 (Li et al. 2010). Analysis of variance with CRD was performed, and the mean values from each treatment were compared based on the least significant difference at $P \leq 0.05$ with Statistix version 8.0 (Analytical Software, Tallahassee, FL, USA). The inoculated fungal isolates were reisolated from the symptoms that developed after inoculation, and the morphological characteristics were checked to confirm their pathogenicity and fulfil Koch's postulates.

Table 2.1 GenBank accession numbers of fungal isolates from soybean in Myanmar

	Species	Isolate	Location	Year	GenBank accession no.					
					ITS	*EF1-α*	*TUB*	*ACT*	*GAPDH*	*CHS-1*
1	*C. plurivorum*	SoyYS1707	Lawksawk	2017	LC383779	_	_	LC440987	LC440999	LC440993
2	*C. plurivorum*	SoyYS1709	Lawksawk	2017	LC383780	_	_	LC440988	LC441000	LC440994
3	*C. plurivorum*	SoyYS1726	Lawksawk	2017	LC383781	_	_	LC440989	LC441001	LC440995
4	*C. truncatum*	SoyYZ1715	Pyinmana	2017	LC360104	_	_	LC440990	LC441002	LC440996
5	*C. truncatum*	SoyYZ1716	Pyinmana	2017	LC360105	_	_	LC440991	LC441003	LC440997
6	*C. truncatum*	SoyYZ1725	Pyinmana	2017	LC360108	_	_	LC440992	LC441004	LC440998
7	*D. endophytica*	SoyYS1732	Lawksawk	2017	LC381426	LC381703	LC381705	_	_	_
8	*D. endophytica*	SoyYS1733	Lawksawk	2017	LC381427	LC381704	LC381706	_	_	_
9	*D. endophytica*	SoyTG18205	Taunggyi	2018	LC480737	LC480745	LC480753	_	_	_
10	*D. endophytica*	SoyTG18210	Taunggyi	2018	LC480738	LC480746	LC480754	_	_	_
11	*D. endophytica*	SoyTG18212	Taunggyi	2018	LC480739	LC480747	LC480755	_	_	_
12	*D. melonis*	SoyYS1701	Lawksawk	2017	LC360096	_	LC377201	_	_	_
13	*D. melonis*	SoyYS1703	Lawksawk	2017	LC360097	LC377208	LC377202	_	_	_
14	*D. melonis*	SoyYS1704	Lawksawk	2017	LC360098	LC377209	LC377203	_	_	_
15	*D. melonis*	SoyYS1706	Lawksawk	2017	LC360099	LC377210	LC377204	_	_	_
16	*D. melonis*	SoyYS1711	Lawksawk	2017	LC360100	LC377211	LC377205	_	_	_
17	*D. melonis*	SoyYS1712	Lawksawk	2017	LC360101	LC377213	LC377206	_	_	_
18	*D. melonis*	SoyYS1713	Lawksawk	2017	LC360102	LC377212	LC377207	_	_	_
19	*D. melonis*	SoyYZ18302	Pyinmana	2018	LC480740	LC480748	LC480756	_	_	_
20	*D. tectonendophytica*	SoyYZ18101	Pyinmana	2018	LC461977	LC461987	LC461981	_	_	_
21	*D. tectonendophytica*	SoyYZ18102	Pyinmana	2018	LC461978	LC461988	LC461982	_	_	_
22	*D. tectonendophytica*	SoyYZ18108	Pyinmana	2018	LC480743	LC480749	LC480758	_	_	_
23	*D. tectonendophytica*	SoyYZ18203	Pyinmana	2018	LC461979	LC461989	LC461983	_	_	_
24	*D. tectonendophytica*	SoyYZ18401	Pyinmana	2018	LC480742	LC480750	LC480759	_	_	_
25	*D. tectonendophytica*	SoyYZ18501	Pyinmana	2018	LC480741	LC480751	LC480760	_	_	_
26	*D. tectonendophytica*	SoyYZ18601	Pyinmana	2018	LC480744	LC480752	LC480761	_	_	_
27	*D. tectonendophytica*	SoyTK18104	Tetkone	2018	LC461976	LC461986	LC461977	_	_	_
28	*D. ueckerae*	SoyHH18301	Heho	2018	LC461974	LC461990	LC461984	_	_	_
29	*D. ueckerae*	SoyHH18501	Heho	2018	LC461975	LC461991	LC461985	_	_	_

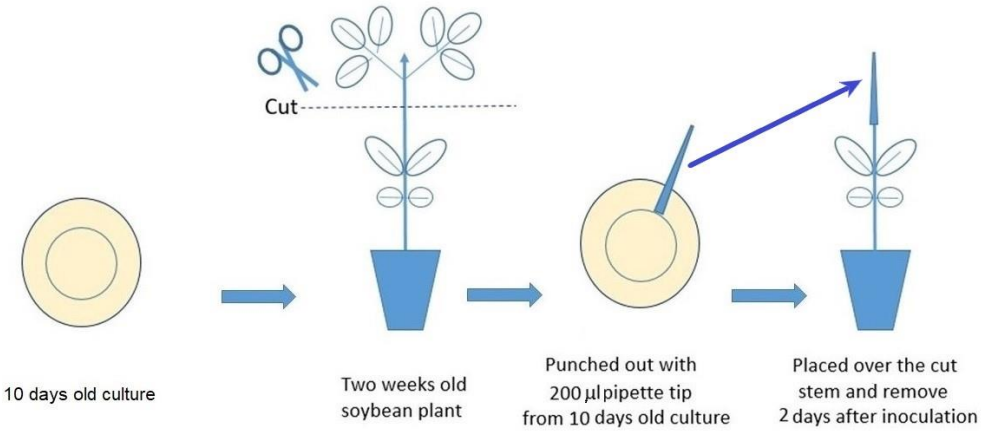

Fig. 2.2 Schematic diagram of inoculation of *Diaporthe* and *Colletotrichum* species on soybean plants (up) and inoculation of the diseases in greenhouse (Biotron Application center, Kyushu University) (down)

Table 2.2 List of primers for phylogenetic analysis

Gene	Primer	Sequence (5´-3´)	Reference
ACT	ACT-512F	ATGTGCAAGGCCGGTTTCGC	(Carbone and Kohn 1999)
	ACT-783R	TACGAGTCCTTCTGGCCCAT	
CHS-1	CHS-79F	TGGGGCAAGGATGCTTGGAAGAAG	(Carbone and Kohn 1999)
	CHS-345R	TGGAAGAACCATCTGTGAGAGTTG	
EF1-α	EF1-728F	CATCGAGAAGTTCGAGAAGG	(Carbone and Kohn 1999)
	EF1-986R	TACTTGAAGGAACCCTTACC	
GAPDH	GDF	GCCGTCAACGACCCCTTCATTGA	(Templeton et al. 1992)
	GDR	GGGTGGAGTCGTACTTGAGCATGT	
ITS	ITS1	TCCGTAGGTGAACCTGCGG	(White et al. 1990)
	ITS4	TCCTCCGCTTATTGATATGC	
TUB	Bt-2a	GGTAACCAAATCGGTGCTGCTTTC	(Glass and Donaldson 1995)
	Bt-2b	ACCCTCAGTGTAGTGACCCTTGGC	

In the pathogenicity test, the two experiments were conducted separately. The first one was examination of the pathogenicity of collected three genera: *Colletotrichum*, *Diaporthe* and *Macrophomina* on soybean cultivar. In this experiment, 3, 6 and 3 representative isolates of *Colletotrichum, Diaporthe* and *Macrophomina,* respectively were selected, and their pathogenicity were examined. Then, the second pathogenicity experiment was conducted for *Diaporthe* spp., and two representative isolates of *D. endophytica*, *D. melonis*, *D. tectonendophytica* and *D. ueckerae* were tested. Moreover, two different species were also inoculated together for their synergetic effect on pathogenicity because two *Diaporthe* spp. were occurred in the same sampling locations.

2.2.4 DNA extraction, PCR amplification and sequencing

Genomic DNA from 29 randomly selected isolates was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) (Table 2.1). After the extraction of template DNA, the samples were stored at -20°C until use.

For the identification of *Diaporthe* spp., the ITS, beta-tubulin (*TUB*) and translation elongation factor 1-α (*EF1-α*) genes were amplified using ITS1 and ITS4, Bt-2a and Bt-2b, and EF1-728F and EF1-986R primers, respectively. For *Colletotrichum* spp., ITS, actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and chitin synthase (*CHS-1*) were amplified by using primer pairs ITS1 and ITS4, ACT-512F and ACT-783R, and GDF and GDR, and CHS-79F and CHS-345R, respectively (Table 2.2).

PCR amplification was carried out in a 25 µl reaction volume containing 2.5 µl of 10× reaction buffer, 2 µl of dNTPs, 1.0 µl of each primer, 0.25 µl of *Taq* polymerase (2.5 U/µl) (Toyobo, Osaka, Japan), 2 µl of template DNA and 16.25 µl of MilliQ water. PCR was performed in a thermal cycler (TProfessional basic Gradient Thermocycler, Biometra, Gottingen, Germany). The PCR conditions for ITS were 95°C for 2 min, 39 cycles of 95°C for 30 sec, 55°C for 50 sec, and 72°C for 1 min, and a final step at 72°C for 5 min: the

Table 2.3 Type strains of *Diaporthe* spp. used in the phylogenetic analysis

No.	Species	Strain no.	Country	Host	GenBank accession number(s)		
					ITS	*TUB*	*EF1-α*
1	*D. angelicae*	CBS111591	Austria	*Heracleum sphondylium*	KC343026	KC343994	KC343752
2	*D. angelicae*	DP0488	Austria	*Angelica sylvestris*	KJ590735	KJ610890	KJ590775
3	*D. arctii*	DP0482	Austria	*Arctium lappa*	KJ590736	KJ610891	KJ590776
4	*D. betatus*	CBS112.21	USA	*Ipomoea batatas*	KC343040	KC344008	KC343766
5	*D. citri*	LGMF 946	Brazil	*Glycine max* (seed)	KC343053	KC344021	KC343779
6	*D. citri*	CBS 199.39	Italy		KC343051	KC344019	KC343777
7	*D. convolvuli*	CBS124654	Turkey	*Convolvulus arvensis*	KC343054	KC344022	KC343780
8	*D. convolvuli*	FAU 649	Canada	*Convolvulus arvensis*	KJ590721		KJ590765
9	*D. cucurbitae*	CBS136.25	Unknown	*Arctium* sp.	KC343031	KC343999	KC343757
10	*D. cucurbitae*	DAOM42078	Canada	*Cucumis sativus*	KM453210	KP118848	KM453211
11	*D. cuppatea*	CBS117499	South Africa	*Aspalathus linearis*	KC343057	KC344025	KC343783
12	*D. endophytica*	CBS 133811	Brazil	*Schinus terebinthifolius*	KC343065	KC344033	KC343791
13	*D. endophytica*	LGMF 948	Brazil	*Glycine max*	KC343072	KC344040	KC343798
14	*D. hordei*	CBS481.92	Norway	*Hordeum vulgare*	KC343120	KC344088	KC343846
15	*D. infecunda*	CBS133812	Brazil	*Schinus terebinthifolius*	KC343126	KC344094	KC343852
16	*D. longicolla*	FAU599	USA	*Glycine max*	KJ590728	KJ610883	KJ590767
17	*D. longicolla*	FAU644	USA	*Glycine max*	KJ590730	KJ610885	KJ590769
18	*D. lusitanicae*	CBS123212	Portugal	*Forniculum vulgare*	KC343136	KC344104	KC343862
19	*D. manihota*	CBS505.76	Rwanda	*Manihot utilissima*	KC343138	KC343106	KC343864
20	*D. melonis*	CBS435.87	USA	*Cucumis melo*	KC343141	KC344109	KC343867
21	*D. melonis*	CMT41	Brazil	*Phaseolus vulgaris*	KP182391	KP182405	KP182382
22	*D. melonis*	FAU626	USA	*Cucumis melo*	KJ590704	KJ610860	KJ590743
23	*D. neoarctii*	CBS109490	USA	*Ambrosia trifida*	KC343145	KC344113	KC343817
24	*D. novem*	CBS127269	Croatia	*Glycine max*	KC343155	KC344123	KC343881
25	*D. novem*	CBS127271	Croatia	*Glycine max*	KC343157	KC344125	KC343883
26	*D. phaseolorum*	AR4203	USA	*Phaseolus vulgaris*	KJ590738	KJ610893	KJ590739
27	*D. schini*	CBS133181	Brazil	*Schinus terebinthifolius*	KC343191	KC344159	KC343917
28	*D. sojae*	FAU636	USA	*Glycine max*	KJ590718	KJ610874	KJ590761
29	*D. sojae*	FAU604	USA	*Glycine max*	KJ590716	KJ610872	KJ590759
30	*D. sojae*	DP0616	USA	*Glycine max*	KJ590715	KJ610871	KJ590758
31	*D. stewartii*	CBS193.36	USA	*Cosmos bipinnatus*	FJ889448	JX275421	GQ250324
32	*D. subordinaria*	CBS101711	New Zealand	*Plantago lanceolata*	KC343213	KC344181	KC343939
33	*D. subordinaria*	CBS464.90	South Africa	*Plantago lanceolata*	KC343214	KC344182	KC343940
34	*D. tecomae*	CBS100547	Brazil	*Tabebuia* sp.	KC343215	KC344183	KC343941

Table 2.3 Continued

No.	Species	Strain no.	Country	Host	GenBank accession number(s)		
					ITS	*TUB*	*EF1-α*
35	*D. tectonendophytica*	LC6623	China	Unknown	KX986795	KX999227	KX999187
36	*D. tectonendophytica*	MFLUCC 13-047	Thailand	*Tectona grandis*	KU712439	KU743986	KU749367
37	*D. terebinthifolii*	CBS133180	Brazil	*Schinus terebinthifolius*	KC343216	KC344184	KC343942
38	*D. ueckerae*	FAU659	USA	*Cucumis melo*	KJ590724	KJ610897	KJ590745
39	*D. ueckerae*	FAU656, CBS139283	USA	*Cucumis melo*	KJ590725	KJ610880	KJ590746
40	*D. ueckerae*	FAU660	USA	*Cucumis melo*	KJ590723	KJ610878	KJ590744
41	*D. ueckerae*	LGMF947, CPC20323	Brazil	*Glycine max*	KC343202	KC344171	KC343929
42	*D. vexans*	CBS127.14	USA	*Solanum melongena*	KC343229	KC344197	KC343955
43	*D. vexans*	FAU597	Dominican	*Solanum* sp.	KJ590734	KJ610889	KJ590774

Table 2.4 Type strains of *Colletotrichum* spp. used in the phylogenetic analysis

No.	Species	Strain no.	Country	Host	GenBank accession number(s)			
					ITS	*ACT*	*GAPDH*	*CHS1*
1	*C. acutatum*	CBS 29467	Australia	*Carica papaya*	FJ972610	FJ907429	FJ972581	
2	*C. aenigama*	ICMP 18608	Israel	*Persea americana*	JX010244	JX009443	JX010044	
3	*C. boninense*	MAFF305972	Japan	*Crinum asiaticum* var. *sinicum*	HM585399	HM582001	HM585386	HM582032
4	*C. boninense*	CBS123755	Japan	*Crinum asiaticum* var. *sinicum*	JQ005153	JQ005501	JQ005240	JQ005327
5	*C. cliviicola*	CBS125375	China	*Clivia miniata*	JX519223	JX519240	JX546611	-
6	*C. cliviicola*	CSSK4	China	*Clivia miniata*	GQ485607	GQ856777	GQ856756	-
7	*C. cliviicola*	CSSS1	China	*Clivia miniata*	GU109479	GU085861	GU085868	-
8	*C. coccodes*	CBS369.75	Netherlands	*Solanum tuberosum*	HM171679	HM171667	HM171673	
9	*C. dematium*	CBS125.25	France	*Eryngium campestre*	GU227819	GU227917	GU228211	-
10	*C. fruticola*	BPDI16	Thailand	*Coffea arabica*	FJ972603	FJ907426	FJ972578	-
11	*C. fruticola*	CBS125397, ICMP18646	Panama	*Tetragastris panamensis*	JX010173	JX009581	JX010032	-
12	*C. fruticola*	CBS238.49	Germany	*Ficus habrophylla*	JX010181	JX009495	JX009923	-
13	*C. gloeosporioides*	CBS953.97	Italy	*Citrus sinensis*	GQ485605	GQ856782	GQ856762	GQ856733
14	*C. gloeosporioides*	IMI356878	Italy	*Citrus sinensis*	JX010152	JX009531	JX010056	JX009818
15	*C. orchidearum*	CBS135131	Netherland	*Dendrobium nobile*	MG600738	MG600944	MG600800	MG600855
16	*C. orchidearum*	MAFF238779	Japan	*Orchidium* sp.	MG600744	MG600950	MG600806	MG600857
17	*C. plurivorum*	CBS125474	Vietnam	*Coffea* sp.	MG600718	MG600925	MG600781	MG600841
18	*C. plurivorum*	LFN0008	Brazil	*Glycine max*	KT696336	KT696275	KT696289	
19	*C. plurivorum*	LJTJ3	China	*Capsicum* sp.	KP748193	KP823738	KP823773	-
20	*C. salsolae*	CBS119296, ICMP 18693	Hungary	*Glycine max* (inoculated)	JX010241	JX009559	JX009917	JX009791
21	*C. scovillei*	CBS126529	Indonesia	*Capsicum* sp.	JQ948267	JQ949588	JQ948597	JQ948928
22	*C. siamense*	ICMP 18578, CBS130417	Thanland	*Coffea arabica*	JX010171	FJ907423	JX009924	
23	*C. simmondsii*	BRIP28519	Australia	*Carica papaya*, fruit	GQ485606	GQ849430	GQ856763	JQ948973
24	*C. sojae*	ATCC 62257	USA	*Glycine max*	MG600749	MG600954	MG600810	MG600860
25	*C. truncatum*	CBS151.35	USA	*Phaseolus lunata*	GU227862	GU227960	GU228254	GU228352
26	*C. truncatum*	CBS195.32	USA	*Glycine max*	GU227865	GU227963	GU228257	GU228355
27	*C. truncatum*	CBS335.75	Indonesia	*Capsicum annuum*	GU227879	GU227977	GU228075	GU228369
28	*C. truncatum*	CBS506.97	Burkina Faso	*Vigna unguiculata*	GU227871	GU227969	GU228263	GU228361
29	*C. truncatum*	CMES1036	Brazil	*Glycine max*	KJ614303		KJ614346	-
30	*C. vittalense*	CBS 181.28	India	*Theobroma cacao*	MG600734	MG600940	MG600796	MG600851
31	*Monilochaetes infuscans*	CBS 869.96	-	-	JQ005780	JQ005843	JX546612	JQ005801

annealing temperature was varied for other genes: 58°C for *TUB* and *EF1-α* (Udayanga et al. 2014), 58°C for *ACT* and *CHS-1*, and 60°C for *GAPDH* (Weir et al. 2012).

The quality of the PCR products was checked by electrophoresis in 1.5% (w/v) agarose stained with ethidium bromide, and the products were purified with a QIAquick PCR kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Then, the purified PCR products were sent to Fasmac Co. (Kanagawa, Japan) for sequencing. The nucleotides sequences of the collected isolates in Myanmar were deposited in GenBank with their respective accession number (Table 2.1)

2.2.5 Phylogenetic analysis

The accession numbers of all sequences that were newly generated in this study are listed in Table 2.1. The sequences of the ITS, *TUB* and *EF1-α* genes of *Diaporthe* species (Table 2.3) were separately aligned with verified sequences published by Udayanga et al. (2015). The reference sequences of the ITS, *ACT*, *GAPDH* and *CHS-1* genes of *Colletotrichum* spp. were obtained from Weir et al. (2012) and Damm et al. (2019) (Table 2.4). The nucleotides of the DNA sequences were aligned via the ClustalW method (Thompson et al. 1994) using MEGA software (version 7), and alignment gaps were treated as gap data. Multilocus phylogenetic analyses of the *Colletotrichum* (based on the ITS, *ACT*, *GAPDH*, and *CHS-1* genes) and *Diaporthe* (based on the ITS, *EF1-α* and *TUB* genes) genera were performed separately by the maximum likelihood (ML) method with a distance matrix based on Kimura's two-parameter correction for multiple hits (Kimura 1980) using MEGA 7 (Kumar et al. 2016). The confidence estimate for tree topologies was determined by bootstrap analysis with 1000 replicates. Additionally, phylogenetic trees for the two genera with Bayesian probabilities were also constructed using the Markov chain Monte Carlo (MCMC) algorithm with MrBayes (version 3.1.2) (Ronquist et al. 2012). Nucleotide substitution models for each gene were selected by using PartitionFinder (version 1.1.1)

(Lanfear et al. 2012). The analysis of MCMC chains was run for 1,000,000 generations, and trees were sampled every 1,000th generation. The first 25% of the trees were discarded, and the posterior probabilities were calculated using the remaining trees. The resulting tree was viewed by using MEGA 7 software.

2.3 Results

In the present study, diseased soybean samples were collected from seven locations in Myanmar in 2017 and 2018, and the causal agents were investigated by morphological characterization, pathogenicity testing and molecular phylogenetic analyses. Total of 98 isolates were obtained and approximately 50% were *Diaporthe* spp. and they are found in all sampling locations except Moemeik. (Table 2.5).

2.3.1 Morphological characteristics of the collected fungal isolates

The observed morphological characteristics indicated that the isolates collected from soybean were *Colletotrichum* spp. and *Diaporthe* spp. Species identification was completed based on the molecular phylogenetic analysis. The morphological characteristics of the four pathogenic fungal species identified in this experiment were as follows.

Diaporthe melonis: Colony colour of the isolates was whitish to grey, and no colour staining was observed on PDA. The growth rate of the isolates was 6.1 ± 0.2 mm/day (n = 12) in the dark at 25°C. Pycnidia on sterilized soybean stems were globose and 189.9 ± 28.2 µm (n = 50) in diameter containing abundant α-conidia. The alpha conidia were hyaline, smooth, ovoid to ellipsoid, biguttulate, and 6.9 ± 0.5 µm × 2.4 ± 0.2 µm (n = 50) in size. Beta conidia were not observed (Fig. 2.3). The morphology of *D. melonis* in this experiment was the same as the taxonomic description according to the report of Udayanga et al. (2015).

Table 2.5 Number of fungal isolates from diseased soybean samples from 7 locations in Myanmar

Location (Region/ State/ Township)	*Diaporthe* spp.	*Colletotrichum* spp.	*Macrophomina* spp.	Total isolate
Nay Pyi Taw (Tetkone)	5	-	2	7
Nay Pyi Taw (Pyinmana)	11	5	3	19
Shan (Heho)	5	5	3	13
Shan (Lawksawk)	8	6	-	14
Shan (Kyaukme)	7	10	5	22
Shan (Moemeik)	-	4	9	13
Shan (Taunggyi)	6	4	-	10
Total isolates	**42**	**34**	**22**	**98**

Diaporthe endophytica: The colony morphology of this fungus was similar to that of *D. melonis*. The growth rate was 5.6 ± 0.3 mm/day on PDA in the dark at 25°C. There was no sporulation on PDA. However, pycnidia and alpha conidia were observed on lesions of the inoculated soybean plant stems. Pycnidia were round to ovoid with ostioles, 135 ± 28.2 µm in diameter, and the morphology of the alpha conidia was similar to those of *D. melonis*, with a size of 6.8 ± 0.5 µm × 2.6 ± 0.3 µm (*n* = 50). Beta conidia were not observed (Fig. 2.4). In the contrast, sporulation of *D. endophytica* was not reported on either media or sterilized host plant tissue by Gomes et al. (2013). In our experiment, the morphology of *D. endophytica* was almost the same as that of *D. melonis.*

***Diaporthe tectonendophytica*:** The pure culture colonies of the isolate from Pyinmana and Tetkone showed whitish with sparse hyphae at the early days of incubation (Fig. 2.5 b and c). The growth rate of the isolates was fast, and average growth rate was 10.2 ± 1.8 mm d^{-1} (*n* = 12) in the dark at 25°C. Ten days after incubation, the colony was changing in colour to yellowish grey in the centre from the upside view. Brown staining to the media was observed at 3 weeks after incubation and the pycnidia were produced on both PDA media and sterilized soybean stem in scattered or aggregated (Fig. 2.5-d). The pycnidia were globosed, elongated or variable in shape and conidial mass were produced at maturity. Both alpha and beta conidia were observed on PDA media and inoculated sterilized soybean stem. Beta conidia were curved or hamate, filiform, hyaline and aseptate, and the size was 6.8 ± 0.7 µm × 2.6 ± 0.3 µm, (*n* = 50) (Fig. 2.5-g). Alpha conidia were 1-2 guttulate, mostly biguttulate, hyaline, ellipsoid. The size of alpha conidia was 5.2 ± 0.7 µm × 2.5 ± 0.2 µm, (*n* = 50) (Fig. 2.5-h).

***Diaporthe ueckerae*:** The colonies of the two isolates from Heho were also whitish in colour with fluffy aerial mycelial growth on PDA (Fig. 2.6 b and c). The growth rates of the isolates were also not obviously different to other species, and it was 9.25±1.9 mm d^{-1} (*n* = 12) in

the dark at 25°C. The conidiomata were produced on PDA at 4 weeks after incubating in scattered or aggregated. The whitish to cream conidial droplets containing thousands of alpha conidia were observed on the pycnidia at 4 to 6 weeks after incubation on PDA. The pycnidia were also formed on the inoculated sterilized soybean stems. Pycnidia were globose, elongated or variable in shape, and formed in single or aggregate (Fig. 2.6 e and f). The alpha conidia were 7.0 ± 0.5 µm × 2.9 ± 0.3 µm, (n = 50) in size (Fig. 2.6 g and h).

Colletotrichum truncatum: The colony on PDA was shown grey to dark grey with white to grey aerial mycelia, and dark staining was observed in the PDA. The colony was thick, and the growth rate was 4.6 ± 0.7 mm/day in the dark at 25°C. Acervuli formed on the inoculated stems, and black setae were dominant, with a width of 208.8 ± 62.1 µm. The conidia were falcate and tapered at each end, 26.2 ± 1.5 × 4.5 ± 0.4 µm (n = 50) (Fig. 2.7). The conidial dimensions were similar to those of *C. truncatum* isolate CBS112998 (Damm et al. 2009).

Colletotrichum plurivorum: The colony was whitish grey initially and later became darker; the aerial mycelia were sparse, and the ascomata (sexual morph) formed clusters at 2 weeks after incubation on PDA at 25°C. Conidia (asexual morph) were rarely found on PDA and were mainly observed on inoculated soybean stems; they were smooth-walled, one-celled, hyaline, and cylindrical to oblong with rounded ends, 15.5 ± 0.9 µm × 5.1 ± 0.3 µm (n = 50). The ascostroma were dark and globose to subglobose, and they contained asci with thin walls; in addition, they were unitunicate and clavated, with dimensions of 45.2 ± 9.8 µm × 8.9 ± 2.1 µm (n = 50). Eight ascospores were arranged in each ascus and slightly curved, and they were narrow at each rounded end, with dimensions of 16.5 ± 3.2 µm × 5.5 ± 0.9 µm (n = 50) (Fig. 2.8). The morphological characteristics of the isolates (SoyYS1707, SoyYS1709, SoyYS1726, SoyYS1730, and SoyYS1731) matched with those of *C. plurivorum* (CBS 125474) (Damm et al. 2019).

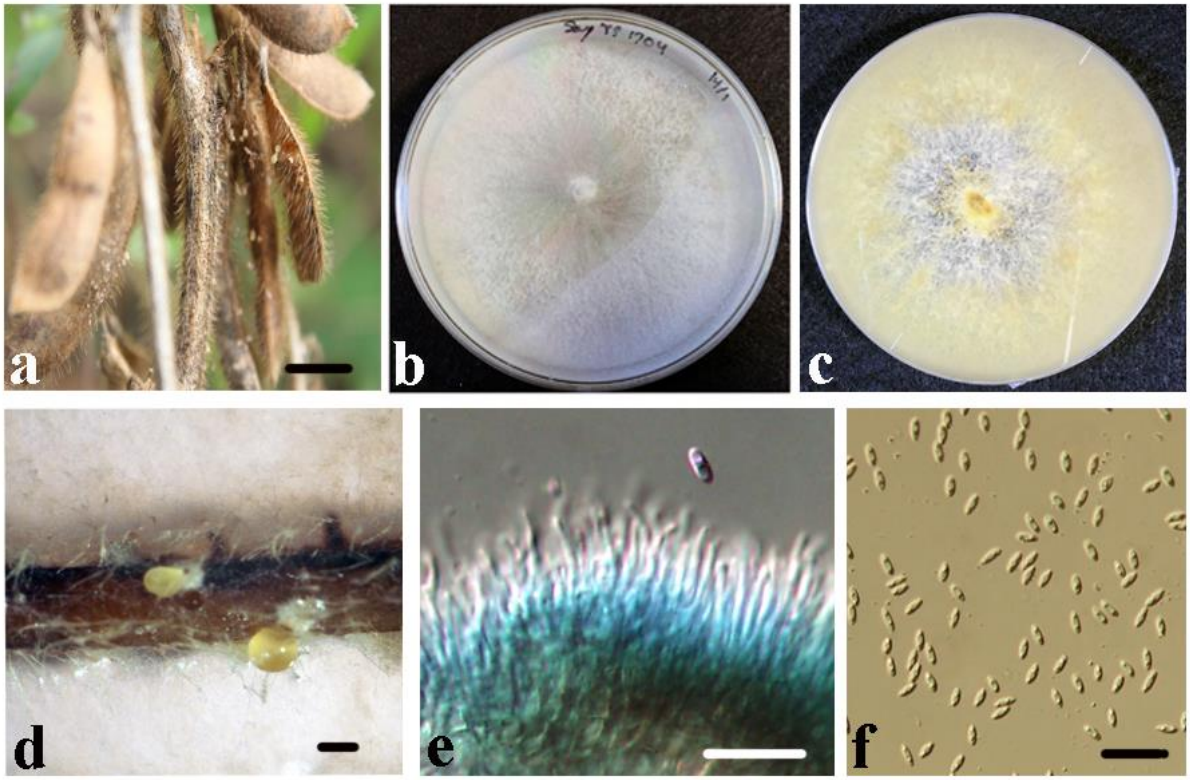

Fig. 2.3 Morphological characteristics of *Diaporthe melonis* (SoyYS1704), a = Symptoms on collected soybean stem sample, b and c = Ten days old colony on PDA (above and below view), d = Pycnidia on inoculated sterilized soybean stem on water agar (bar = 1mm), e = Conidiophores (bar = 15 µm), f = Alpha conidia (bar = 20 µm)

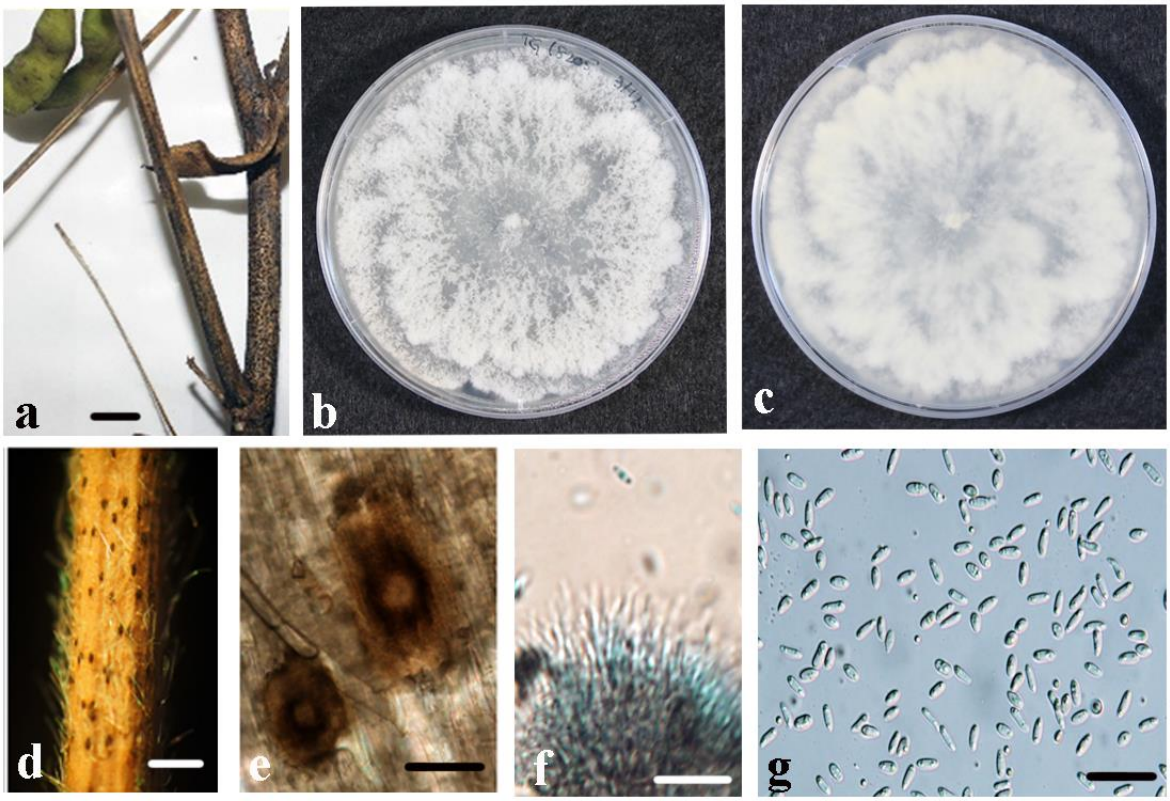

Fig 2.4 Morphological characteristics of *Diaporthe endophytica*, (SoyYS1733); a = Field symptoms on collected soybean stem sample, b = Ten days old colony on PDA, d = Pycnidia on sterilized soybean stem (bar = 2mm), e = Pycnidia in epidemic of inoculated soybean stem (bar = 50 µm), f = Conidiogenous cells and alpha conidia (bar = 20 µm), g = Alpha conidia (bar = 20 µm)

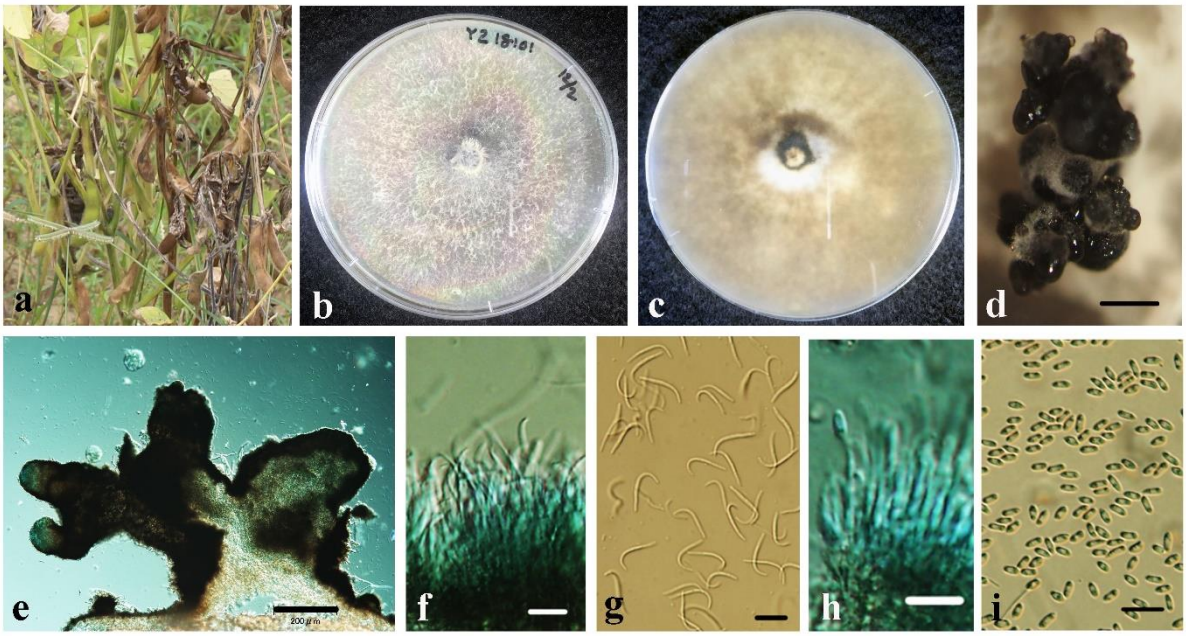

Fig. 2.5 Morphological characteristics of *D. tectonendophytica* (SoyYZ18101); a = Symptoms in field, b and c = Ten days old colony on PDA (above and below view), d = Conidiomata on PDA (bar = 500 µm), e = Cross section of conidiomata (bar = 200 µm), f = Conidiogenous cells and beta conidia (bar 10 µm), g = Beta conidia (bar = 10 µm), h = Conidiogenous cells and alpha conidia (bar 10 µm), j = Alpha conidia (bar = 10 µm)

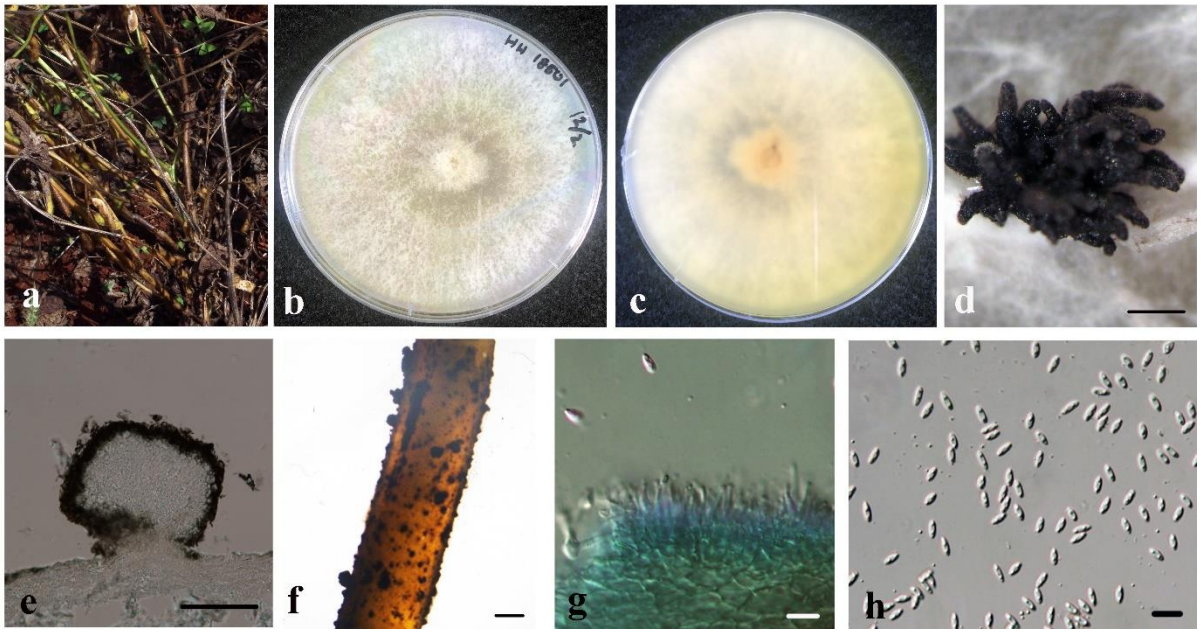

Fig. 2.6 Morphological characteristics of *Diaporthe ueckerae* (SoyHH18501); a = Symptoms in field, b and c = Ten days old colony on PDA (above and below view), d = Conidiomata on PDA (bar = 500 µm), e = Cross section of conidiomata (bar = 200 µm), f = Conidiomata on sterilized soybean stem (bar 1mm), g = Conidiogenous cells and alpha conidia (bar = 10 µm), h = Alpha conidia (bar = 10 µm)

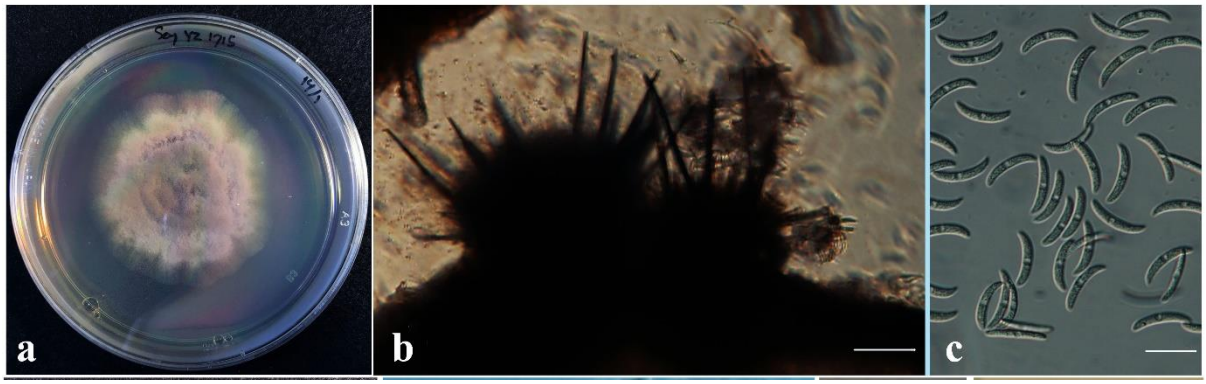

Fig. 2.7 Morphological characteristics of *Colletotrichum truncatum* (SoyYS1715); a = Colony on PDA at 7 days after incubation, b = Acervulus on sterilized soybean stem (Bar = 20 μm), and c = Conidia (Bar = 20 μm)

Fig. 2.8 Morphological characteristics of *Colletotrichum plurivorum* (SoyYS1733)**;** a = Colony on PDA at 10 days after incubation, b = Ascocarp on inoculated soybean stem (Bar = 50 µm), c = Ascus and ascospores (Bar = 10 µm), and d = Conidia (Bar = 20 µm)

2.3.3 Pathogenicity test

In the first experiment, the pathogenicity of *Diaprothe* spp. were significantly higher than those of *Colletotrichum* spp. and *Macrophomina* spp. The inoculated symptoms of the representative fungal isolates of *Colletotrichum* spp., *Diaporthe* spp. and *Macrophomina* spp. are shown in Fig. 2.9. There were no significant differences of lesion length (%) among the *Diaporthe* spp. but the lesion length (%) was significantly different among *Colletotrichum* spp in LSD all-pairwise comparisons test at $P \leq 0.05$. The lesion length (%) of *C. plurivorum* was significantly lower than those of *C. truncatum*. In this experiment, *Macrophomina* spp. and *C. plurivorum* were lower level of virulent disease reaction on the soybean stem inoculation (Table 2.6).

In the second experiment, the pathogenicity of two representative isolates of four *Diaporthe* spp., namely, *D. melonis, D. endophytica, D. tectonendophytica* and *D. ueckerae,* were tested on soybean cultivar using stem cutting inoculating method. Two different species were also inoculated together for their synergetic effect on pathogenicity because infection of two *Diaporthe* spp. was occurred in the same sampling locations. In this experiment, the lesion length (%) ranged from 28.4% to 44.3% in inoculation treatments and 1.4% in *control,* and there were significant differences between the inoculations and *control* in analysis of variance with completely randomized design. However, there was no significant difference ($P = 0.10$) among the other signal species inoculation and two species coinoculations. Additionally, the highest lesion length (44.3%) was observed in coinoculation of *D. endophytica* + *D. ueckerae,* but it was not significant different to the other coinoculations and single species inoculation (except *D. tectonendophytica*) in LSD all-pairwise comparisons test at $P \leq 0.05$ (Table 2.7).

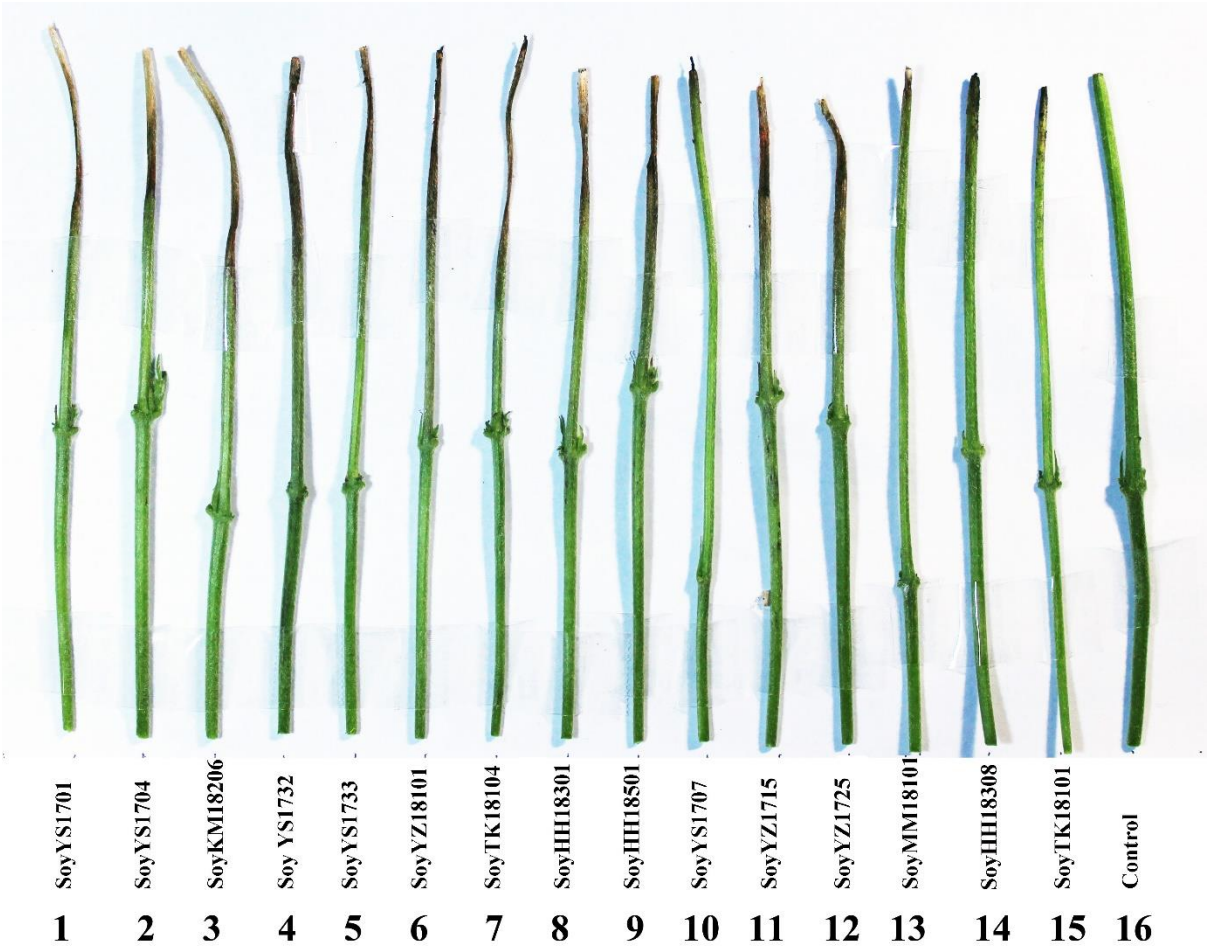

Fig. 2.9 Inoculated symptoms of different fungal isolates on soybean cultivar (Yezin-10) at 10 days after inoculation (1-9 = *Diaporthe* spp., 10-12 = *Colletotrichum* spp. and 13-15 = *Macrophomina* spp.)

Table 2.6 Lesion length (%) based on the stem length of soybean (cultivar Yezin-10) at 10 days after inoculation with different fungal species

Isolate	Species	Lesion length (%) (mean ± SE)*
SoyYS1701	*Diaporthe melonis*	45.2 ± 2.1 a
SoyKM18206	*Diaporthe melonis*	42.3 ± 1.9 a
SoyYZ18101	*Diaporthe tectonendophytica*	48.2 ± 2.4 a
SoyTK18104	*Diaporthetectonendophytica*	39.5 ± 3.3 a
SoyYS1732	*Diaporthe endophytica*	42.7 ± 2.8 a
SoyHH18301	*Diaporthe ueckerae*	28.6 ± 3.1 ab
SoyYS1707	*Colletotrichum plurivorum*	6.2 ± 1.0 c
SoyYZ1715	*Colletotrichum trucatum*	23.2 ± 2.3 b
SoyYZ1716	*Colletotrichumtrucatum*	24.3 ± 2.1 b
SoyMM18101	*Macrophomina* sp.	10.1 ± 1.5 c
SoyHH18308	*Macrophomina* sp.	5.9 ± 1.4 c
SoyTK18101	*Macrophomina*sp.	3.4 ± 1.0 c
Mean		26.6
CV		18.9

* Mean values of lesion length (%) were calculated from 5 observations. Means followed by the same letters are not significantly different by the least significant difference (LSD) all-pairwise comparisons test at $P \leq 0.05$.

Table 2.7 Means lesion length (%) on soybean stems inoculated with isolates of genus *Diaporthe* in single-inoculation and coinoculation tests

Species	Lesion length (%) (mean ± SE)*
Diaporthe melonis	33.3 ± 2.8 ab
Diaporthe endophytica	36.1 ± 1.7 ab
Diaporthe tectonendophytica	28.4 ± 3.6 b
Diaporthe ueckerae	39.4 ± 2.4 ab
D. melonis + *D. endophytica*	40.8 ± 3.2 ab
D. melonis + *D. tectonendophytica*	36.1 ± 3.5 ab
D. melonis + *D. ueckerae*	39.0 ± 4.0 ab
D. endophytica + *D. tectonendophytica*	35.4 ± 4.9 ab
D. endophytica + *D. ueckerae*	44.3 ± 3.3 a
D. tectonendophytica + *D. ueckerae*	42.1 ± 3.9 ab
Control	1.4 ± 0.3 c
Grand mean	34.2
CV	19.1

* Mean values of lesion length (%) were calculated from 8 observations that included 2 randomly selected isolates of each species with 4 replicates. Means followed by the same letters are not significantly different by the least significant difference (LSD) all-pairwise comparisons test at $P \leq 0.05$.

2.3.3 Phylogenetic analysis

Phylogenetic analysis of *Diaporthe* spp. based on ITS, *EF1-α* and *TUB* sequences of 66 strains, including closely related reference strains of *Diaporthe* spp. Gene boundaries were as follows: ITS: 1-502, EF1-α: 503-878, and TUB 879-1326. *D. vaccinia* (DP5032) was used as an outgroup. For the Bayesian analysis, the substitution models used were as follows: GTR+I+G for the ITS and HKY+G for the *EF1-α* and *TUB* genes. The topologies of the phylogenetic trees obtained via the maximum likelihood method by using MEGA7 software and Bayesian analysis were similar, and the parsimony bootstrap (≥70%) and Bayesian posterior probability (≥0.90) values are shown on the branches in Fig. 2.10. Eight isolates obtained from two locations (Lawksawk and Pyinmana) were closely related to *D. melonis* (CBS435.87). Other eight isolates from Pyinmana and Tatkone were formed a single clade with the *D. tectonendophytica* ex-type isolates MFLUCC130471 and LC6623, where Tatkone isolate (SoyTK18104) is more similar to the ex-type isolates. However, the morphological characteristics were not clearly different among the eight isolates. Two isolates from Heho were similar to *D. ueckerae* (FAU656). Lastly, other five isolates from Lawksawk and Taunggyi were formed in a same cluster with *D. endophytica* (LGMF948 and CBS133811). Total tested 23 isolates from soybean disease stems collected from five locations of Myanmar were phylogenetically similar to four species *D. endophytica*, *D. melonis*, *D. tectonendophytica* and *D. ueckerae*, respectively. Two different *Diaporthe* spp. (*D. endophytica* and *D. melonis*, and *D. melonis* and *D. tectonendophytica*) were occurred in a same sampling location of Lawksawk and Pyinmana, and a single species was observed in Heho, Tatkone and Taunggyi, respectively.

For phylogenetic analysis of *Colletotrichum* spp., the sequences of the ITS, *GAPDH*, *CHS-1* and *ACT* genes of the three representative isolates (SoyYZ1715, SoyYZ1716 and SoyYZ1725) from Nay Pyi Taw and three representative isolates (SoyYS1707, SoyYS1709,

and SoyYS1726) from Lawksawk were aligned with closely related verified sequences for phylogenetic analysis. The alignment contained 37 taxa, and *Monilochaetes infuscans* (CBS 869.96) served as the outgroup. The gene boundaries, including gaps, were as follows: ITS, 1-519; *GAPDH*, 520-826; *CHS-1*, 827-1116; and *ACT*, 1117-1364. The substitution models selected for Bayesian analysis were GTR+G for ITS, HKY+I for *GAPDH* and HKY+G for the *CHS-1* and *ACT* genes. The results showed that the SoyYZ1715 and SoyYZ1716 isolates were identical to the CBS195.32 and CMES 1036 sequences, and the three isolates obtained in this study were also closely related to the *C. truncatum* type strain (CBS151.35). The other three isolates (Soy YS1707, SoyYS1709 and SoyYS1726) were belonged to the same clade as *C. plurivorum* (CBS125474 and LFN0008) (Fig. 2.11).

2.4 Discussion

The symptoms of soybean stem diseases caused by the *Diaporthe* species complex are described as black blotching with or without pycnidia on the stem (Kmetz et al. 1978). Moreover, groups of *Colletotrichum* species are also associated with the stem and cause anthracnose on soybeans (Yang et al. 2014), and characteristics among the *Colletotrichum* species were also similar. Consequently, we differentiated the collected isolates only to the genus level as *Colletotrichum* spp. and *Diaporthe* spp. based on the morphological characteristics.

The pathogenicity measured with the stem cutting inoculation method reproduced necrotic lesions with fruiting bodies on the cut stems of soybean. This method is mainly used for pathogenicity testing and varietal resistance screening experiments for phomopsis diseases, sclerotinia stem rot, and charcoal rot disease in soybean (Kull et al. 2007; Li et al. 2001; Shan et al. 2013; Twizeyimana et al. 2012). However, the inoculation test with the isolates of *Colletotrichum* spp. also reproduced necrotic lesions on cut stems, and we assumed that these species were fungal pathogens of soybean. Therefore, isolates of these

two genera were identified separately by molecular phylogenetic analysis. The pathogenicity tests of four species of *Diaporthe* genus were performed not only to fulfil Koch's postulates but also to identify the pathological consequences of the cooccurrence of two pathogenic species within the *Diaporthe* disease complex in soybean. The pathogenicity of two species of *Diaporthe* was examined via coinoculation in this study because the two species were found in the same field in Lawksawk and Nay Pyi Taw. In the case of the single-species inoculation of *D. tectonendophytica*, the disease lesion (%) was significantly lower than that associated with *D. ueckerae*, but it was not significantly different from those associated the other two species, *D. melonis* and *D. endophytica*. On the other hand, when the pathogenicity resulting from coinoculation and single-species inoculation was compared, no significant differences were observed. The effect of the coinoculation of two pathogens (*Mycosphaerella pinodes* and *Phoma medicaginis* var. *pinodella*) of the Ascochyta blight complex on the same organ of pea plants limited disease development and reproduction, but synergism was observed when the plants that had been previously inoculated were subsequently inoculated with other species (Le May et al. 2009). In my study, we used the stem cutting inoculation technique and inoculated two *Diaporthe* spp. together on the same tip of a stem, and no obvious differences were found between the single-species inoculations and the coinoculations of two species. However, further experiments involving the coinoculation of *Diaporthe* sp. and other fungal genera that frequently infest soybean will be necessary to understand the pathogenicity resulting from the occurrence of two or more different species well.

Currently, molecular tools such as the sequencing of different genes or intergenic regions have been shown to be applicable and more accurate for the identification of fungal species (Ash et al. 2010; Gomes et al. 2013; Yang et al. 2014). In strategies involving the use of genetic barcodes of different genes for species delimitation, the ITS region of r-DNA

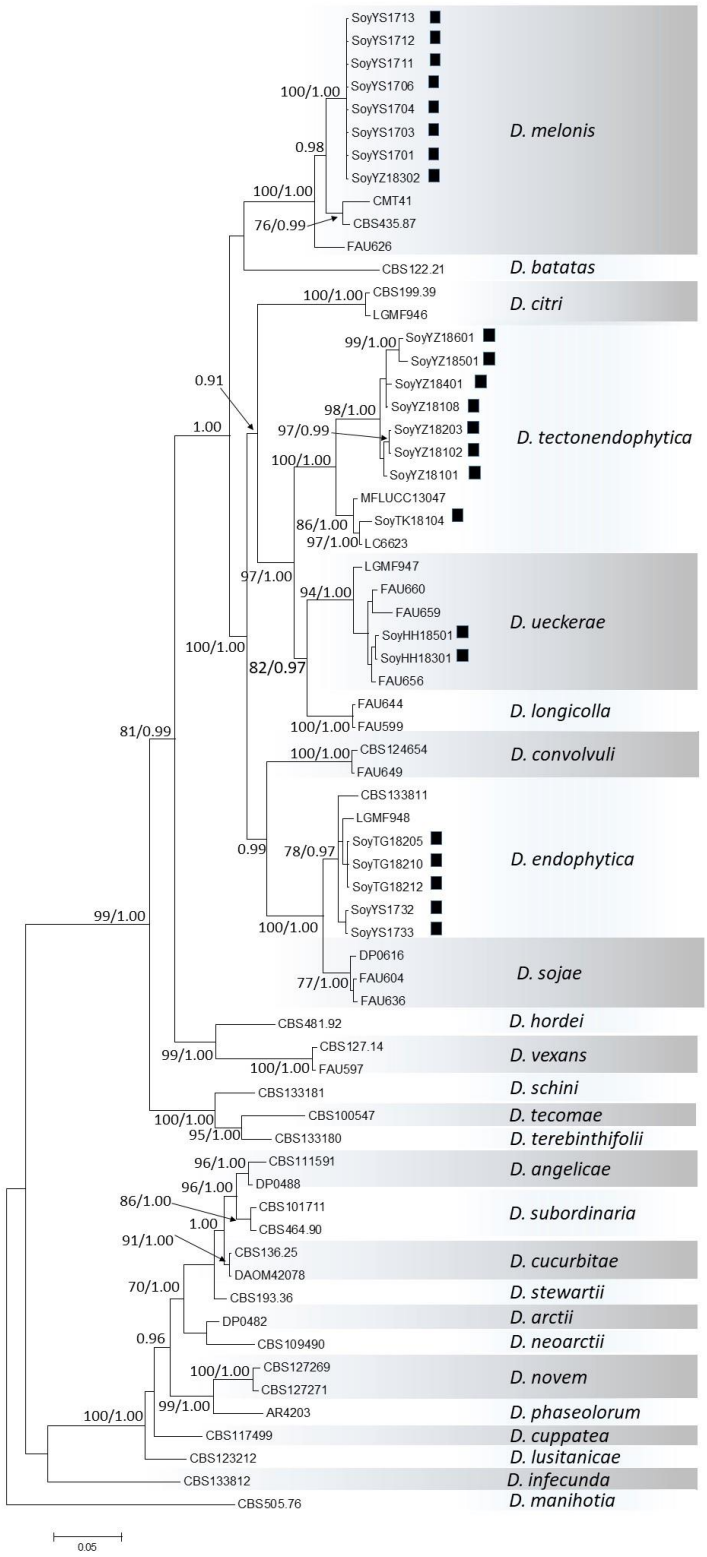

Fig. 2.10 A maximum likelihood tree of *Diaporthe* spp. collected from Myanmar and other closely related species of *Diaporthe* based on a multilocus dataset (ITS, *EF1-α* and *TUB*) genes. *D. manihotia* was designated as outgroup. Bootstrap values (≥ 70%)/ Bayesian posterior probability (≥0.90) are shown at the branch. Black square (■) follow by the isolate name = isolate in the present study

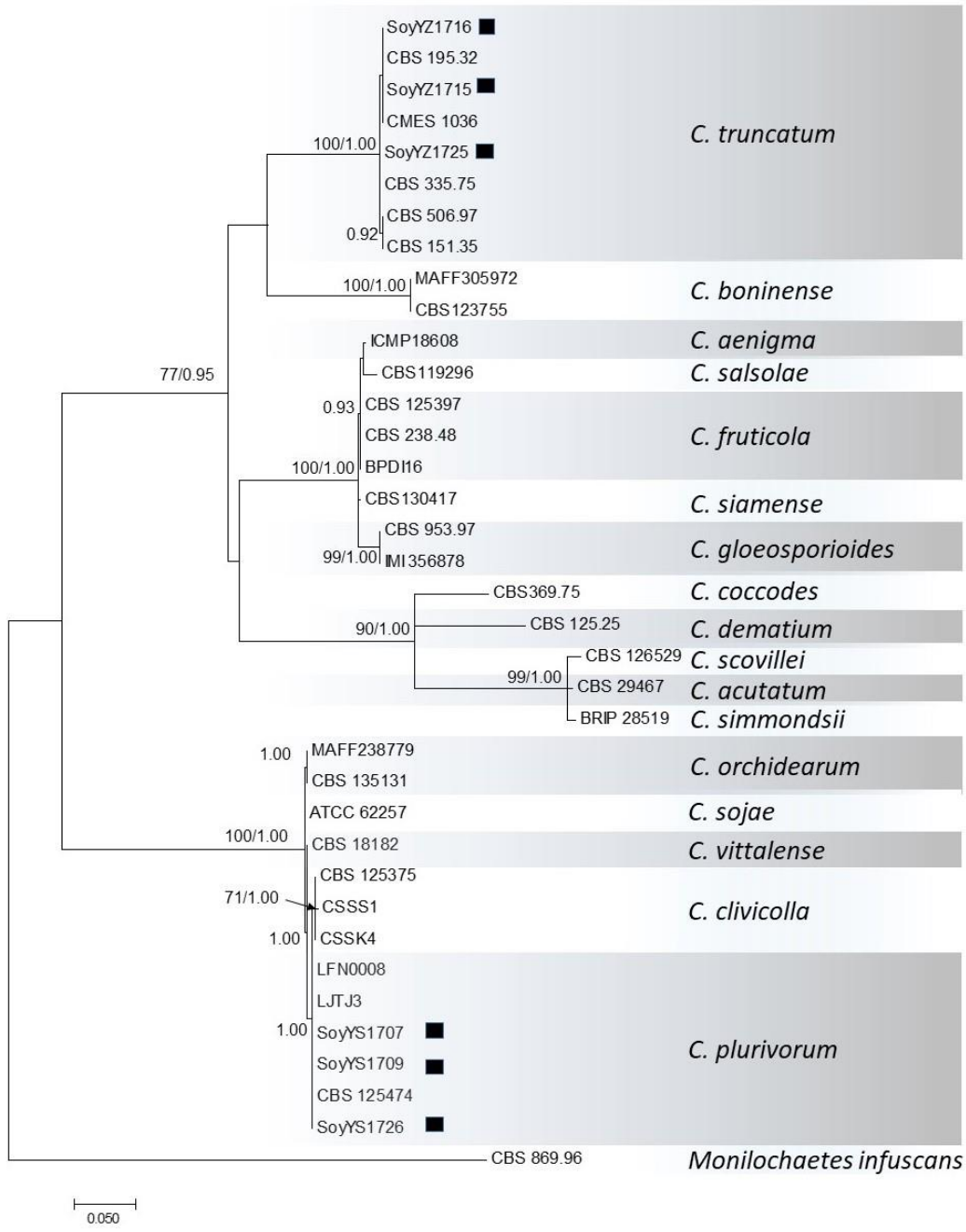

Fig. 2.11 Molecular phylogenetic trees inferred from the ITS, *ACT*, *GAPDH* and *CHS-1* genes of *Colletotrichum* species using the maximum likelihood method. Bootstrap support values (≥70%)/Bayesian posterior probabilities (≥0.90) are displayed at each branch, including detailed descriptions of the symbol as per Fig. 2.10.

has been widely applied (Gardes and Bruns 1993; Schoch et al. 2012; Shishido et al. 2006). Similarly, many *Colletotrichum* species cannot be identified using only the ITS (Weir et al. 2012). Therefore, other fungal barcoding genes were aligned with other sequences of verified reference isolates, and a phylogenetic study was conducted. According to the multilocus phylogenetic analysis, the isolates of *Diaporthe* from the Lawksawk, Pyinmana, Taunggyi and Tetkone area were similar to *D. endophytica* and *D. melonis, D. tectonendophytica* and *D.* ueckerae. The *Colletotrichum* isolates from Lawksawk and Pyinmana were identified as *C. plurivorum* and *C. truncatum,* respectively.

Known hosts of *Diaporthe melonis* include *Annona squamosa*, *Carapa guianensis*, *Cucumis melo* and *Glycine max* (Dissanayake et al. 2017), and this species is phylogenetically closely related to *D. longicolla* and *D. sojae* (Udayanga et al. 2015). *D. endophytica* has been isolated from seeds of *Glycine max*, as an endophyte on the leaves of *Schinus terebinthifolius* and on the petioles of *Maytenus ilicifolia* in Brazil (Gomes et al. 2013). In our pathogenicity tests, *D. endophytica* and *D. melonis* were pathogenic on soybean plants. On the other hand, there has been no previous report of the infestation of *D. tectonendophytica* in soybean plants. *D. tectonendophytica* first occurred in teak tree (*Tectona grandis*) in Northern Thailand as an endophytic fungus (Doilom et al. 2017). Almost 50% of the total natural teak forest area is within Myanmar (Kollert and Cherubini 2012). Additionally, natural and planted teak trees occur near soybean fields in the Pyinmana area. Therefore, I assume that the original source of the *D. tectonendophytica* identified in this study was natural or planted teak trees from which it spread and infect to soybean plants. Finally, *D. ueckerae* has been reported as a ubiquitous pathogen that can infect cucumber and soybean in Brazil (Udayanga et al. 2015). The observed morphological characteristics of the three *Diaporthe* species; *D. melonis*, *D. tectonendophytica* and *D.*

ueckerae were similar to those described in previous reports (Udayanga et al. 2015; Doilom et al. 2017).

C. truncatum is a common cause of anthracnose disease in major soybean-growing countries (Wrather et al. 1997, 2010) and has been distributed to the neighbouring country of Myanmar (CAB-International 2001). However, *C. plurivorum* has been reported on soybean in Brazil and Japan (Barbieri et al. 2017; Damm et al. 2019) and belongs to the *C. orchidearum* species complex (Damm et al. 2019). This species was recently reported as a pathogen that is associated with anthracnose disease on chili in China and the Andaman and Nicobar Islands (Liu et al. 2016; Sakthivel et al. 2018), on papaya in Taiwan (Sun et al. 2019), and on *Pyrus* spp. in China (Fu et al. 2018). In the present study, we revealed that *C. plurivorum* was weakly virulent, whereas *C. truncatum* was strongly virulent on soybean.

I identified isolates of *C. plurivorum*, *D. endophytica, D. melonis, D. ueckerae* in five locations, Shan State, and only one species, *C. truncatum, D. melonis* and *D. tectonendophytica* were isolated from the diseased samples from the Nay Pyi Taw area. Relatively more diverse fungal plant pathogen species were found in southern Shan State, which seems to be facilitated by mild temperatures (not very hot or cold) and humid weather. TeKrony et al. (1983) reported that disease incidence caused by *Diaporthe* species was significantly related to relative humidity and air temperature and that seed infection by these fungal pathogens is more dependent on moisture. The maximum disease incidence of soybean anthracnose caused by *C. truncatum* occurred at 28.4°C and 76% relative humidity in September 1995 in India (Singh et al. 2001).

In Myanmar, 15 other species of *Diaporthe* and 16 species of *Colletotrichum* have been reported on different host plants (Thaung 2008). Additionally, infection of soybean by *C. plurivorum, C. truncatum, D. endophytica, D. melonis, D. tectonendophytica* and *D. ueckerae* in Myanmar was never checked before this study. During the field survey of two

major soybean-growing areas, fungal diseases were prominent and severely affected the yield and income of the farmers. To my knowledge, this is the first report of the characterization of fungal pathogens on soybean in Myanmar, and effective control measures are urgently needed by farmers. We hope that the results of this investigation will support further experiments on the development of disease control and management strategies for soybean production.

CHAPTER 3

Isolation and Characterization of Antagonistic Beneficial Fungus *Trichoderma virens* Tv911 and Its Effects on Plant Growth

3.1 Introduction

Application of synthetic agro-chemicals for plant growth enhancement and weeds, pest and disease control would cause negative impacts on biodiversity, environment and human health (Debenest et al., 2010; Geiger et al., 2010). Accordingly, organic agriculture has become popular with rising trend throughout the world (Willer et al., 2009). Nowadays, many microbial products are available for pest and disease management in crop production (Berg, 2009), and also available for plant growth promotion as a biofertilizer (Rodríguez and Fraga, 1999; Vessey, 2003).

The genus *Trichoderma* (teleomorph *Hypocrea*) has attracted an increasing attention and interest because of their economic value, and it is widely used not only as a biocontrol agent especially for soil borne plant pathogenic fungi (Qualhato et al., 2013) but also for enzyme production in industrial usage (Ahamed and Vermette, 2008). Additionally, it has the potential to degrade environmental hazardous chemical residue from the contaminated agricultural soil (Arfarita et al., 2013).

Antagonist mechanisms of *Trichoderma* spp. are competition for nutrient and space, and direct mycoparasitism with antibiosis such as cell wall degrading enzymes to inhibit the growth of plant pathogens (Benítez et al., 2004). Moreover, these species are common and persistent fungi in the rhizosphere soil microbial community in a natural ecosystem (Lidia et al., 2011). Among them, *T. asperellum*, *T. hamatum*, *T. harzianum, T. koningii* and *T. virens* were colonizing to the roots and rhizosphere and some strains of each species have been identified as a plant growth promoter of the early growth stage of bean plants (Hoyos-

Carvajal et al., 2009). The morphological and cultural properties of *T. harzianum* and *T. vriens* were not obviously different (Sharma and Singh, 2014), and it can be accurately identified by molecular sequencing of ITS gene (White et al., 1990).

In our previous study, several *Trichoderma* species isolated from waste paper sludge compost abandoned by paper manufacturing company in Saga, Japan during 2014-2016 were characterized by their paper degradation ability. As the results, these isolates are potentially responsible for production of cellulose degrading enzyme (cellulase) and total crude protein in treated paper wastes (Peng et al., 2016). Remarkably, there is no report concerned to the characterization of *Trichoderma* spp. derived from such paper sludge compost and possibility of usage of these isolates as a biological resource of plant growth promotion and antagonist of the plant pathogenic fungi. Although, cellulose is not a major component in cell wall of plant pathogenic fungi (Bartnicki-Garcia, 1968), we assumed that degradation of cellulose residue in soil, and colonization of those saprophytic fungi in soil is helpful for plant growth and disease suppression. Additionally, *Trichoderma* is a potential microbial inoculant to decompose crop residues, and it increase soil fertility status, physicochemical and microbial condition of the soil for sustainable agricultural production (Devika et al., 2019; Sharma et al., 2012).

The objectives of this research in this chapter are (1) to isolate and identify the antagonistic fungal resources from the waste paper sludge compost, (2) to evaluate their antagonistic effects on plant pathogenic fungus *in vitro*, and (3) to determine the plant growth promotion ability of selected biological agent on some vegetables, namely Japanese mustard spinach, radish and tomato under greenhouse condition.

3.2 Materials and Methods

3.2.1 Isolation

The fungus resources were newly isolated from waste paper sludge compost obtained from Saga Prefecture, Japan (Fig. 3.1) by serial dilution method for this experiment. Briefly, 1 g of waste paper sludge compost was dissolved in 1 ml of sterilized distilled water and shaken on rotary shaker (EYEL4, Multishaker MMS) at 150 rpm speed for 30 min and 10 times serial dilution was performed to obtained 10^{-4} concentration of the original solution. Consequently, the diluted suspension was spread on the sterilized potato dextrose agar (PDA) (Merck Millipore, Germany) plates amended with streptomycin (Sigma-Aldrich, USA). One mL of 10% (w/v) streptomycin solution was sterilized by using Minisart® Syringe Filter (Sartorius Stedim Biotech GmbH, 37070, Goettingen, Germany), and added to 1 L of warm (approximately 50°C) PDA medium after autoclaved sterilization. The PDA plates were incubated at 25°C for 48 hours. The grown fungal colony was transplanted on new PDA plates, and their genus level identification was conducted by visual observation using light microscope (Leica DM 2500) mounted with an Olympus DP70 camera (Olympus, Tokyo, Japan) based on morphological characteristics. Single spore culture was prepared to obtain pure culture for further investigations.

The isolates obtained from this experiment were deposited in the Laboratory of Institute of Tropical Agriculture, Kyushu University.

3.2.2 Identification of antagonistic fungal resources

For species identification, representative 6 isolates were selected, and genomic DNA of each isolate was extracted by using DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. ITS regions of rDNA was amplified by using ITS1 and ITS4 primers (White et al., 1990) (Table 2.2). PCR was performed in total volume of 25 µl, containing 2.5 µl of 10× reaction buffer, 2 µL of dNTPs (2 mM each),

Fig. 3.1 Paper sludge compost, the waste product of paper production plant of Kotobuki paper Co. Ltd in Saga Prefecture, Japan

1 µl of each primer (10 µM), 0.25 µl of *Taq* polymerase (2.5 units/ µl) (Toyobo, Osaka, Japan), 2 µl of template DNA and 16.25 µl of sterilized distilled water. This PCR reaction was conducted in thermal cycler (Biometra TProfessional basic Thermalcycler gradient) programmed for 95°C for 2 min, 39 cycles of 95°C for 30 sec, 55°C for 50 sec, and 72°C for 1 min, and 72°C for 5 min for final extension. The quality of PCR products was checked with ethidium bromide stained gel electrophoresis using 1.5% (w/v) agarose gel. PCR products were purified with QIAquick® PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacture instructions. The purified PCR products were sent to Fasmac Co. (Kanagawa, Japan) for sequencing. Resultant sequences were queried to the BLAST in http://ddbj.nig.ac.jp/blast/blastn, and the sequences were deposited in GenBank with the accession number LC269252 to LC269257.

3.2.3 Antagonistic effects of collected fungal resources against FOLy *in vitro*

I assessed the biocontrol activities of the collected fungal materials by using a slightly modified dual culture technique proposed by Mbarga et al. (2012). Concisely, the fungal isolates of *Fusarium oxysporum* f. sp. *lycopersici* (FOLy) (MAFF 103045), *Trichoderma* spp. (Tv811, Tv911 and Th621) and *Clonostachys* spp. (Cr322, Cr411 and Cr15-3-1) were separately grown on PDA plates, and incubated for 5 days at 25°C. Five mm mycelial disc of FOLy and each tested fungal isolate was placed in opposite side at 50 mm apart to each other on PDA plate for the assessment of direct antagonistic effect. Moreover, antagonistic effect by volatile compound of tested fungal isolates was also examined by using the seal plate method (Aydi Ben Abdallah et al., 2015). In this method, 5 mm mycelial disc of FOLy and each tested isolate of *Trichoderma* spp. and *Clonostachys* spp. were put on PDA separately, the plate containing FOLy was inverted over the tested isolate plates, and seal with parafilm to avoid escaping of the volatile compound. The data were collected at 1 week after incubation at 25°C. The effectiveness of biological agents was considered according

to their inhibition rate (IR) with the following formula. IR (%) = [(colony diameter of control – colony diameter presence of tested antagonist)/colony diameter of control] × 100. This experiment was carried out with 5 replications, and colony diameter was mean value of two perpendicular measurements of FOLy colony.

3.2.4 Plant growth promotion effects of Tv911 on some vegetables

Trichoderma virens, isolate Tv911 was used as a resource of plant growth promotion test on three kinds of vegetables: Japanese mustard spinach (*Brassica rapa* var. *perviridis*), tomato (*Solanum esculentum* cv. Monterosa) and radish (*Raphanus sativus* cv. Saradakomachi) in this experiment. The tested crops were grown in two types of soil, namely Farmer use nursery soil (FNS) and Home garden soil (HGS), commercial products of Oishi Bussan Co. Ltd., Fukuoka, Japan. These soils were named by the company according to usage of the customers, where FNS is used by the farmers and HGS is mainly used in home garden, and the trade name is Monsaku and HB-101, Baiyoudo, respectively.

Firstly, the inoculum was prepared from 10 days old culture of Tv911 on PDA by harvesting conidial suspension with sterilized distilled water. The conidial suspension was adjusted 10^7 conidia/mL concentration and mixed thoroughly with the soil materials using soil mixer machine with the rate of 1 mL spore suspension kg^{-1} of soil. The inoculated soil materials were put in 20 L plastic bag, and it was sealed and incubated for one month at room temperature to obtain stable survival fungal population at plant sowing time. Two kg of inoculated and non-inoculated soil were put in pots and the seeds were sown at 1 cm depth. The experiment was performed with three replications and each replication had 5, 3, and 4 plants for Japanese mustard spinach, radish and tomato and respectively. Plant height and fresh shoot and root weight were measured at one month after sowing for tomato and Japanese mustard spinach, and at 6 weeks after sowing for radish under greenhouse conditions, respectively.

3.2.5 Statistical analysis

Data obtained in this experiment were analyzed in one-way analysis of variance (ANOVA) using Statistix software (Version 8.0) (Analytical Software, 2105, Miller Landing Rd, Tallahassee, FL, 32312, USA). Mean values were compared least significant difference (LSD) all-pairwise comparisons test at $P \leq 0.05$.

3.3 Results

3.3.1 Identification of fungal isolates from waste paper sludge

This study discovered a total of three fungal species from the waste paper sludge compost (Table 3.1). Firstly, they were differentiated into genera (*Trichoderma* and *Clonostachys*) based on the morphological characteristics. The colony of *Trichoderma* was whitish green, and the conidiophores were long, erect and bare phialides with greenish spherical conidia. The colony of *Clonostachys* sp. was whitish in colour with pale yellow in reverse, and conidiophores were erect, long and verticillium-like phialides with oval shaped conidia (Fig. 3.2). Then, the studied isolates could be identified as the following species by performing the molecular characterization. The nucleotides sequences of ITS regions rDNA of isolate, Tv811 and Tv911 were 100% identical to the *Trichoderma virens* (GenBank accession JX908731), three isolates of Cr322, Cr411 and Cr15-3-1 were 99% identical to *Clonostachys rosea* (GenBank accession: KY365588) and isolate Th621 was 99% identical to *Trichoderma harzianum* (GenBank accession: KY750364), respectively (Fig. 3.3).

3.3.2 Antagonistic effect of fungal isolates on mycelial growth of FOLy *in vitro*

The antagonistic effect of obtained isolates against soil borne plant pathogenic fungus, FOLy was determined with inhibition rate (IR) in two experiments of direct metabolites and volatile compound *in vitro* (Fig. 3.4 and Fig. 3.6). The mean values of IR of tested isolates on FOLy in both direct metabolites and volatile compound tests were

significantly differed among the isolates ($P \leq 0.05$), but the growth inhibition by direct metabolites was higher than the inhibition by volatile compound at 7 days after incubation. In the direct growth inhibition, the highest IR (64.27 %) was observed in Tv911 and there were no significant differences among the *Trichoderma* isolates. IR of *Trichoderma* isolates was significantly higher than that of *C. rosea* isolates; Cr322, Cr411 and Cr15-3-1 in both tests (Table 3.1). Consequently, *T. virens*, Tv911 isolate was selected for the next experiments. The growth inhibition zone of direct antagonistic test was observed by light microscope, where mycelia of *Trichoderma* were coiling to those of host FOLy (Fig. 3.5).

3.3.3 Plant growth promotion effect of Tv911

In the case of growth promotion effect of Tv911, plant heights of Japanese mustard spinach in treated FNS and HGS were significantly higher than in non-treated controls at $P \leq 0.05$ (Fig. 3.7-a). Additionally, the plant height increased by 16.22% and 9.84% in Tv911 treated FNS and treated HGS, respectively (Table 3.2). The plant heights of tomato plant were significantly different among the treated and non-treated soils, where treated FNS was significantly higher than non-treated FNS at $P \leq 0.01$ (Fig. 3.7-b). The plant heights of tomato were promoted at 50.26% and 7.00% in both treated FNS and HGS, respectively (Table 3.2). In the experiment of effect of Tv911 on the growth of radish plants, fresh shoot and root weight were measured. There were no significant differences in the fresh shoot weight among the treatments. However, the root weights were significantly different between treated and non-treated soils at $P \leq 0.05$ (Fig. 3.7-c), and the average root weight in treated FNS and HGS were 58.86% and 64.45% increased over non-treated FNS and HGS, respectively (Table 3.2).

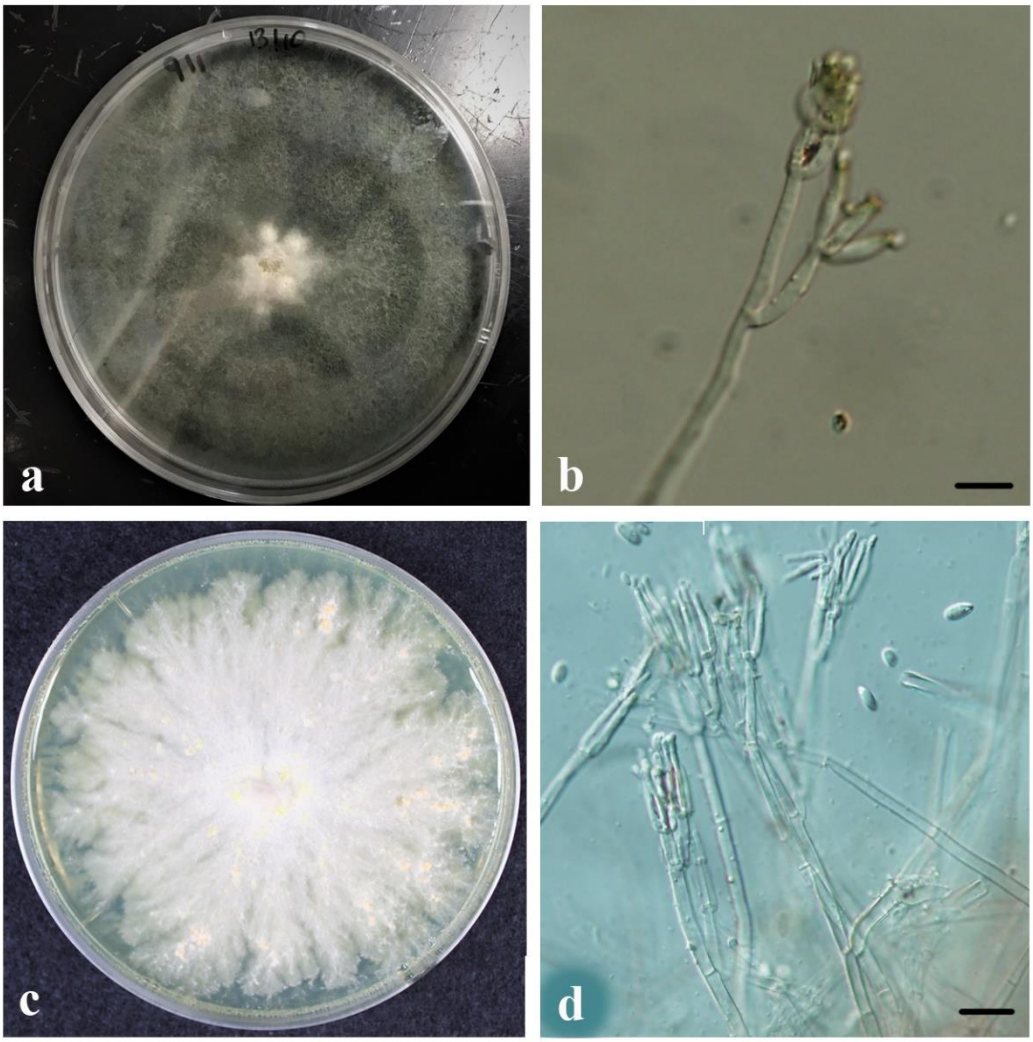

Fig. 3.2 Morphological characteristics of fungal resources obtained from waste paper sludge compost, a = colony of *Trichoderma virens* on PDA, b = conidia and conidiophores of *Trichoderma* sp. (bar = 10 µm), c = colony of *Clonostachys rosea* on PDA, d = conidia and conidiophores of *Clonostachys rosea* (bar = 10 µm)

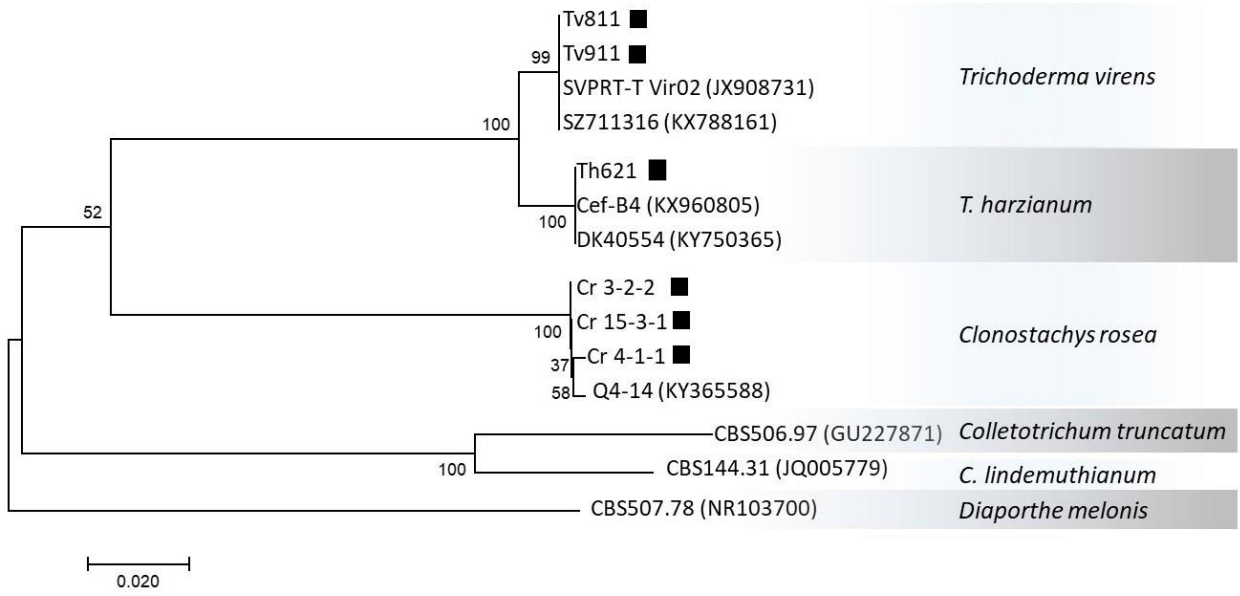

Fig. 3.3 Phylogenetic analysis of the fungal resources isolated from paper sludge compost inferred by ITS gene of rDNA using Neighbor-joining method with MEGA 7. (■represents the isolates in this study)

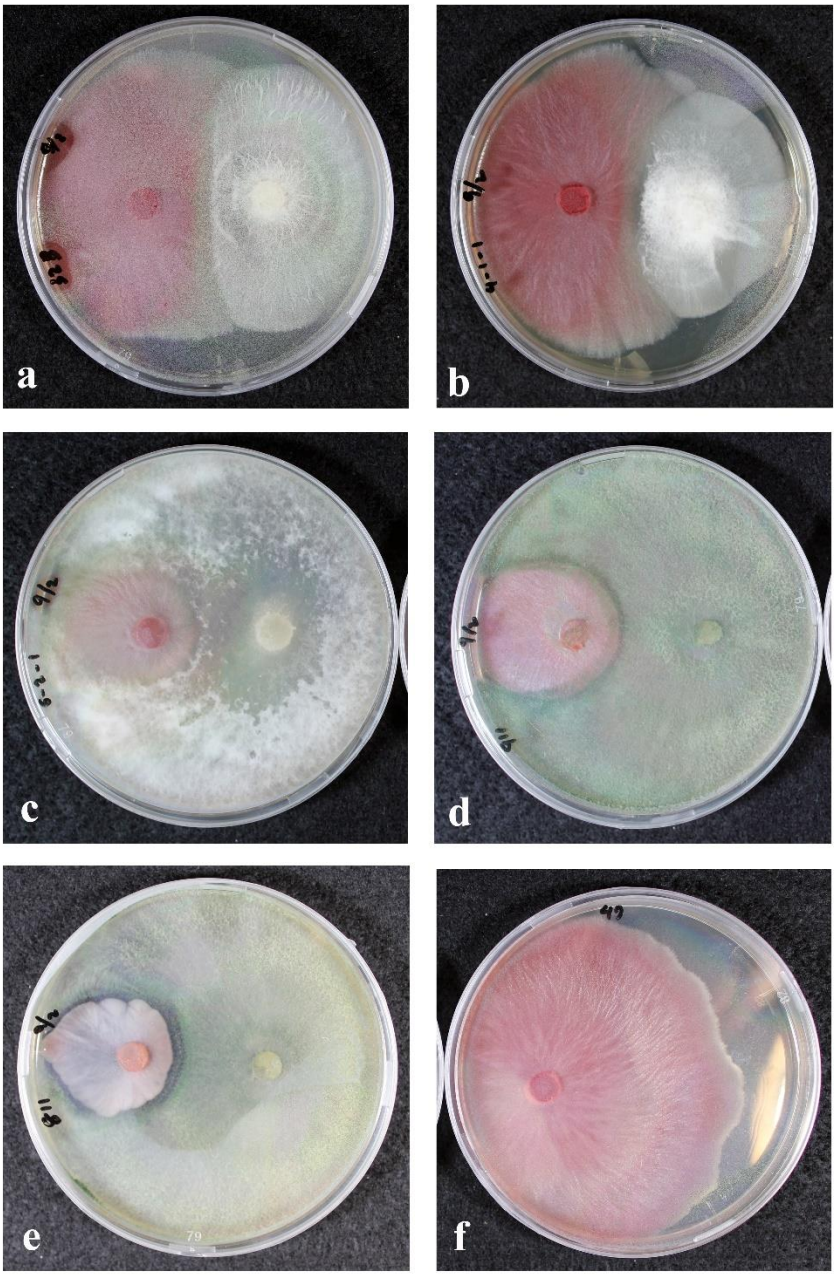

Fig. 3.4 Antagonism (direct metabolites test) of *Clonostachys rosea* and *Trichoderma* spp. against the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOLy), where, a = Cr322, b = Cr411, c = Th621, d = Tv811, e = Tv911 and f = Control check

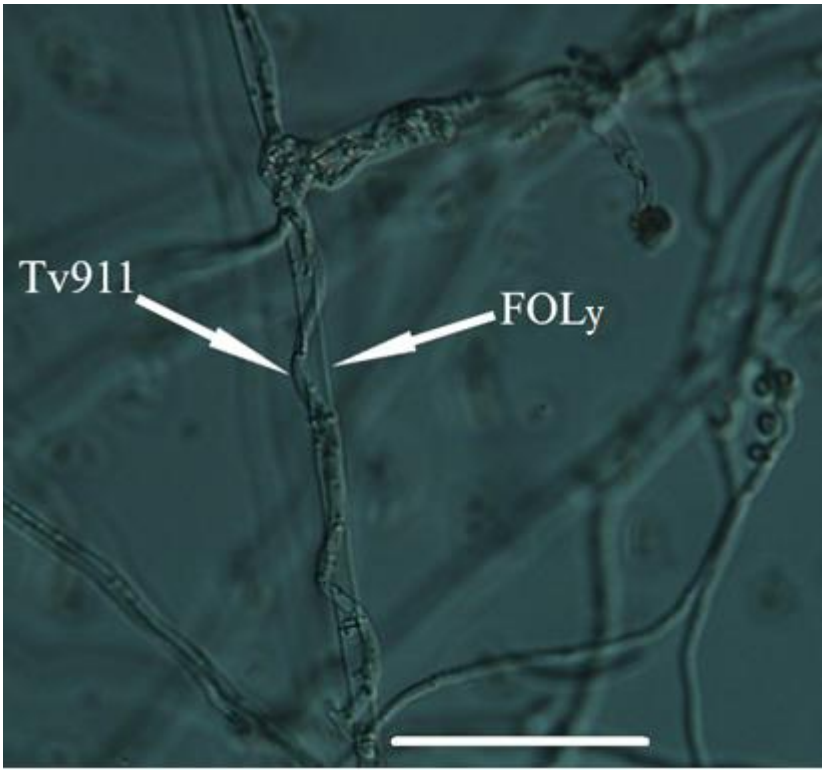

Fig. 3.5 Mycelial coiling of *Trichoderma virens* (Tv911) around the host *F. oxysporum* f. sp. *lycopersici* (FOLy) in the inhibition zone between the growth of two fungi (Bar = 50 µm)

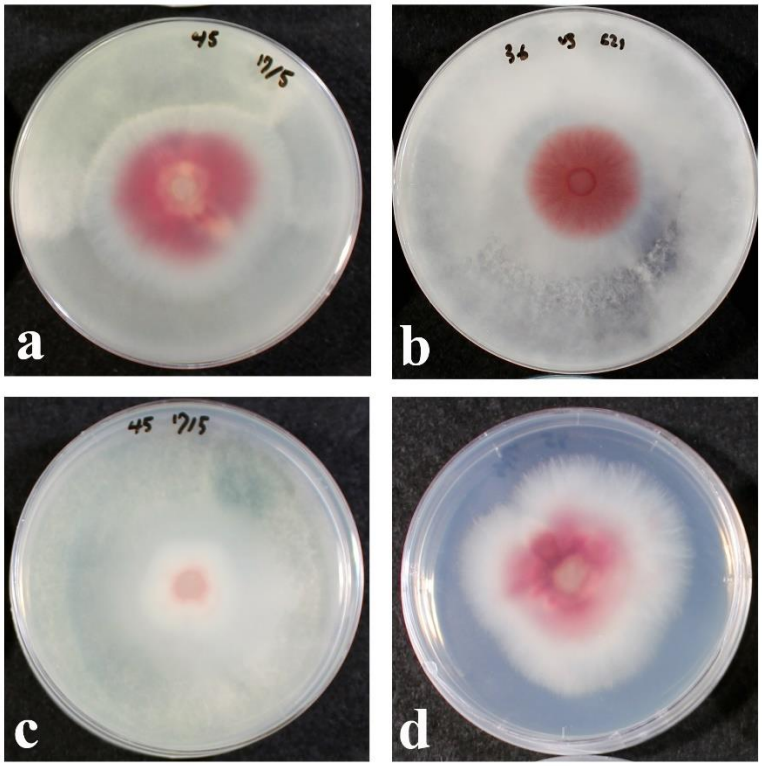

Fig. 3.6 Antagonism (volatile compound test) of *Trichoderma* spp. and *C. rosea* against the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOLy) a = Cr322, b = Th621, c = Tv911 and d = Control check

Table 3.1 Inhibition rate (IR (%)) of fungal isolates against the growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOLy) *in vitro*

Species	Isolate	GenBank accession no.	IR (%) by direct *	IR (%) by volatile *
Trichoderma virens	Tv811	LC269256	60.55 a	19.53 b
T. virens	Tv911	LC269257	64.27 a	27.41 a
T. harzianum	Th621	LC269254	59.06 a	18.08 b
Clonostachys rosea	Cr322	LC269252	9.68 b	7.00 c
C. rosea	Cr411	LC269253	10.92 b	3.50 c
C. rosea	Cr15-3-1	LC269255	9.19 b	4.37 c

* Number followed by the same letter do not differ significantly by the least significant difference (LSD) all-pairwise comparisons test at $P \leq 0.05$.

3.4 Discussion

Trichoderma species have been generally recognized as a cellulose decomposer, biological control agents and plant beneficial symbiotic fungus for plant growth promotions (Ahamed and Vermette, 2008; Contreras-Cornejo et al., 2009; Harman et al., 2004). In the present study, we obtained fungal isolates from the waste paper sludge compost and studied their competitiveness to one of the harmful soil fungal plant pathogen *in vitro. Clonostachys rosea* has shown the antagonistic effects on the fungal plant diseases, such as *Alternaria* spp., *Fusarium culmorum* and *Sclerotinia sclerotiorum* in the previous studies (Jensen et al., 2004; Roberti et al., 2008; Rodriguez et al,. 2011). The isolates of *C. rosea*, Cr322, Cr411 and Cr15-3-1 showed fewer effect on the mycelial growth of FOLy in this experiment. These results are consistent with the previous study of *in vitro* assay that *C. rosea* showed lower level of fungal growth inhibition (1-23%) than *Trichoderma* strains (40-68%) against *Fusarium circinatum* (Moraga-Suazo et al., 2011). In the present study, *Trichoderma* isolates effectively inhibited the growth of FOLy in both tests of direct metabolites and volatile compound, but lower inhibition rate (IR) was found in the latter. In the antagonistic activities, *Trichoderma* spp. produce antibiotics, carbon and nitrogen permeases, hydrolytic enzymes and siderophores (Benítez et al., 2004). At 7 days after incubation, the cessation of the growth of FOLy by Tv911 isolates and direct parasitism of mycelial coiling at the inhibition zones were observed in this experiment (Fig. 3.5). In another study, different species of *T. viride* inhibited the mycelial growth of *F. oxysporum* f. sp. *adzuki* and *Pythium arrhenomanes* effectively *in vitro* (John et al., 2010).

Trichoderma spp. have been well known as biological agents to promote the plant growth either indirectly, by modifying rhizosphere environment, increasing nutrient availability, enhancing the crop defensive mechanisms, or directly by mycoparasitism to plant pathogenic fungi (Benítez et al., 2004; Matarese et al., 2012; Qualhato et al., 2013). In

the current research, isolate of *T. virens* Tv911 increased the growth of three types of vegetables from 7.00% to 64.45% compared to non–treated control in two types of soil, FNS and HGS. There were differences in effects of Tv911 on plant growth promotion between FNS and HGS in the greenhouse pot experiment. The enhancement of Tv911 in plant height of Japanese mustard spinach and fresh shoot weight of radish between FNS and HGS is not obviously different, whereas those of plant height of tomato in FNS was clearly higher than HGS. In another study, *T. harzianum* increased the dry weight of cucumber by 17% in vermiculite and 43% in sandy loam soil at 15 days after planting, the root colonization of *T. harzianum* also varied between 40 and 90% depending on the test vegetables (Kleifeld and Chet, 1992). Moreover, *T. harzianum* enhanced 23.8% and 17.2% in the seedling height of cucumber and pepper at 18 to 30 days after planting, respectively (Inbar et al., 1994). *T. viride* had plant growth promotion effects on soybean including shoot and root system, and fruit yield at 12 weeks after planting in the pot experiment (John et al., 2010). Contreras-Cornejo et al. (2009) also demonstrated that plant growth promotion activities through the auxin-related compounds produced by *T. virens* in *Arabidopsis* seedlings. Application of *Trichoderma* to the growing media in the nursery has beneficial effects on the crop growth and biological control activities to soil borne pathogens (Inbar et al., 1994).

In conclusion, two species of *Trichoderma*; *T. harzianum* (Th621) and *T. virens* (Tv911 and Tv811) have significantly suppressed the growth of FOLy, the causal organism of fusarium wilt of tomato by means of direct confrontation *in vitro*. Additionally, Tv911 isolate has plant growth promotion effects on the growth of these vegetables (Japanese mustard spinach, radish, and tomato) in two types of growing soil, FNS and HGS in greenhouse pot experiment. Therefore, Tv911 has the potential for plant growth promotion and disease control as a biological agent for organic crop production.

Further investigations of plant disease control ability *in vivo* and extracellular enzymes of Tv911, which seem to be involved in antagonistic mechanism to the plant pathogenic fungi, and enhancement of nutrient uptake ability from soil to plants are necessary.

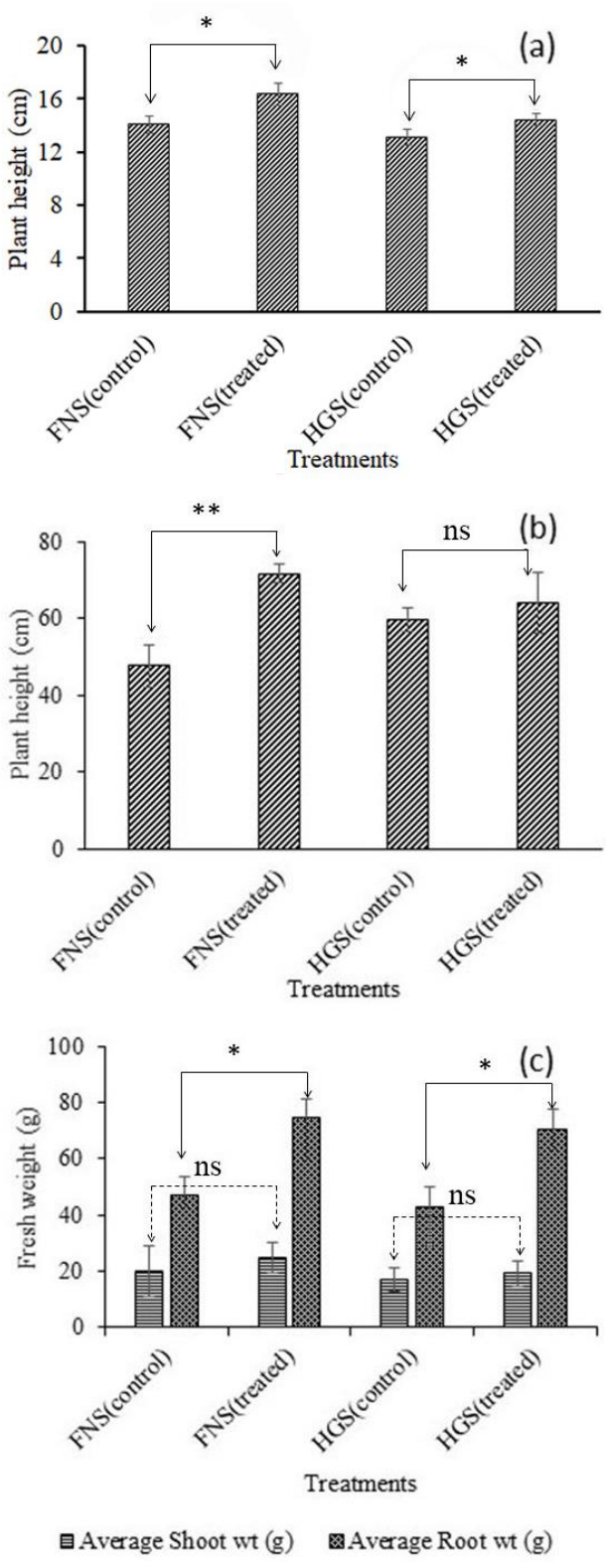

Fig. 3.7 (a) Plant height of Japanese mustard spinach, (b) Plant height of tomato and (c) Fresh shoot and root weight of radish in *Trichoderma virens*, Tv911 treated and non-treated Farmer use nursery soil (FNS) and home garden soil (HGS) at 4, 4 and 6 weeks after sowing, respectively. Symbols of *, ** and *ns* mean significantly different at $P<0.05$, $P<0.01$ and not significantly different, respectively.

Table 3.2 Summery statistics of the effects of *Trichoderma virens* (Tv911) on the growth of three vegetables

Treatment	Japanese mustard spinach plant height (cm)[x]		Tomato plant height (cm)[y]		Radish fresh shoot weight (g/plant)[z]		Radish fresh root weight (g/plant)[z]	
	FNS	HGS	FNS	HGS	FNS	HGS	FNS	HGS
Treated	16.3±1.9	14.4±1.0	71.8±2.4	64.0±8.0	24.8±5.4	19.1±4.4	81.1±14.4	70.5±7.0
Control	14.1±1.4	13.1±1.3	47.8±5.6	59.8±3.2	20.0±11.7	16.9±5.9	46.8±12.3	42.9±13.8
Increased over control (%)	16.22%	9.84%	50.26%	7.00%	23.83%	12.77%	58.86%	64.45%

[x] Mean values of three replications, and each replication contained 5 plants

[y] Mean values of three replications, and each replication contained 4 plants

[z] Mean values of three replications, and each replication contained 3 plants

CHAPTER 4

Plant Disease Control Effects of *Trichoderma virens* Tv911 Against Fusarium Wilt of Tomato and Lettuce

4.1 Introduction

Fusarium wilt diseases of tomato and lettuce are caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hans (FOLy) and *Fusarium oxysporum* Schlechtendal f. sp. *lactucae* J.C. Huber et Gerik (FOLa), respectively and they are very destructive and remarkable plant diseases in vegetable production.

F. oxysporum is an economically important species that induce wilt diseases and show a high level of host specificity and, they are classified into more than 120 *formae speciales* and race based on the plant species (Fravel et al. 2003). Broad-spectrum chemical fungicides such as methyl bromide, carbendazim, benomyl and benzimidazoles are used to control fusarium wilt diseases caused by *F. oxysporum* (Ajilogba and Babalola 2013). However, due to many negative impacts of chemical fungicides on the environmental and human health concerns, many researchers tried to develop the other safe strategies of plant disease management, such as biological control using antagonistic beneficial microorganisms (Larkin and Fravel 1998; Amer and Utkhede 2000; Cotxarrera et al. 2002; Fravel et al. 2003; Akköprü and Demir 2005; Moretti et al. 2008; Anitha and Rabeeth 2009; Hend A. Alwathnani 2012; Innocenti et al. 2015; Meena et al. 2017).

The objective of this chapter is to demonstrate the effects beneficial biological agent, *Trichoderma virens* (Tv911) against fusarium wilt diseases of lettuce and tomato caused by *F. oxysporum* f. sp. *lactucae* and *F. oxysporum* f. sp. *lycopersici*, respectively, under greenhouse conditions.

4.2 Materials and Methods

4.2.1 Disease suppression effects of Tv911 against tomato fusarium wilt

In this experiment, tomato cv. Monterosa was used as the test plant, and seeds were surface sterilized by 2% (w/v) NaOCl solution for 3 min and washed three times with sterilized water and sown in home garden soil (HGS) soil provided by Oishi Bussan Co. Ltd., Fukuoka, Japan. Two weeks old seedlings were transplanted into pots containing 1.5 kg of autoclaved sterilized HGS soil. The fungal isolates of FOLy and *Trichoderma virens* were the same as previous experiments in chapter 3. The treatments were non-inoculated control (mock), inoculated with FOLy only, FOLy+Tv911 and Tv911 only. Ten ml of spore suspension of FOLy and Tv911 were inoculated, where the concentration was adjusted to obtain 1×10^6 conidia / ml. The experiment was done as completely randomized designed (CRD) with four replications and each replication contained three plants. Irrigation was carried out, as necessary.

The disease severity was recorded as index of leaf damage (ILD) at 4 weeks after transplanting with the formula, ILD= Σ grades/max grade where the grades are from 0 to 4 score scale (0= asymptomatic leaves, 1 = leaves wilted, 2 = leaves moderate yellowing, 3 = leaves with necrosis and 4 = dead leaves) according to the method of Selim et al. (2014).

4.2.2 Disease suppression effects of Tv911 against lettuce fusarium wilt

Fusarium wilt of lettuce disease samples were collected from Tarou-hara cho, Kurume city, Fukuoka Prefecture, Japan, and the causal fungal pathogens from discoloured roots were isolated as described in chapter 2. The molecular identification was conducted using partial sequence of ITS gene using ITS1 and ITS4 primers as shown in previous experiments. The sequence of FOLa isolate, LR1903 was deposited in GenBank with the accession number, LC507102. The pathogenicity of the collected isolate was determined by

inoculation to lettuce cultivar via soil inoculation as describe in Scott et al. (2010). The original inoculated fungal organism was reisolated to fulfill Koch's postulates.

In this experiment, seeds of lettuce cultivar were surface sterilized with 3% NaOCl (w/v) and rinse three times with sterilized distilled water. The treatments, experimental design, layout and inoculation were also applied as previous experiments. The data of disease severity was scored for symptoms development as a 1 to 4 score scale (Scott et al. 2010) at 3 weeks after transplanting, where 1 = no symptoms, 2 = mild stunting, 3 = severe stunting and some leaf yellowing and/or death and 4 = dead plants. The data were recorded at 3 weeks after transplanting.

4.2.3 Determination of colonization of Tv911, FOLy and FOLa in roots and soil in both experiments using qPCR

The colonization of Tv911 was estimated by using quantitative real-time PCR (qPCR) using DNA binding dye including standard melt curve method. The species specific primers were designed base on ITS gene sequence of Tv911 in Primer Blast (https://www.ncbi. nlm.nih.gov/tools/primer-blast/). Two primer sets; Tv911-1F (5´-CGT GGG CGT TTC GAA AAT GA-3´) and Tv911-1R (5´-GGG TTG AAA TGA CGC TCG GA-3´), and Tv911-2F (5´-GTT GGG GAT CGG CCC TTT AC-3´) and Tv911-2R (5´-CAG CGG GTA TTC CTA CCT GA-3´) were selected based on the dissimilarities to DNA sequences of other fungi.

The species specific primers for FOLy and FOLa were also designed based on the *EF1-α* and ITS genes as mentioned above. The two primer set; FolaEF-F (5´- GCC CAT CGA TTT CCC CTA CG -3´) and FolaEF-R (5´- TGG TAA GAG GGC AAA CGC TC -3´), and FolaITS-F (5´- GAG GAC CCC TAA AAC TCT GTT TCT -3´) and FolaITS-R (5´- TCG CAT TTT GCT GCG TTC TT -3´) were used for this experiment.

Several conventional PCR trials with *Taq* polymerase (Toyobo, Osaka, Japan) were performed to determine the selectivity and sensitivity of the primer sets to target species using template genomic DNA of *Clonostachys rosea, Diaporthe melonis*, *Phomopsis asparagi*, *Trichoderma harzianum* and *T. virens*.

The fungal colonizations in rhizospheric soil and root tissue were estimated via the DNA quantity of target species from the unknown environmental DNA samples. The measurements were conducted at 1, 2 and 4 weeks after transplanting for the first experiment, effect of Tv911 against the fusarium wilt of tomato. The measurement was also taken every week in the next experiment, effect of Tv911 against the fusarium wilt of lettuce till 3 weeks after transplanting. Total genomic DNA from 100 mg of washed and blotted dried fresh plant root tissue and 500 mg of soil were extracted by using DNeasy Plant mini kit (Qiagen, Hilden, Germany) and ISOIL DNA extraction kit (Nippon Gene, Tokyo, Japan), respectively.

The standard curve for DNA calibration was constructed by 10 times serial dilutions (from 4×10^{1} ng to 4×10^{-5} ng concentration) of known concentration of DNA from Tv911, FOL and FOLa pure culture, which were simultaneously run with subjected samples in each qPCR assays, respectively. The PCR reaction mixture was prepared to total 10 µl volume containing 5 µl of 2× SYBR Green Supermix, 100 nM of forward primer, 150 nM of reversed primer, 1 µL of template DNA, and sterilized distilled water for volume makeup. The assay was performed in the condition of 1 cycle of 96°C for 3 min, followed by 40 cycles of 95°C for 20 sec, 60°C for 25 sec, and 72°C for 25 sec (Agilent Technologies, AriaMx qPCR System). The melt curve obtained by 0.5°C resolution with 5 sec soak time for data acquisition from 65°C to 95°C. Data were analyzed using AriaMx Software version 1.6.

4.2.4 Statistical analysis

Data obtained in these experiments were analyzed in one-way analysis of variance (ANOVA) using Statistix software (Version 8.0) (Analytical Software, 2105, Miller Landing Rd, Tallahassee, FL, 32312, USA). Mean values were compared least significant difference (LSD) all-pairwise comparisons test at $P \leq 0.05$.

4.3 Results

4.3.1 Effect of Tv911 against fusarium wilt of tomato

The index of leaf damage (ILD) in FOLy and FOLy+Tv911 was significantly higher than mock and Tv911 treatments. FOLy+Tv911 was significantly lower ILD than the FOLy only. Moreover, the plant height of FOLy+Tv911 was significantly higher than FOLy only, and the Tv911 reduced the amount of disease damage by the FOLy (Fig. 4.1 and Table 4.1).

The colonization of Tv911 in rhizospheric soil and root tissue were detected by quantification PCR products with fluorometric qPCR at 1, 2 and 4 weeks after transplanting. For this experiment, the primers set (Tv911-2F/Tv911-2R) was selected as the species specific primers, and the expected PCR products (171 bp) were amplified only in the *T. virens* template DNA. The nucleotide position of the primers on the ITS gene of rDNA of Tv911 is shown in Fig. 4.2. The primer set did not amplify to the template DNAs of *C. rosea*, *D. melonis*, *P. asparagi*, and *T. harzianum*. Primer dimers were not also observed in non-template DNA control.

In the PCR assay using above primer sets, the quantification of the PCR products was carried out based on the standard curve (Fig. 4.3). The amount of input template DNA was linearly correlated to the cycle threshold, Cq (ΔR) as expressed by the equation $Cq = -3.34x+15.36$, ($R^2 = 0.99$) where x is input amount (ng) of template DNA of Tv911. The results revealed that the detection limit of the assay using this primer set was approximately 4×10^{-5} ng of template DNA per sample. The primer dimers and amplification of non-target

regions were not generated, and there was a single peak in melt-curve (Fig. 4.3). In this experiment, the significant fluorescence signal was not observed in extracted DNA from both soil and root samples of mock and FOLy treatments, respectively. There was no significant difference between FOL+Tv911 and Tv911 treatments in each sampling time. However, amount of DNA of Tv911 in rhizospheric soil was not obviously changed over the one month growing period of tomato, and those of Tv911 in root tissue as endophytes was increasing about four times from 0.05 pg/(mg root tissue) to 0.19 pg/(mg root tissue) in FOL+Tv911 and 0.06 pg/(mg root tissue) to 0.23 pg/(mg root tissue) in Tv911 treatments (Table 4.1).

The results of the testing of selectivity of the two primer sets to the template DNA of different species showed that the primer set (FolaITS-F and FolaITS-R) was suitable for the qPCR assay for *F. oxysporum*. However, it cannot discriminate between the forma species (f. sp) – *lycopersici* and *lactucae,* that is this primer set detect the species *F. oxysporum*. However, the PCR result showed no primer dimer and no amplification of other tested species. The qPCR condition as mentioned above was applicable for this experiment and the results showed that the melting temperature of the target PCR product was 79.5° C. In qPCR amplification, the amount of input template DNA was linearly correlated to the cycle threshold, *Cq (ΔR)* as expressed by the equation $Cq = -3.23x+18.47$, ($R^2 = 0.99$) where *x* is input amount (ng) of template DNA of *F. oxysporum* (Fig. 4.4). The DNA concentration of FOLy in the soil was going downward trend in the soil, but it was increasing in the root tissue of tomato. Tv911 treatment reduced the amount of colonization of FOLy in both lettuce root and soil in this experiment, and this result was consistent with the disease severity values (Table 4.1).

4.3.2 Effect of Tv911 against fusarium wilt of lettuce

The morphological characteristics of the collected isolates from the wilt disease of lettuce in the Kurume city, Fukuoka Prefecture, Japan were as followings. The colony on PDA was whitish pink in colour, thick with sparse mycelia, and the growth was 7.8±2.1 mm/day at 25° C. Only abundant microconidia were observed on PDA and they are ovoid to oblong, 5.2 to 9.8 µm in length and 3.9 to 4.2 µm in width. The spherical thick walled chlamydospores were observed on old culture and 9.8±2.4 µm in diameter (Fig. 4.5).

According to the phylogenetic analysis inferred from the partial sequence of ITS genes, the isolate, LR1903 was identical to the reference sequence of *Fusarium oxysporum* f. sp. *lactucae*, isolate MAFF 744086 (GenBank accession of partial ITS = KU840918) (Fig. 4.6). This isolate was collected from Fukuoka prefecture, Japan in 2002 and identified as race 3 (Nishimura 2003). In the pathogenicity test, the isolates using in this study were virulent and causing root rot symptoms on lettuce cultivar.

In the experiment of effect of Tv911 against FOLa, the disease symptoms were noticeable at 2 weeks after transplanting. The disease severity values of T2, (FOLa only) and T3 (FOLa+Tv911) were significantly higher than those of other treatments at 3 weeks after transplanting. However, the disease severity of T3 (FOLa+Tv911) was significantly lower (53.3 %) than the T2 (FOLa only) (Fig. 4.7 and Table 4.2).

The amount of DNA of Tv911 and FOLa in lettuce root and rhizospheric soil was estimated via qPCR with DNA binding dye method with the primer sets of Tv911-2F and Tv911-2R primers, and FolaITS-F and FolaITS-R primers, respectively. The results of standard curve and equation for calculating the initial DNA concentration of unknown samples were the same as the previous experiment, the effect of Tv911 against fusarium wilt of tomato caused by FOLy.

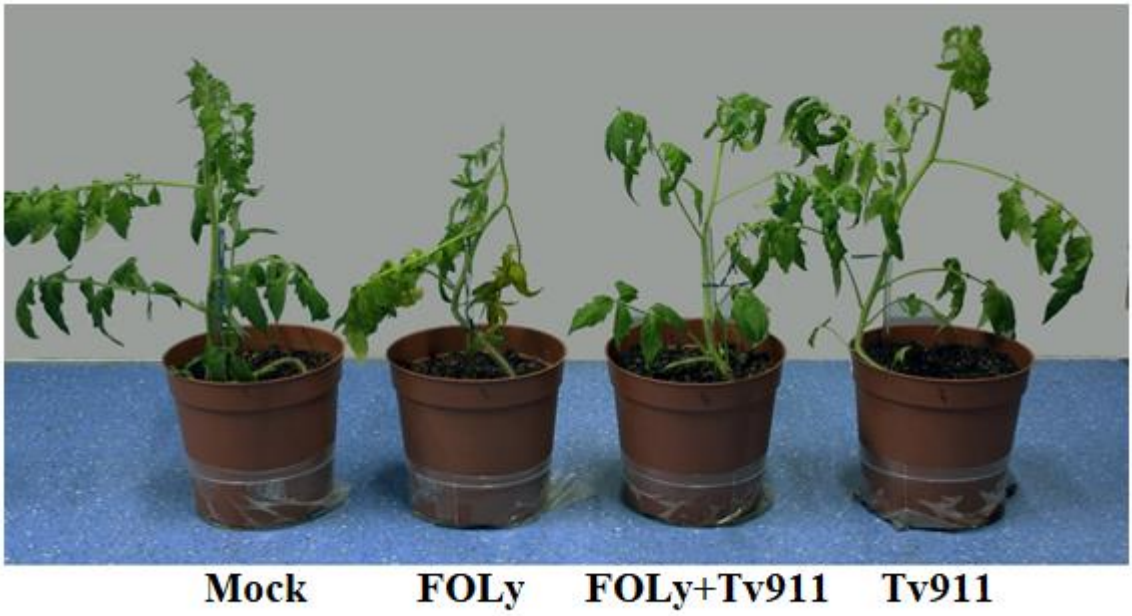

Fig. 4.1 Effect of Tv911 on the fusarium wilt of tomato in green house experiment at 4 weeks after transplanting

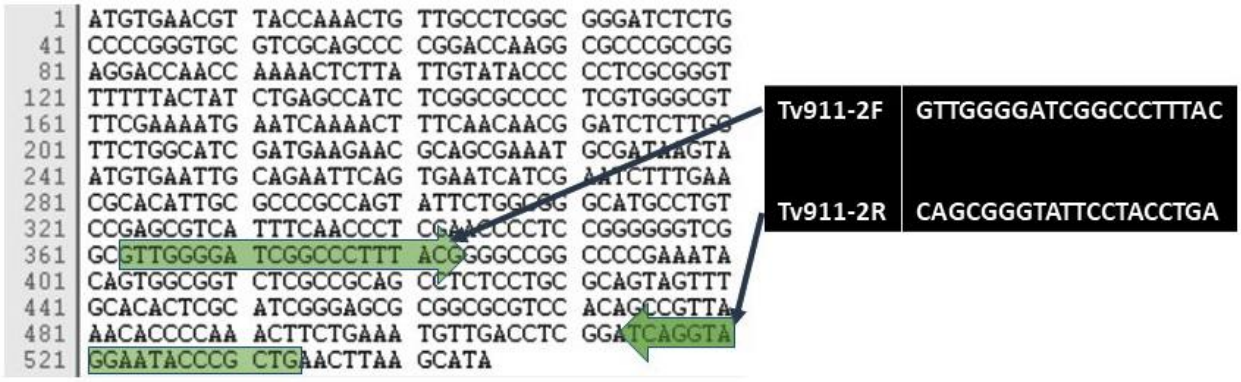

Fig. 4.2 Nucleotide position of the primers (Tv911-2F and Tv911-2R) on the partial sequence of ITS gene of *T. virens* strain Tv911 (5´ to 3´)

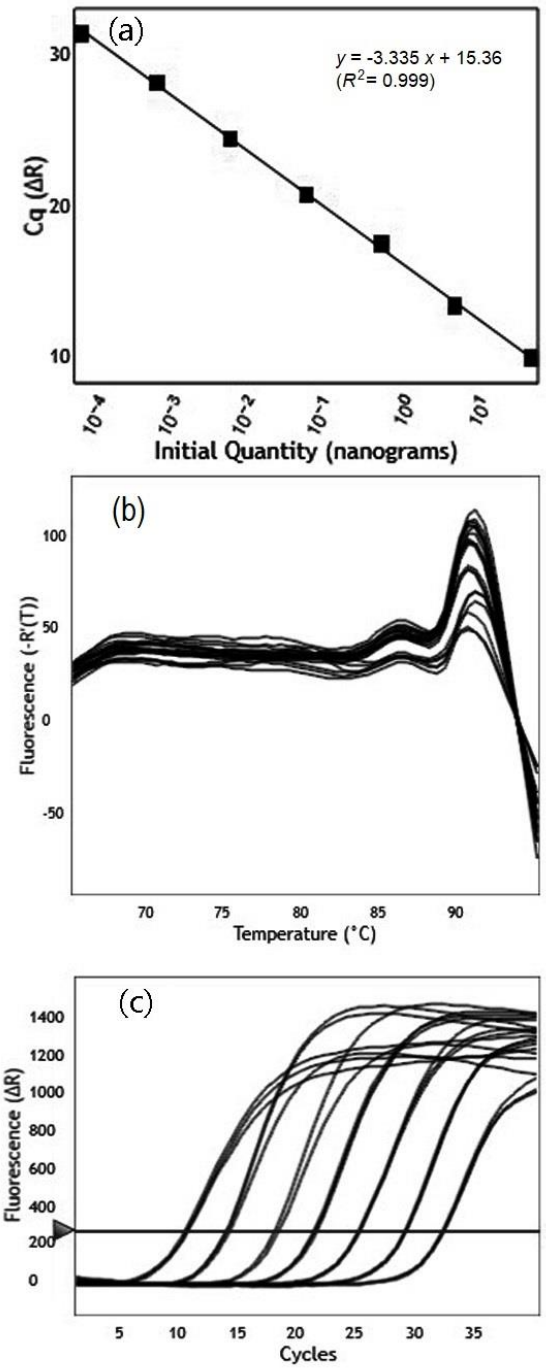

Fig. 4.3 Sensitivity of qPCR using Tv911-2F/Tv911-2R primers with DNA binding method, assessed by 10 times dilutions (from 4×10^{1} to 4×10^{-5} ng) series of genomic DNA of Tv911 extracted from pure culture. (a) = Standard curve based on relationship between cycle threshold values and DNA concentration of Tv911, (b) = Melting curve (fluorescence vs. temperature), and (c) = Real-time amplification curve of different concentration of Tv911 DNA

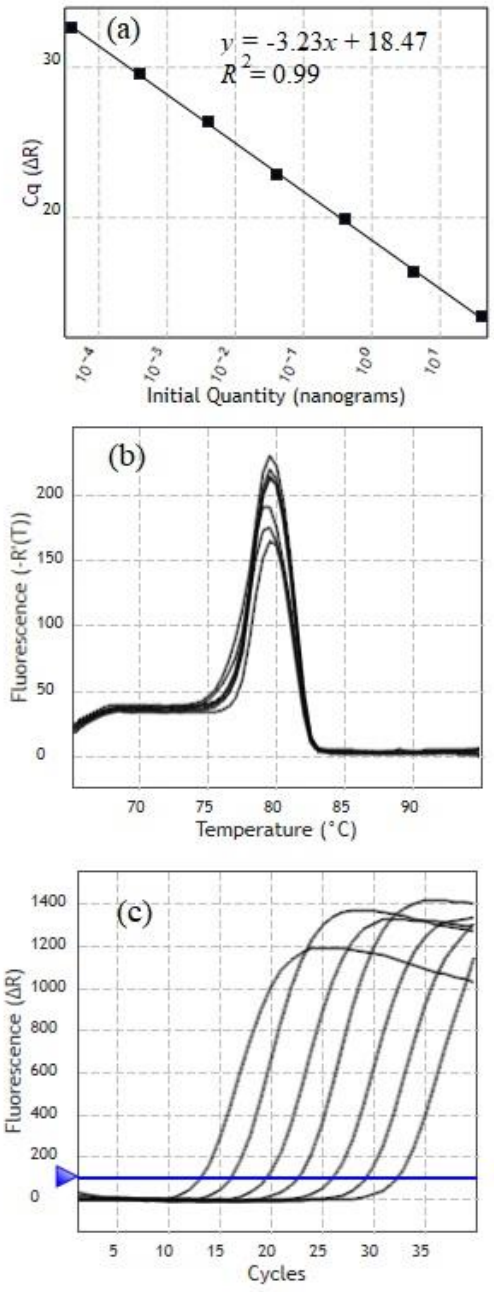

Fig. 4.4 Sensitivity of qPCR using FolaITS-F/FolaITS-R primers with DNA binding method, assessed by 10 times dilutions (from 4×10^{1} to 4×10^{-5} ng) series of genomic DNA of Tv911 extracted from pure culture. (a) = Standard curve based on relationship between cycle threshold values and DNA concentration of Tv911, (b) = Melting curve (fluorescence vs. temperature), and (c) = Real-time amplification curve of different concentration of Tv911 DNA

Table 4.1 Effect of Tv911 on Fusarium wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOLy) at 4 weeks after transplanting

Treatment	ILD [a]	Plant height (cm) [b]	pg DNA of Tv911/mg soil [c] (mean±SD)			pg DNA of Tv911/mg root [c] (mean±SD)			pg DNA of FOLy/mg soil [d] (mean±SD)			pg DNA of FOLy/mg root [d] (mean±SD)		
			1 WAT	2 WAT	4WAT	1 WAT	2 WAT	4WAT	1 WAT	2 WAT	4WAT	1 WAT	2 WAT	4WAT
Mock	0.04±0.02 c*	48.1±4.8 b*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
FOLy	0.45±0.05 a	33.4±3.5 c	NA	NA	NA	NA	NA	NA	3.67±1.89	4.13±0.71	4.41±0.78	1.33±0.91	9.96±2.9	12.79±3.9
FOLy+Tv911	0.17±0.03 b	54.5±1.5 ab	6.66±0.5	5.80±0.4	5.03±1.0	0.05±0.01	0.12±0.01	0.19±0.02	2.18±1.27	2.52±0.26	2.46±0.22	0.06±0.06	0.15±0.11	0.57±0.29
Tv911	0.02±0.02 c	57.0±2.6 a	6.13±0.6	6.37±0.9	5.75±0.9	0.06±0.02	0.12±0.02	0.23±0.08	NA	NA	NA	NA	NA	NA

[a] Mean of Index of leaf damage at 4 weeks after transplanting (WAT) from four replications

[b] Mean value of plant height at 4 WAT from four replications

[c] DNA of Tv911 was quantified by qPCR using SYBR Green and Tv911-2F/Tv911-2R primers after extraction from soil and root tissue at 1 WAT, 2 WAT and 4WAT, respectively. NA= not detectable fluorescence signal in PCR reaction

[d] DNA of Tv911 was quantified by qPCR using SYBR Green and FolaITS-F/FolaITS-R primers after extraction from soil and root tissue at 1 WAT, 2 WAT and 4WAT, respectively. NA= not detectable fluorescence signal in the PCR reaction

*Number Followed by the same letter do not differ significantly by LSD all-pairwise comparisons test at $P \leq 0.05$.

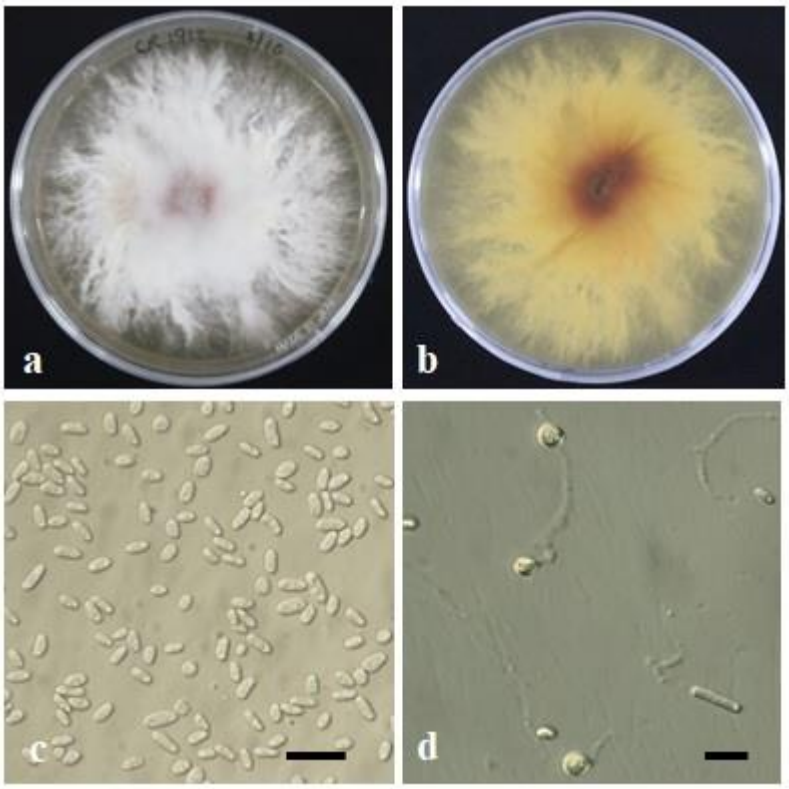

Fig. 4.5 Morphological characteristics of *Fusarium oxysporum* f. sp. *lactucae* (FOLa), a = Colony on PDA at 10 days after incubation (upside view), b = Colony (down side view), c = Micro-conidia (bar = 20 µm) and d = Chlamydospores (bar = 20 µm)

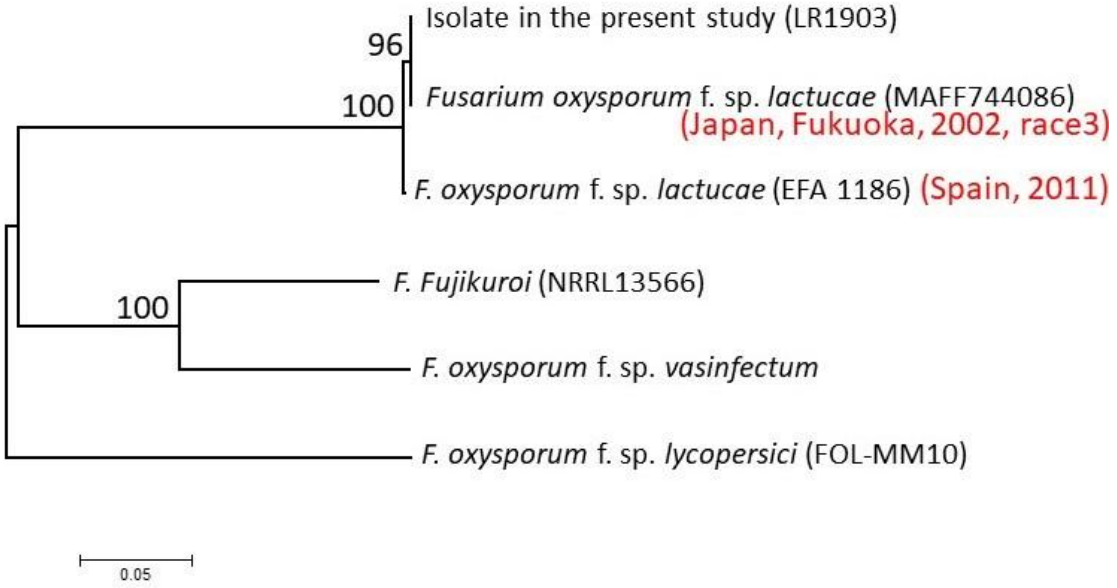

Fig. 4.6 Phylogenetic tree of *Fusarium oxysporum* f. sp. *lactucae* (FOLa) inferred from partial sequence of ITS gene by using neighbouring method

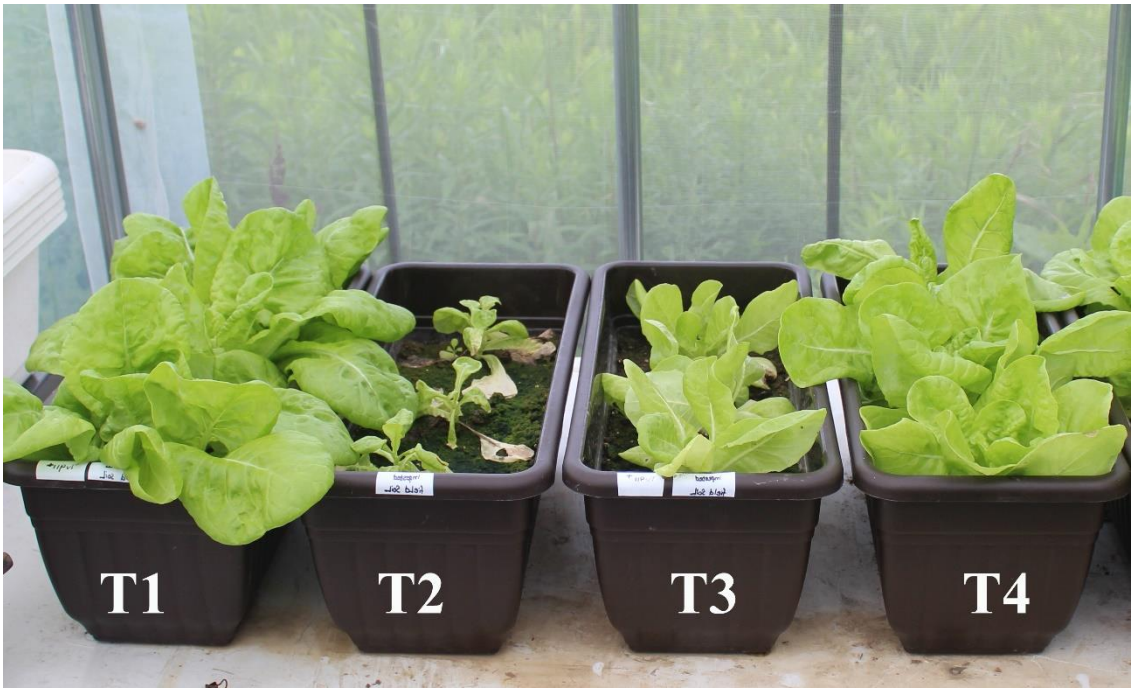

Fig. 4.7 Effect of Tv911 against fusarium wilt of lettuce caused by FOLa at 3 weeks after transplanting under greenhouse condition, where T1 = mock, T2 = FOLa only, T3 = FOLa+Tv911 and T4 = Tv911 only

Table 4.2 Effect of Tv911 on Fusarium wilt of lettuce caused by *Fusarium oxysporum* f. sp. *lactucae* (FOLa) at 3 weeks after transplanting

Treatment	Severity [a]	pg DNA of Tv911/mg soil [c] (mean±SD)			pg DNA of Tv911/mg root [c] (mean±SD)			pg DNA of FOLa/mg soil [d] (mean±SD)			pg DNA of FOLa/mg root [d] (mean±SD)		
		1 WAT	2 WAT	3WAT	1 WAT	2 WAT	3WAT	1 WAT	2 WAT	3WAT	1 WAT	2 WAT	3WAT
Mock	1.0±0 c*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
FOLa	3.8±0.5 a	NA	NA	NA	NA	NA	NA	10.4±3.02	9.8±2.42	24.2±7.83	64.2±15.21	75.9±16.24	118.4±25.41
FOLa+Tv911	1.8±0.3 b	5.15±1.62	6.45±2.04	7.11±2.44	0.25±0.07	0.42±0.12	0.72±0.36	4.2±1.22	4.2±2.13	7.2±2.58	12.7±5.22	69.3±17.31	82.1±22.13
Tv911	1.0±0 c	6.94±2.33	8.65±2.81	7.85±2.44	0.34±0.07	0.57±0.11	1.25±0.38	NA	NA	NA	NA	NA	NA

[a] Mean value of Index of leaf damage at 4 weeks after transplanting (WAT) from four replications

[b] Mean value of plant height at 4 WAT from four replications

[c] DNA of Tv911 was quantified by qPCR using SYBR Green and Tv911-2F/Tv911-2R primers after extraction from soil and root tissue at 1 WAT, 2 WAT and 3WAT, respectively. NA= not detectable fluorescence signal in PCR reaction

[d] DNA of Tv911 was quantified by qPCR using SYBR Green and FolaITS-F/FolaITS-R primers after extraction from soil and root tissue at 1 WAT, 2 WAT and 4WAT, respectively. NA= not detectable fluorescence signal in PCR reaction

*Number Followed by the same letter do not differ significantly by LSD all-pairwise comparisons test at $P \leq 0.05$.

In this experiment, the DNA of Tv911 in the root tissue of lettuce was slightly increasing during the 3 weeks growing period and it was more or less stable in soil. In T3 (FOLa+Tv911), the amount of DNA of FOla and Tv911 in soil were not significantly different, but the amount of FOLa DNA was significantly higher than those of DNA of Tv911 in root tissue of lettuce. Additionally, the higher quantity of FOLa DNA was detected in the root tissue of lettuce plant in both T2 (FOLa only) and T3 (FOLa+Tv911). However, Tv911 significantly reduced the FOLa DNA approximately from 118.4 pg/mg root tissue to 82.1 pg/mg root tissue (Table 4.2).

4.4 Discussion

Trichoderma spp. have been interested as a biocontrol agent for many soil born plant pathogens in disease management practices (Elad 1980; Benítez et al. 2004; Dubey et al. 2007; Moraga-Suazo et al. 2011; Matarese et al. 2012; Mbarga et al. 2012). In this study, Tv911 significantly reduced the disease severity of the fusarium wilt of lettuce (53.3%) and tomato (62.2%) in the pot experiments under greenhouse condition. In previous study, *T. harzianum* (Strain T22) decreased disease severity of lettuce wilt caused by *F. oxysporum* f. sp. *lattucae* (strain 365.07) in comparison to infected control by 75 to 78 % in different moisture regimes (Innocenti et al. 2015). *T. harzianum* (ANR-1) treatment also showed least disease incidence in tomato caused by FOly (Sundaramoorthy and Balabaskar 2013).

The colonizations of the fungi including plant pathogens and beneficial symbionts were estimated by using the conventional ways such as the counting of root infection percent and colony forming unit (CFU) through culturing on growing media (Akköprü and Demir 2005; Scott et al. 2010, 2014). In this study, the colonization of Tv911 and *Fusarium* spp. in roots and soil were quantified by using ITS sequenced based species selective primers with qPCR assay. Such primers and methodology were simple and effectively used for

quantification of specific fungal species, such as *Phomopsis sclerotioides,* in the plant and soil samples (Shishido et al., 2010, 2013).

The colonization of Tv911 in rhizospheric soil of tomato roots was not significantly fluctuated at the tested period, and it was increased approximately 4 times in tomato roots and 3 times in lettuce roots. The similar phenomenon of colonization of FOLy and FOLa in the roots of tomato and lettuce, respectively and in the rhizospheric soil were observed in both experiments. However, the significant higher quantity of FOLa DNA was detected in the roots of lettuce than in the roots of tomato, and we can assume that the tested lettuce cultivar could be very susceptible to the fungal pathogen, FOLa. Tv911 significantly reduced the colonization of the tested plant pathogenic fungi in the soil and plant roots.

In conclusion, Tv911 has the potential for disease control as a biocontrol agent for fusarium wilt of tomato and lettuce. However, further study of the effective formulation of *Trichoderma virens* for the disease control under various environmental conditions and investigation of extracellular enzymes of Tv911, which seem to be involved in antagonistic mechanism to the plant pathogenic fungi, and enhancement of nutrient uptake ability from soil to plants are required. Moreover, it is necessary to conduct the effect of Tv911-derived metabolites on the metabolic changes of host crop under field condition before commercialization.

CHAPTER 5

Application of *Trichoderma virens* Tv911 to Control the Soybean Anthracnose Caused by *Colletotrichum truncatum* at the Seedling Stage in Myanmar

5.1 Introduction

Anthracnose is an important disease in soybean (*Glycine max* (L.) Merr.), and it is mainly caused by *Colletotrichum truncatum* (Schwein.) Andrus & Morre. It been reported as a destructive plant disease of the aerial plant parts wherever soybeans are cultivated (Khan and Sinclair 1992; Bowers and Russin 1999; CABI and EPPO 2001). The anthracnose of soybean disease caused by *C. truncatum* caused severe damage at the maturity stage of the plants in Nay Pyi Taw area, central area of Myanmar in 2017 (Zaw et al. 2019) (In Chapter 2).

The common disease symptoms of soybean anthracnose are black irregular lesion especially on pods, petioles and stems (Dias et al. 2018). This disease is mainly transmitted by seed and causes lesion on cotyledons and hypocotyl, and damping-off of soybean seedlings at the V1 and V2 developmental stages (Hartman et al. 2015). The disease is severe under warm and high relative humidity weather conditions especially in seedling, late reproductive stages and senescence (Singh et al. 2001).

The integrated disease management strategies for soybean anthracnose include disease free seeds, using resistant cultivars and spraying chemical fungicide, and seed treatment to control the disease for early growth stages (Bowers and Russin 1999). Moreover, applications of foliar fungicides were commonly recommended for controlling

the disease (Gawade et al. 2009). However, due to the negative impacts of fungicides such as emerging of fungicide resistant strains, environmental polluted issues, and health problems of animal and human, ecological friendly integrated disease management strategies were formulated to control the anthracnose of soybean (Begum et al. 2008a; Shovan et al. 2008; Gawade et al. 2009; Kale B.G. 2016).

The objectives of this study are to evaluate the biocontrol activities of *T. virens* strain Tv911 against the seed transmitted soybean anthracnose disease caused by *C. truncatum* and to detect the colonization activities of the fungal pathogen and biocontrol agent in root tissue and rhizospheric soil by using quantitative real-time PCR (qPCR) with the species selective primers.

5.2 Materials and Methods

5.2.1 Microorganism source and test plant cultivar

The antagonistic fungal resources *Trichoderma virens* strain Tv911, the causal organism of soybean anthracnose, *C. truncatum* (SoyYZ1716), and soybean cultivar (Yezin 10) were used in this experiment.

5.2.2 Designing species specific primers of *C. trancatum* for qPCR analysis

The species specific primers of *C. truncatum* was designed based on the partial sequences of ITS gene as mentioned in the previous chapter. The two primer sets; Ct1716-1F (5´-GCC TGT TCG AGC GTC ATT TC-3´), Ct1716-1R (5´-AGA GTC CCT CCG AAT CCC AA-3´), and Ct1716-2F (5´-GTT GGG GCT CTA CGG TTG AC-3´), Ct1716-2R (5´-TGG GGG TTT TAC GGC TAG AG-3´) were carefully chosen for this experiment. Selectivity and sensitivity of the primers were checked with conventional PCR using the template DNA of *C. plurivorum*, *Diaporthe melonis*, *Trichoderma harzianum* and *T. virens*.

5.2.3 Pot experiment of effect of Tv911 against *C. truncatum*

The experiment was conducted in the greenhouse, Institute of Tropical Agriculture, Kyushu University. The effect of Tv911 against anthracnose of soybean disease caused by *C. truncatum* was examined at the germination and seedling stages. There were four treatments in this experiment: 1) T1 = mock, 2) T2 = *C. truncatum* (only) inoculation, 3) T3 = *C. truncatum* + *T. virens* inoculation and 4) T4 = *T. virens* (only) inoculation.

Soybean anthracnose pathogen, *C. trancatum,* isolate SoyYZ1716 was cultured on PDA and incubated at 25° C for 20 days. The spores were washed off with sterilized distilled water, and the spore concentration was adjusted to obtain 10^8 spores/ml by counting on the hemocytometer. The antagonistic fungus, *T. virens* (Tv911) was also cultured on PDA and incubated at 25° C for 10 days. The spores were harvested with sterilized distilled water and the concentration of the spore suspension was adjusted to 1×10^8 spores/ml. Tween 20 was added to the spore suspensions at 0.02 % (v/v) concentration.

Seed inoculation of the two fungus was performed as described in Begum et al. (2008b) with slight modification. Briefly, soybean seeds were surface sterilized with 10% (w/v) NaOCl solution for 3 min and rinsed three times with sterilized distilled water. Then, the seeds were dried in clean bench for 1 h, and then, soaked for the treatments. The seeds were soaked in sterilized distilled water, *C. truncatum* spore suspension, mixture (1:1) of *C. truncatum* and *T. viren* spores suspension, and *T. virens* spore suspension as T1, T2, T3 and T4 treatments, respectively, and were kept on rotary shaker (EYEL4, MULTI SHAKER MMS) at 150 rpm speed for 1 h. After that, the seeds were dried in the clean bench for 3 h, and 13 seeds were sown in a pot (18 cm diameter and 20 cm height) containing 1.5 kg of sterilized home garden soil (HGS) (Oishi Bussan, Co.). Each treatment contains 3

replications and each experiment was arranged in completely randomized design in greenhouse.

5.2.4 Collected data and analysis

Data of seed germination, pre- and post-emergence damping off and survival of seedling were recorded at 7 days and 14 days after sowing. Moreover, the colonization of the pathogen, *C. truncatum* and antagonistic fungus, Tv911 was detected by using qPCR. The genomic DNA from plant tissue (root and hypocotyl) and rhizospheric soil were extracted by using DNeasy plant mini kit (Qiagen, Hilden, Germany) and ISOIL DNA extraction kit (Nippon Gene, Tokyo, Japan), respectively. The standard curve for qPCR with DNA binding dye method was constructed by 10 times serial dilutions (from 10 ng to 10^{-5} ng concentration) of known DNA concentration from the pure culture of each species. The standard concentrations of DNA of each species were simultaneously run with unknown subjected samples in every qPCR assay. The PCR reaction mixture and PCR condition were performed as described in the Chapter 4.

The resulted data were analyzed in one-way analysis of variance (ANOVA) Satatistix software (Versiton 8.0) (Analytical Software, 2105, Miller Landing Rd, Tallahassee, FL, 32312, USA). Mean comparison was performed by using least significant difference (LSD) value at $P \leq 0.05$.

5.3 Results

5.3.1 *C. trancatum* species specific primers for qPCR

In the conventional PCR results, Ct1716.1-F and Ct1716.2R primer set amplified the target amplicon (164bp) band in *C. truncatum* template DNA, but there were no primer dimers and no PCR amplification other tested template DNA of *C. plurivorum*, *Diaporthe*

melonis, *Trichoderma harzianum* and *T. virens* (Fig. 5.1). Therefore, this primer set was selected for qPCR to detect the fungal DNA of *C. truncatum* in soil and plant tissue.

5.3.2 Effects of Tv911 against seed inoculated soybean anthracnose disease

The germination rate of the tested soybean seeds was 89.7% in untreated mock, and there were significant differences among the treatments (Fig 5.2). The lowest germination was observed in T2 (*C. truncatum* (only) inoculation), and Tv911 significantly eliminate the disease damage caused by the fungal pathogen. The pre- and post-emergence death of T3 (*C. truncatum* + Tv911) was significantly lower than those of T2 (*C. truncatum* only) at $P \leq 0.05$ (Table 5.1).

The fungal DNA of both fungi were detected in hypocotyl tissue root and soil in qPCR assay. The estimation of colonization of Tv911 was performed as the previous chapter, and in the detection of DNA of *C. truncatum*, the quantity of target DNA in unknown samples was calculated based on the standard curve (Fig. 5.3). The input of template DNA was linearly correlated to the cycle threshold, Cq (ΔR) as expressed by the equation $Cq = -3.08x + 12.82$ ($R^2 = 0.996$), where x is input amount (ng) of template DNA of *C. truncatum*.

The colonization of *C. truncatum* in hypocotyl is higher than root tissue. In hypocotyl of some infected plant, discolored tissue was observed (Fig. 5.2), and no symptoms were observed in root. On the other hand, the colonization of Tv911 was significantly higher in root tissue, and only a trace amount (0.04 pg/ mg fresh tissue) was detected stem tissue.

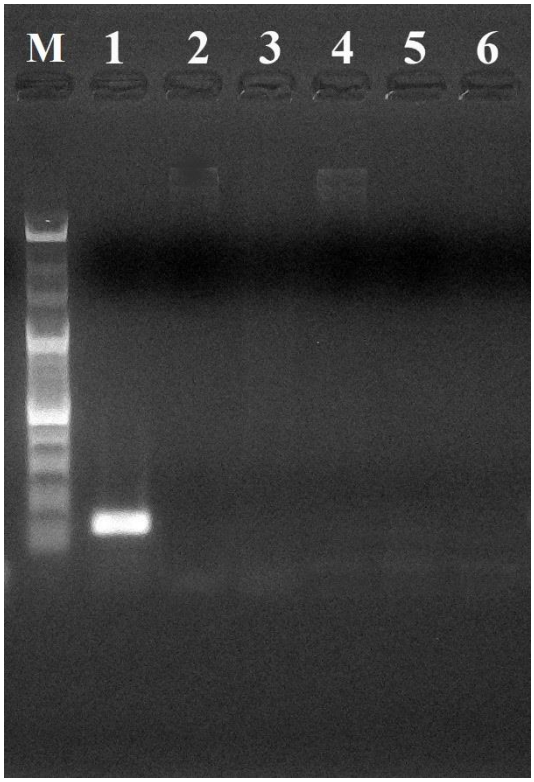

Fig. 5.1 PCR amplification of template DNA of different fungal species using Ct1716-1F and Ct1716-2R primer set, where M = 100 bp DNA ladder, 1 = *C. truncatum*, 2 = *C. plurivorum*, 3 = *Diaporthe melonis*, 4 = *T. harzianum*, 5 = *T. virens*, 6 = Non-template control

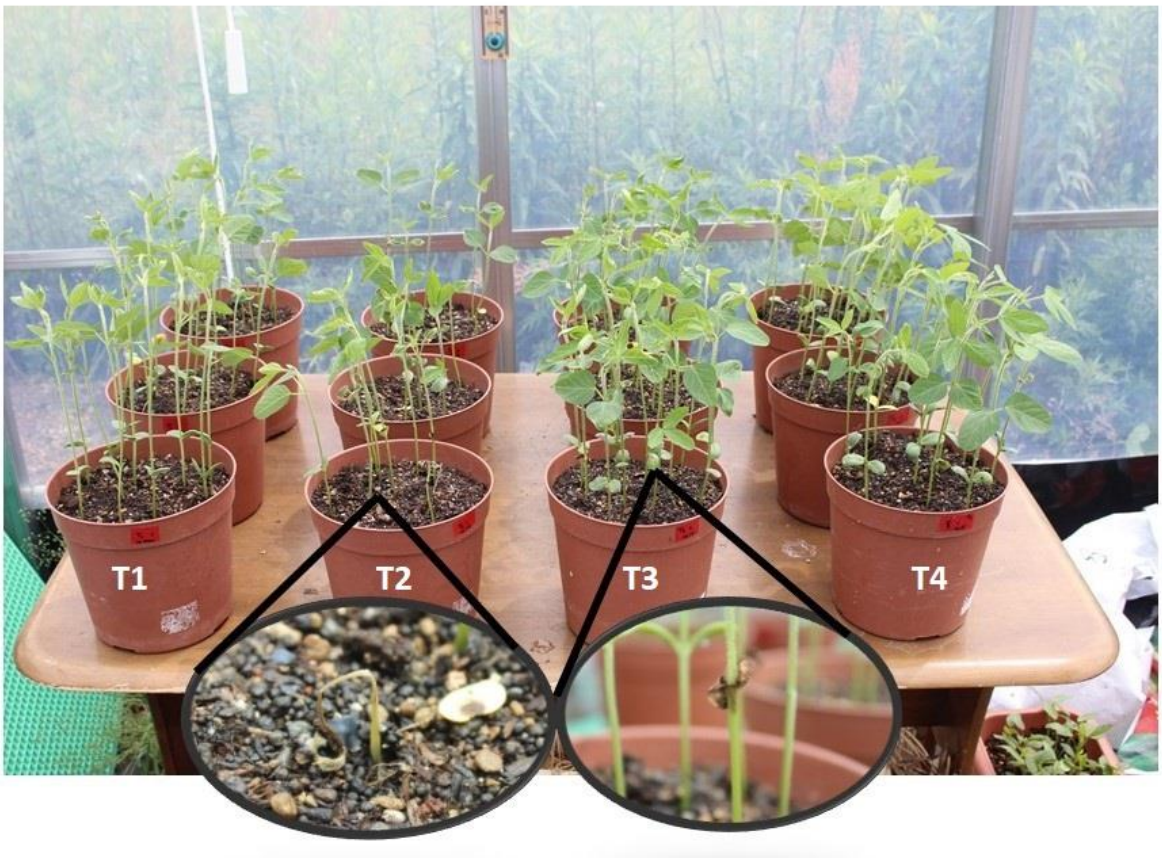

Fig. 5.2 Effect of Tv911 on germination of soybean (Yezin 10) inoculated with *Colletotrichum truncatum* at 14 days after sowing, where T1 = mock, T2 = *C. truncatum* (only) inoculation, T3 = *C. truncatum*+Tv911 inoculation and T4 = Tv911 (only) inoculation, and magnified symptoms of inoculation of *C. truncatum*

Table 5.1 Effect of Tv911 on germination of soybean (Yezin 10) which was inoculated by *Colletotrichum truncatum* at 14 days after sowing

Treatment	Germination (%)*	Survival seedling (%)*	Pre-emergence death (%)*	Post-emergence death (%)*
T1 (mock)	89.7 a	89.7 a	10.3 b	0.0 c
T2 (*C. truncatum* only)	59.0 b	43.6 b	41.0 a	15.4 a
T3 (*C. truncatum*+Tv911)	89.7 a	87.2 a	10.3 b	2.6 b
T4 (Tv911 only)	92.3 a	92.3 a	7.7 b	0.0 c

* Number followed by the same letter do not differ significantly by LSD all-pairwise comparisons test at $P \leq 0.05$.

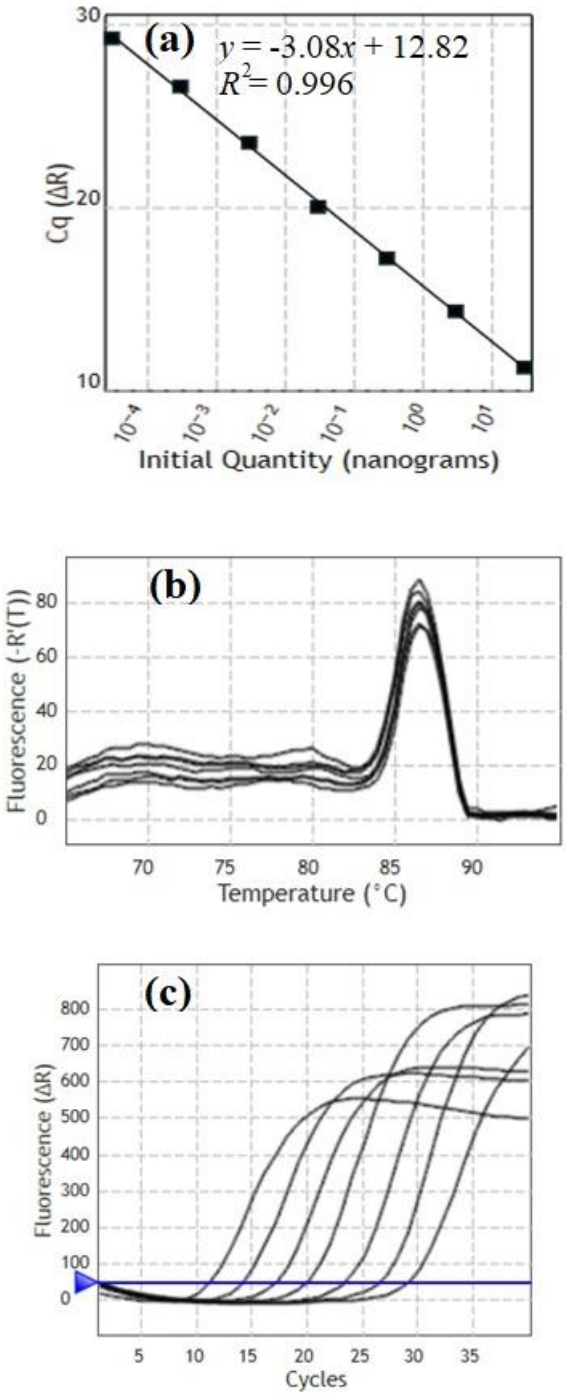

Fig. 5.3 Sensitivity qPCR using Ct1716-1F and Ct1716-2R primer set with DNA binding dye method, where (a) = Standard curve, (b) = Melting curve and (c) = Real-time amplification curve at different initial concentration of *C. truncatum* DNA: lines correspond to 10-fold dilutions of *C. truncatum* DNA (10 to 10^{-5} ng).

Table 5.2 Estimation of colonization of *Colletotrichum truncatum* and Tv911 in rhizospheric soil, root and stem (hypocotyl) of soybean at 14 days after sowing

Treatment	*C. truncatum* DNA concentration (mean±SD)*			Tv911 DNA concentration (mean±SD)*		
	pg/mg soil	pg/mg root	pg/mg stem	pg/mg soil	pg/mg root	pg/mg stem
T1 (mock)	NA	NA	NA	NA	NA	NA
T2 (C. *truncatum* only)	0.22±0.09	1.45±1.10	33.72±9.22	NA	NA	NA
T3 (C. *truncatum*+Tv911)	0.12±0.09	0.03±0.09	5.08±3.98	16.21±2.36	1.85±0.14	0.039±0.02
T4 (Tv911 only)	NA	NA	NA	37.69±6.74	2.38±0.84	0.036±0.03

*DNA of *C. truncatum* and Tv911 were quantified by qPCR using SYBR Green and the species specific primer sets after extraction from the soil, root and stem (hypocotyl) tissue at 14 days after sowing. NA = not detectable fluorescence signal in PCR reaction

5.4 Discussion

Trichoderma spp. have been widely used as biocontrol agents for many fungal diseases in agricultural production (Berg 2009), and well known since at least 1920s for their ability such as mycoparasitism, antibiosis, and competition for resources and space (Harman 2005). In the present study, the antagonistic effect of fungal biocontrol agent, *T. virens* Tv911 against soybean anthracnose caused by *C. truncatum* were tested at the seedling stage of soybean under greenhouse condition. The germination rate of soybean was significantly different among the treatments, and the highest one was observed in Tv911 treated. Moreover, T3 (*C. truncatum*+Tv911) significantly reduced the disease damage (pre-emergence death and post emergence death) than T2 (*C. truncatum*) only. Begum et al. (2008b) stated that *T. harzianum* (UPM40) and *T. virens* (UPM23) enhanced the germination rate and seedling establishment compare to non-treated check, and they had stronger antagonistic activities to inhibit the radial growth of the pathogen of *C. truncatum*. Moreover, in another experiment, *T. harzianum* significantly reduced the disease incident of soybean pathogens including *Glomerella glycines* (anamorph: *Colletotrichum* sp.) (Fernandez 1992). The pre- and post-emergence damping off and seedling mortality were highest in T2, but the remaining plants were growing up as a healthy plant because the micro-environmental condition of the greenhouse was not favourable for disease development. The soybean anthracnose caused by *Colletotrichum* sp. cause damage to the above-ground parts at any growth stages, but the symptoms generally conspicuous at either during early seedling stage or late reproductive stages, and the infection remains latent in vegetative stages (Sinclair 1991; Khan and Sinclair 1992).

The colonization of two fungi, *C. trancatum* and *T. virens* Tv911 was detected via qPCR (DNA binding dye method) with species specific primers. This method is effective

and relatively quick to estimate the amount of DNA of target species in unknown environmental genomic DNA form soil and diseased plant parts (Shishido et al. 2010, 2013). The DNA of *C. truncatum* in hypocotyl was higher than those in root and soil. The fungal pathogen was inoculated in seeds and infection took place from the seed coat and cotyledon. Then it was continuous to the hypocotyl because we observed the damping off symptoms on hypocotyl in some inoculated plants. However, DNA concentration of Tv911 in soil was significantly higher than that in the root tissue, and only a trace amount of Tv911 DNA was detected in hypocotyl. This may be the reason that *Trichoderma* is a common soil inhabitant and root endophyte as an avirulent plant symbiont (Harman et al. 2004; Contreras-Cornejo et al. 2016).

In this experiment, I demonstrated the beneficial effect of Tv911 against the soybean anthracnose caused by *C. truncatum*. As a result, I consider that it can effectively control the disease damage and increase the germination and seedling vigour. Moreover, we can design the *C. truncatum* species selective primer set for qPCR for estimation of the target DNA content in the environmental genomic DNA sample, and the colonization behaviour of the two fungi has been understood in this study. Therefore, *T. virens,* Tv911 isolate has a potential for controlling the seedborne fungal disease (anthracnose) of soybean. However, further investigations of possible effects of its metabolites on the crop and environment under field conditions are necessary.

CHAPTER 6

General Conclusions

Soybean fungal diseases are one of the major constraints and cause a significant yield loss throughout the world. Myanmar's soybean yield is approximately one third of world average yield, and the survey and research on fungal diseases of soybean have not been conducted before. Therefore, this study was conducted to identify the fungal causal pathogen of stem diseases of soybean in Myanmar and to develop environmental friendly biocontrol measure using effective biological agent, *Trichoderma* spp.

In the chapter 2, I obtained a total of 78 fungal isolates from 7 locations of two major soybean cultivating areas, Nay Pyi Taw and Shan State. According to morphological characteristics, the collected isolates were identified into the genus level as *Colletotrichum*, *Diaporthe* and *Macrophomina.* Isolates of *Colletotrichum* and *Diaporthe* showed virulent reaction in inoculation test to fulfill Koch's postulates, and the isolates of *Macrophomina* in this study were not high pathogenic to soybean. Therefore, the occurrence of *Colletotrichum* and *Diaporthe* in 2017 and 2018 was important for the disease management practices and these two fungal genera were major causal pathogens of soybean stem diseases in farmer's field of two areas of Myanmar. The species level identification and coinoculation of different species of *Diaporthe* were conducted to understand the fungal species community existence and involvement in stem disease of soybean. Based on the molecular phylogenetic analysis using ITS, *ACT, CHS-1* and *GAPDH* genes, the collected *Colletotrichum* isolates were identified as *C. plurivorum* and *C. truncatum*. The virulence in *C. truncatum* isolates was higher than that in *C. plurivourm* in the inoculation tests. The phylogenetic analysis of 23 isolates of *Diaporthe* spp. was also conducted using ITS, *EF1-α* and *TUB* genes, and they are homology to *D. endophytica*, *D. melonis*, *D. tectonendophytica* and *D. ueckerae*, respectively. Therefore, a total of six pathogenic fungal

species were observed in this study. Among them, *D. tectonendophytica* has not been reported as a causal pathogen of soybean. It has been reported as a pathogen of teak tree in Thailand which is the neighbouring country of Myanmar. Consequently, I can assume the conidial flow of *D. tectonendophytica* between the teak tree and soybean field because there are many natural and planted teak trees around the soybean fields in Nay Pyi Taw.

Two *Diaporthe* spp. were discovered in the same location except Heho, Shan State. The effect of coinoculation of two species was not significantly different from that of single species inoculation, and I can understand that existence of one or two species of *Diaporthe* is not clearly related to the disease severity of soybean stem disease. However, further experiments of coinocualtion with other fungal genera that frequently isolated from the disease soybean plant should be conducted to understand the correlation between the occurrence of different fungal species and disease severity.

In the chapter 3, the biocontrol agents were isolated, and their antagonistic abilities were assessed to control the fungal plant pathogen. Representative six fungal strains were collected form the waste paper sludge compost, and species identification was performed via ITS sequencing and phylogenetic analysis. The species were *Clonostachys rosea*, *Trichoderma harzianum* and *T. virens.* The antagonistic effectiveness of the isolates against plant pathogenic fungi, *Fusarium oxysporum* f. sp. *lycopersici* (FOLy) was observed *in vitro* tests. *T. virens* isolate, Tv911 was selected for the experiments for evaluation of plant growth promotion and disease suppression. Tv911 inhibited 64.27% and 27.41% of the growth of FOL in direct inhibition test and inhibited by volatile compound test, respectively. *T. virens*, isolate Tv911 was selected for the next experiments. Then, plant growth promotion ability of *T. virens,* Tv911 was evaluated under greenhouse conditions. Tv911 promoted the plant height of tomato and Japanese mustard spinach into 16.22% and 50.26% in treated Farmer used nursery soil (FNS) and 9.84% and 7.00% in treated Home garden

soil (HGS) in comparison with non-treated control, respectively, for one month. Fresh root weight of radish also increased significantly in tested soil at 6 weeks after sowing. I can assume that the metabolites of Tv911 are beneficial for the growth of the tested vegetables. Therefore, the selective biological agent, Tv911 has a potential for plant growth promotion ability as a biofertilizer in organic agricultural production.

In the chapter 4, antagonistic ability of Tv911 for the fusarium wilt of tomato and lettuce, the most harmful soilborne fungal diseases, was examined in greenhouse conditions. The disease severity caused by FOLy and FOLa was significantly reduced in Tv911 treated soil. The colonization of Tv911 was estimated using qPCR with specific primers set. In this experiment, the species specific primer sets were newly designed for qPCR, and this is efficient technique for quick detecting and quantifying target species in unknown environmental genomic DNA sample. In the results of qPCR experiment, Tv911 colonized a stable condition in the soil, and an increase (approximately 4 times) in the root tissue over one month growing period. In the experiment of effect of Tv911 against fusarium wilt of lettuce caused by FOLa, the similar results obtained. Although Tv911 cannot completely eliminate the disease incidence caused by FOLa, it significantly reduced the disease severity and pathogenic fungal colonization in root tissue in comparison with non-treated control (FOLa only). Therefore, Tv911 isolates would be a prospective biological resource for plant growth promotion and suppression of fungal plant diseases in future organic agricultural production.

In the chapter 5, I demonstrated the biocontrol activities of Tv911 against soybean anthracnose in the seedling stage under greenhouse condition. Anthracnose of soybean caused by *C. truncatum* as a destructive fungal plant disease and causes heavy yield loss especially under hot and humid weather conditions in Myanmar. Tv911 increased the germination and seedling vigour and reduced the pre- and post-emergence mortality. The

colonization of *C. truncatum* and Tv911 was also checked via qPCR. The estimation of Tv911 in hypocotyl and root tissue of soybean and in soil was performed as in the previous section. For *C. truncatum* colonization, the new primer set was designed based on the partial sequences of ITS gene. However, the DNA concentration of the two targeted fungi was measured only one time at 2 weeks after sowing. Tv911 DNA concentration in root was higher than in hypocotyl, but the concentration of *C. truncatum* DNA in root tissue was significantly lower than that in hypocotyl tissue. The colonization of *C. truncatum* in hypocotyl tissue in Tv911 treatment was lower than non-treated check. Therefore, *T. virens*, Tv911 is an effective biological control agent for the seedborne fungal disease caused by *C. truncatum* at the germination and early growth stage of seedling of soybean.

According to the findings of the present study, the field symptoms of premature leaf falls and black blotching on the stems in Myanmar were caused by six fungal species, namely *Colletotrichum plurivorum*, *C. truncatum, Diaporthe endophytica*, *D. melonis, D. tectonendophytica* and *D. ueckerae*. The information on occurrence and distribution of these fungal species would support for determining the disease management strategies for soybean cultivation in Myanmar. Then, *T. virens* (Tv911) promote the plant growth of some vegetables and it has antagonistic activities to the *Fusarium oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *lactucae* and *C. truncatum* which are the causal organisms of fusarium wilt of tomato and lettuce, and soybean anthracnose, respectively. I hope that the findings of this study would fulfil the knowledge gaps of soybean fungal plant diseases in Myanmar and the biocontrol agent, Tv911 also would be useful for environmental friendly organic crop production. However, further investigations of effect of Tv911-derived metabolites on the crop and environment under field conditions are necessary before commercial use.

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