Forest Diseases; FTA Cards and DNA Sampling

by

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DEDICATION

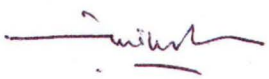
This thesis is dedicated to

Henri Supriyanto and Abiyyudha Hemiwarsanto,

a beloved husband and son

DECLARATIONS

This thesis does not contain any material which has been accepted for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge, this thesis contains no material previously published or written by another person except where due acknowledgment is made.



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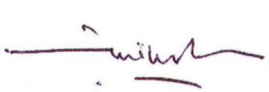

Purnamila Sulistyawati

ABSTRACT

The identification of plant pathogens often requires rapid, effective and reliable sampling techniques for pathogens or infected plant tissues. The first and critical step to a PCR-based identification from the processing of sampled tissues is the extraction and purification of template DNA of suitable quality for PCR. Many DNA extraction techniques for plant and fungal DNA are time-consuming and/or require sophisticated laboratory equipment. Whatman International Ltd from Flinders Technology Associates (FTA) has patented cards which offer a simple and rapid method for the room temperature collection, transport and storage (short and long term) of DNA. Direct capture of plant pathogen DNA in the field, achieved by squashing infected tissue (either symptomatic or asymptomatic) and/or pathogen structures onto cards, will facilitate the detection and identification of the pathogen. DNA sampling with these cards provides many advantages for a plant pathologist such as increasing the number of samples that can be collected, stored and transported in the field, especially in remote locations. It circumvents any requirement for travelling with collection containers, cumbersome equipment or labile buffers. It is particularly useful when the isolation of a pathogen is either not possible, as for an obligate pathogen, or only achieved with a low rate of success.

The aims of the research in this thesis were to investigate the applicability of FTA cards as a new method for DNA sampling from fungal and infected plant material associated with forest diseases. After DNA sampling and capture on the card, the DNA extractions were subjected to PCR, DNA sequencing and species-specific PCR. There were three main sources of material squashed onto the FTA cards; fungal material (cultures, fruitbodies and spores); asymptomatic or symptomatic plant material (root, leaves, and seeds); water and soil that were likely to contain infective propagules.

DNA was easily obtained from fungal cultures squashed onto cards and the DNA thus harvested is suitable for PCR, DNA sequencing and species-specific PCR. With the latter type of PCR, caution must be exercised when using the card in order to avoid contamination. In case studies of forest diseases involving the squashing of infected material onto FTA cards, several fungal pathogens were identified based on sequencing of the PCR product obtained from the DNA captured by the FTA card; *Fusarium oxysporum*, *Cylindrocladium* spp., *Phoma* spp., and *Phytophthora* spp. were detected and identified. The use of FTA cards to sample the DNA of certain fungal propagules such as rust spores or the fungal propagules contained in soil or water did not prove very successful.

These preliminary results clearly demonstrate the potential of FTA cards to assist forest pathologists in disease detection and identification. Further modifications to FTA card sampling techniques are discussed so that the DNA of a wide range of forest pathogens can be successfully obtained from plant tissue, soil or water.

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In the first place I would like to say my prayer and thanks to Allah Almighty for all the strength, patience and ability given to me so that I was able to finish my research and my thesis. *"RABBI YASSIR WA LAA TU'ASSIR"*. Thank you Allah, for all your kindness, your blessings and everything which happens to me in the past, present and the future, be it good or bad, YOU have YOUR reasons.

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TABLE OF CONTENTS

TABLE OF TABLE

TABLE OF FIGURE

CHAPTER 1

Introduction to the molecular diagnostics of fungal plant diseases

INTRODUCTION

Fungal plant diseases

Fungi are eukaryotic organisms devoid of chlorophyll that obtain nutrients by absorption, and reproduce by spores. Fungi therefore require preformed organic compounds produced by other organisms such as plants and their exploitation of different types of substrates varies greatly according to species. The majority are saprophytic and obtain nutrients only from dead organic material such as plant residues producing enzymes or organic acids capable of softening and dissolving cellulose. Some species are parasites and utilise nutrients from living host tissue such as plants. Such fungi may produce toxins, poisons which are capable of killing the living cells of a host. There are many fungal species which are able to grow saprophytically or parasitically.

Many fungal genera contain different species which result in significant economic loss across a wide range of crops e.g. *Fusarium* and *Phytophthora* (the latter is not a true fungus but fungus-like). Fusarium species are probably the most important of fungal plant pathogens, causing a variety of blights, root rots or wilts on nearly every species of economically important plants including forest trees; during 1991-1997 USA wheat producers suffered cumulative losses of 1.3 billion dollars from Fusarium head blight (Johnson *et*

al. 1998); *Fusarium circinatum*, causes pitch canker of Radiata pine and is not present in Australia (Cook and Matheson 2008). If biosecurity measures are effective in delaying the entry and spread of pitch canker to Australia by as little as 2-3 years then this will produce an economic benefit over time of AU$ 13 million. The financial loss caused by the potato late blight pathogen *Phytophthora infestans* has been estimated at more than US$ 2.7 thousand million in the developing countries of the world alone (Hausladen 2006).

According to the Department of Conservation and Land Management Western Australia (2003), the estimation of annual losses caused by several *Phytophthora* species in WA based on a Rural Resources Development Corporation Survey in 1993 totalled AU$ 1,200,000 for horticulture and AU$ 500,000 for floriculture. In California, *Phytophthora citricola* has been calculated to affect between 60-75% of avocado orchards and cause losses in excess of USD 40 million annually (Coffey 1992).

In order to minimize disease problems it is first necessary to accurately and rapidly identify the cause of the disease as this can enable timely and effective intervention. Many fungal diseases are not apparent in the early stages of infection and methods to detect the infection at this cryptic stage may require immunological or molecular diagnostic technology as the diseases progresses symptoms and signs become more obvious and are used for diagnosis.

Morphological/conventional techniques in fungal identification

Conventional techniques in fungal identification rely heaviliy upon the accurate recognition and description of fungal morphological characteristics. Many fungi can be readily recognised in the field by marcomorphology (sporocarp shape, texture, colour, odour, taste and spore print), but the identity of many can only be confirmed by the macroscopic examination of spores or other features. These discriminant features, whether macroscopic or microscopic will vary among fungi (Bougher and Syme 1997) and fungal morphology is extremely diverse. Often identification depends on the isolation of fungi into pure culture and the subsequent observations of cultural traits. Knowledge of fungal ecology is also important to the identification of fungi and may indicate to which genera they belong; fungal species of the same genus may have similar ecology. For example: members of the genus *Agaricus* are decomposers of soil organic matter, where members of the genus *Amanita* are mycorrhizal partners of plants (www.fungibank.csiro.au).

Good field guides which contain keys, pictures, and descriptions of individual species or diseased specimens are needed for morphological based identifications. Keys have been used for a long time and remain the most reliable way of identifying fungi. Keys consist of series of mutually choices which when followed through in sequence, leads down to a small set of candidate names. Where possible, choices are based on readily visible features. However keys do not always include the fungal species targeted for identification, they may be difficult to use, especially to those less expert in the

knowledge of macroscopic or microscopic fungal structures. Often keys identify fungi only to genus level with any certainty (Hood 2003). Molecular diagnostic tools add another significant level of information to keys to assist in accurate identification.

Molecular techniques in fungal diagnostics

Polymerase Chain Reaction (PCR) amplification is arguably one of the most common molecular techniques used to assist the detection and/or identification of microorganisms. The Polymerase Chain Reaction (PCR) method was developed by Mullis and Faloona in 1986 (Sambrook and Russell 2001) and since then PCR has already been used all over the world, greatly facilitating the molecular identification and detection of many organisms including plant diseases (Neumaler 1991; Woods 2004; Michailides *et al.* 2005).

PCR is a method for exploiting enzymes to amplify a specific region of DNA that is located between two given nucleotide sequences of approximately 20 bp. In a chain reaction, copies are themselves copied, so that the total number of product molecules increases exponentially with time, resulting in over a million-fold amplification. Thus, a fragment from a single molecule of DNA can be amplified in an hour or two to produce many millions of copies, facilitating down-stream processing such as DNA sequencing.

One of the most important developments in PCR technology was the discovery and use of the thermostable *Taq* DNA polymerase enzyme from the thermophilic bacterium *Thermus aquaticus*. By 1990, standard reagents for

PCR i.e PCR buffer, dNTP, $MgCl_2$ and *Taq* DNA polymerase were commercially available for PCR assays (Lauerman 2004). Improvements in buffers have given greater stability and longer activity for reagents and enzymes, which has contributed to improved PCR and reverse transcription PCR techniques. Other polymerase enzymes have been isolated and evaluated from a number of thermophilic organisms having a variety of activities different from *Taq* DNA polymerase. This allows longer segments of DNA to be produced as PCR amplicons with greater accuracy.

Primers are designed to be complementary to the ends of the DNA fragment to be copied. The double stranded sample DNA is first denaturated, by heating, to produce two single strands, and the reaction is then cooled to allow the two primers to hybridize to complementary sequences on the two DNA strands. The enzyme DNA polymerase, then binds the 3' ends of the hybridized primers and extends them by adding nucleotides that are complementary to the original DNA. In the first round of synthesis the new chains grow as long as the enzyme can proceed in the time alotted for the reaction. However, when these chains are copied in the next round, the polymerase comes to the end of the chain and stops, producing molecules of a fixed length. In the succeeding rounds of amplification, the fixed length molecules become the predominant species.

For some purposes, e.g. detection of a particular pathogen using species-specific primers, PCR amplification is sufficient, with gel electrophoresis the

only down-stream processing required. In other instances, further analyses of PCR products such as PCR-RFLP (Restriction Fragment Length Polymorphism, (Krokene 2004)) , DNA sequencing or SSCP (Single Strand Conformation Polymorphism, (Kjøller and Rosendahl 2000; Alguacil *et al.* 2009; Krüger *et al.* 2009; Miyazaki *et al.* 2009)) are required.

Several variations of PCR amplification have been used widely such as Random Amplification of Polymorphic DNA (RAPD, (Zeng *et al.* 2005; Séré *et al.* 2007)), Amplified Fragment Length Polymorphism (AFLP, (Abdel-Satar *et al.* 2003; Cipriani *et al.* 2009; Horisawa *et al.* 2009; Lima *et al.* 2009; Zheng *et al.* 2009)), Reverse Transcription PCR (RT-PCR, (Brody and Maiyuran 2009; Genre *et al.* 2009; Lo´pez and Go´mez-Go´mez 2009; Tollot *et al.* 2009)), real-time PCR (Alaei *et al.* 2009; Huang *et al.* 2009; Luo *et al.* 2009; Maciá-Vicente *et al.* 2009; Yin *et al.* 2009) and nested PCR (Jørgensen *et al.* 2005; Kim *et al.* 2009; Krüger *et al.* 2009)

DNA sequencing analysis is the procedure of determining the order of nucleotides in a given DNA fragment. The DNA sequencing approach to fungal identification has many advantages. Results can be obtained rapidly, the approach is very sensitive and specific, it is possible to start with a small quantity of DNA and sequencing can be done directly from PCR products (Petti 2007).

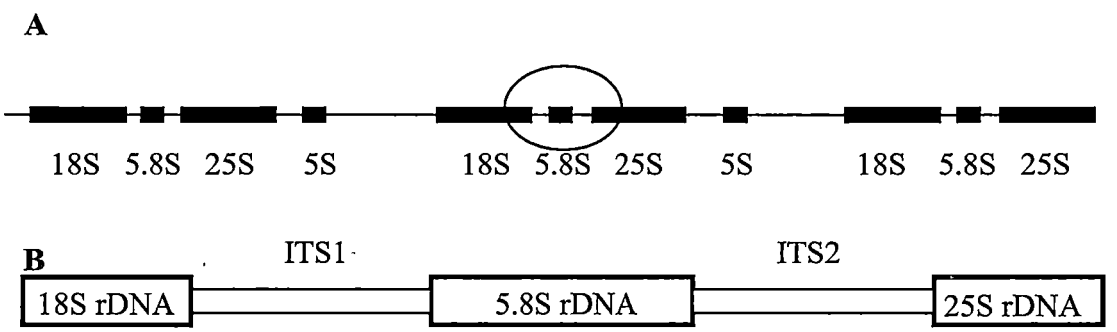


Figure 1.1 A. Schematic representation of the organisation of the rRNA gene repeats indicating the location of the internal transcribed spacers (ITS). B. An enlarged view of the circled section in A. Primer sites for DNA amplification from a broad range of fungi are located in the 18S and 25S rRNA genes.

DNA regions coding for ribosomal RNA (Figure 1) have been widely used in mycological studies. These genes occur as tandem repeats, making them easier to amplify than single-copy genes. This rDNA region typically contains genes for the Small Sub Unit (SSU) 17S, 5S, 5.8S and Large Sub Unit (LSU) 25S, ribosomal sub units, Internal Transcribed Spacers (ITS), and Intergenic Spacers (IGS). To the outside of the small and large rRNA sequences are the external transcribed spacer region (ETS) and the Intergenic spacers (IGS). The 5.8S nuclear rDNA gene lies between ITS 1 and ITS 2.

The ITS regions are highly conserved within most species (with intra specific similarities usually > 99 %) but are variable between species, making it suitable for use in taxonomy (Gomes *et al.* 2002; Xu 2006; Petti 2007). It has the resolving power to place unknowns to the species level or at least within a species group (Kurtzman 1994; Hamelin *et al.* 1996; Mishra *et al.* 2000;

Landeweert *et al.* 2003; Redecker *et al.* 2003; Engkhaninun *et al.* 2005). The interspersion of highly conserved and highly variable sequences in the ribosomal DNA facilitates the design of primers with a range of specificities, from almost universal to species-specific.

In fungi, the ITS region, including the 5.8S gene is typically about 450-700 bp in size. It can be amplified by the universal primer pair ITS1 and ITS 4 (White *et al.* 1990; Gardes *et al.* 1991), or a fungal specific forward primer, ITS1-F (Gardes and Bruns 1993), can be combined with either ITS4 or a basidiomycete specific reverse primer ITS4-B (Gardes and Bruns 1993). Many species-specific primers have also been designed from ITS sequences (Belbahri *et al.*, 2007, Hseu *et al.*, 1996, Lim *et al.*, 2005, Maxwell *et al.*, 2005, Silvar *et al.*, 2005, Tyler *et al.*, 1995)

The primers ITS1 and ITS4 may amplify a wide range of fungal targets and work well to analyze DNA isolated from individual organisms, but do not effectively exclude the host plant sequences in environmental samples or mixed plant/microbial samples often used in studies of plant-associated microbiota. Subsequently, the plant-excluding primers ITS1-F and ITS4-B came into wide use for preferential amplification of fungal ITS sequences from mixed DNA samples.

Accurate classification of gene sequences to a particular genus or species requires analysis with a high- quality, comprehensive reference library. The

European Molecular Biology Laboratory/EMBL (www.embl.org), the National Center for Biological Information/NCBI (www.ncbi.nlm.nih.gov) and GenBank (www.ncbi.nlm.nih.gov/Genbank), MicroSeq, Ribosomal Database Project, Ribosomal Differentiation of Microorganisms, and SmartGene are all useful databases. Reference databases are largest for 16S rDNA (bacteria) and ITS1/2 (fungi) sequences, but sequences from other gene targets are increasing rapidly (Petti 2007).

A fast way to take advantage of the ITS data currently deposited is to search GenBank or EMBL using software designed to find highly similar DNA sequences. Computer programs such as FASTA (Pearson 1998) and BLAST (www.ncbi.nlm.nih.gov/BLAST/, Altschul *et. al* (1990)) facilitate the retrieval of highly similar DNA sequences from the database. Additional software, e.g. Phylip (http://www.phylip.com/, Felsenstein (1989) and Tuimala (2006)), PAUP (http://paup.csit.fsu.edu/) is available to determine the phylogenetic relationships between the unknown fungus and identified species and therefore help confirm its taxonomic status. The program can assist in assessing confidence in species identification by scoring percent probabilities; similarity index value and the sequence pattern for the species are listed (Woods 2004).

However there are many species and groups of species whose DNA sequences are not represented in the databases. These species are often assumed to be new species when the ITS sequence of the unknown fungus has less than 98% similarity with any known species (Brasch and Graser, 2005, Decock *et al.*,

2006, Henry *et al.*, 2000, Li *et al.*, 2008, Takamatsu *et al.*, 2008). Caution must be exercised in using these publicly available resources as there is no quality control on either the DNA sequencing or the putative identification of the isolate from which the sequence was derived.

In the future the barcoding of fungi should accelerate the construction of a comprehensive, consistent reference library of DNA sequences. DNA barcode sequences are a standardized approach to identifying animals and plants by minimal sequences of DNA. DNA barcoding is based on a simple observation: patterns of sequence diversity in short, standardized gene regions ('DNA barcodes') allow specimens to be assigned to known species or to new ones. In addition, because of their digital format, DNA barcode libraries will allow fully automated identifications for most specimens. Automation will massively improve the ability to monitor, understand, and manage fungal biodiversity with substantial scientific, forensic, epidemiological and economic benefits (http://www.DNAbarcoding.org)

Conventional versus molecular diagnostics

The identification of fungal diseases has conventionally relied upon the observation of diseased tissue; both macroscopic and microscopic, and often required the isolation of fungal pathogens into culture. Such methods depend on the ability of the pathologist to identify the signs and symptoms of a fungal disease, cultures and their colony morphology.

According to Atkins and Clark (2004), the accuracy and reliability of conventional identification methods is highly dependent on the experience and skills of the person who makes the diagnosis. Problems in conventional identification may result in an incorrect diagnosis and therefore any subsequent treatment may not be effective. Whatever their experience the conventional diagnostician faces certain problems. Environmental factors can influence the expression of those traits which allow the identification of fungal pathogens and resulting in confusion over diagnosis (Atkins and Clark 2004; Woods 2004).

There are some plant diseases caused by fungi that are difficult to differentiate using conventional taxonomic identification methods as they cause similar symptoms in the plant host or have a nearly identical morphology in culture. Some fungi, for example *Armillaria* and *Cylindrocladium* (Péréz-Sierra and Henricot 2002), are difficult to culture, or are obligate pathogens and cannot be cultured, so that, diagnosis based on the culture/colony of the fungi is either impossible or very tedious.

Other fungal pathogens may be relatively easy to culture but do not produce the reproductive structures that are required for accurate identification. For example, when members of the genus *Colletotrichum* grow in culture, they do not generally create their characteristic conidiomata. The fungi are familiar with its appresoria, however, for medical research if there is one specific character not present, the identification will be difficult (Cano *et al.* 2004).

Molecular diagnostics are sensitive and need only use very small amounts of fungal or infected plant material. PCR based techniques are available that detect single or multiple pathogens in symptomatic and/or asymptomatic plant tissue e.g. multiplex PCR. Rapid processing of samples means that diagnosis can be based on a larger number of samples with a wider and more accurate investigation of disease incidence and distribution. Molecular tools developed for diagnostics may be applied in other fields of plant pathology e.g. epidemiological investigations, population genetics, resistance screening, ensuring disease-free certification, quarantine, assessing the effectiveness of control treatments.

In summary, molecular diagnostics approaches have overcome some problems with conventional methods in that they are more accurate, quicker, highly sensitive, reliable and adaptable to high throughput methods. Since they offer quick and accurate results they allow timely and effective disease management (Martin *et al.* 2000; Lévesque 2001; Atkins and Clark 2004; Woods 2004).

The improvement of DNA based plant disease diagnostics; Whatman FTA Card Technology

It must always be remembered that molecular techniques are only tools and their application should be complementary to conventional diagnostic procedures and/or expert interpretation. The actual molecular based diagnosis should be able to be performed by a person who does not have any experience

with a particular plant disease (McCartney *et al.* 2003). Rapid, reliable and standardized sampling techniques for the DNA of plant diseases carried out in the field would also mean that a farmer or field manager could collect DNA to be sent for testing.

Whatever the sampling methodology, the first and critical step is the extraction and purification of template DNA of suitable quality for PCR. There are many DNA extraction techniques for plant and fungal DNA but most of these are time-consuming and/or require sophisticated laboratory equipment. These methods may include grinding samples with a mortar and pestle, blending samples with a commercial homogenizer, or sometimes freezing samples with liquid nitrogen. Conventional methods may also require equipment such as water baths and centrifuges. Obviously, it is difficult to bring this equipment to the field especially in remote locations where field experiments may be conducted.

Whatman International Ltd from Flinders Technology Associates (Moscoso *et al.* 2004) have patented FTA Card which provides a simple and rapid method for the room temperature collection, transport and storage of DNA. Using FTA Card as one new sampling method provides an easier way for DNA movement, which may be suitable for biosecurity applications.

The Whatman FTA Card is a potential alternative to these conventional methods because it is designed for sample collection in the field and storage at

room temperature (Crabbe 2003). FTA paper is impregnated with chelators, denaturants, and free radical traps, which inhibit enzymes, microbes and chemicals that may degrade the DNA or RNA in the fungal sample. This allows nucleic acids to be stored at ambient temperatures for long periods on the FTA Card (Rogers and Burgoyne 1997).

FTA card contains chemicals that lyse cells, denature protein and inactivate viral contaminants. These chemicals also protect nucleic acids from nucleases and ultraviolet damage. In addition, the FTA card rapidly inactivates micro-organisms and prevents their growth.

The development of FTA Card as a field sampling technique should facilitate the detection and identification of the pathogen whilst avoiding laborious and often unsuccessful attempts at isolation. For example, identification of root-rot pathogens is necessary to allow an assessment of the risk at a plantation site of root rot and the implementation of appropriate measures to manage the disease.

According to Whatman International Ltd (www.whatman.com), some features and benefits of FTA card technology are: easy capture of nucleic acids; nucleic acids collected on FTA cards are stable for several years at room temperature; storage at room temperature before and after sample collection, reducing the need for laboratory freezers; a colour change upon sample collection to facilitate the handling of colourless samples; suitability for virtually any cell

type and available in a variety of configurations to meet application requirements.

FTA Card is used to collect the DNA directly from the organisms *in situ.* It has been developed for the rapid isolation of human DNA without the need for bulky equipment and used extensively in forensic science (Belgrader and Marino, 1996, Biruš *et al.*, 2003, Børsting and Morling, 2006, Chomeczynski and Rymaszewski, 2006, Forrest *et al.*, 2004, Fujita and Kubo, 2005, Kline *et al.*, 2002, Plaia *et al.*, 2007, Prieto *et al.*, 2006, Schmalzing *et al.*, 1997, Sitaraman *et al.*, 1999, Tack *et al.*, 2007).

The card has also been used in human biotechnology for identifying and detecting diseases (Beck *et al.* 2001; Dobbs *et al.* 2002; Kuboki *et al.* 2003; Becker *et al.* 2004; Li *et al.* 2004; Guio *et al.* 2006; Milne *et al.* 2006; Dictor *et al.* 2007); human genes (He *et al.*, 2007, Lema *et al.*, 2006) and pharmacology (Mas *et al.* 2007).

Recently, FTA card has been used widely in many other types of research, for example in animal biotechnology for the detection of insects and their distribution (Harvey 2005; Owens and Szalanski 2005); fish population genetics (Livia *et al.* 2006); animal genetics (Bendezu *et al.*, 2005, Crabbe, 2003, Gutierrez-Corchero *et al.*, 2002) and animal diseases (Inoue *et al.*, 2007, Moscoso *et al.*, 2005, Perozo *et al.*, 2006, Purvis *et al.*, 2006, Rensen *et al.*, 2005).

Several studies used the FTA Card technology for identification and detection of protozoa (Orlandi and Lampel 2000; Hide *et al.* 2003; Chu *et al.* 2004), while others used the card for capturing the DNA from bacteria (Rajendram *et al.*, 2006, Rogers and Burgoyne, 1997).

Originally developed for collecting DNA from blood, bacteria and animal tissue samples, the kits for collecting plant DNA are now available. In plant biotechnology, it was reported that FTA cards have been used for large-scale sampling of plant DNA (Mbogori *et al.*, 2006, Tsukaya *et al.*, 2005).

In addition, the card technology has been used to identify viral plant pathogens (Ndunguru *et al.* 2005). FTA card has also been applied to plant genetic studies (Bendezu, 2004, Drescher and Graner, 2002, Lin *et al.*, 2000, Natarajan *et al.*, 2000).

The application of FTA cards to sampling fungal DNA associated with plant and forest diseases

These have also been used, with some success, for fungal DNA (Suzuki *et al.* 2006). The technique appears to have potential to facilitate the field sampling of fungal DNA particularly in remote locations, although some types of fungal cells may not be compatible with the card. Since fungal cells are extremely diverse and some undergo thickening, are less susceptible to degradation and

may not readily release DNA, e.g. teliospores or woody sporocarps. It is therefore necessary to determine the limits of FTA card suitability for a range of applications (i.e. type of fungal structure or infected tissue) before relying on it as a sampling method.

Plant biosecurity measures to prevent the arrival of new plant diseases and pests must be stringent. The rapid movement by air of plant products, plants and people around the world facilitates the inadvertent release of associated fungal propagules into new environments, where they can spread and infect other the transfer of plant pathogen propagules. One advantage of sampling with the FTA Card is avoiding the transfer of viable plant pathogens or fungi from one place to another.

The sampling of fungal DNA by FTA cards could facilitate investigations required for effective disease management in forestry in a number of ways, especially in respect to countries with less well developed logistical infrastructure.

- permit the harvesting of DNA from a larger number of samples than could be attained if material had to be transported back to a laboratory
- reduce the need to transport fungal or diseased material which could pose a biosecurity risk or be difficult and costly to transport from the more remote locations often associated with forestry

- allow the immediate harvest of DNA from fresh samples in remote locations – tissue that is transported or kept for several days may become contaminated.

RESEARCH OBJECTIVES

The overall objective of this research was to assess the applicability of FTA Card technology as a new sampling method to collect and store fungal plant pathogen DNA directly from the field.

This master's research was supported by the Australian Centre of International Agricultural Research and associated with a project investigating developing management strategies to reduce the impact of fungal root-rot in *Acacia mangium* plantation in Indonesia. Testing of the FTA cards was carried out in the context of a field trip to Indonesia and therefore much of the fungal or plant material sampled revolves around diseases of plantation or nursery *Acacia mangium*, especially root rot.

The specific aims of this research are to investigate the applicability of FTA Card as a new method in DNA sampling for fungal material by

1. Testing the ability of FTA card to bind DNA from various fungal propagules (mycelium, sporocarps, basidiospores, urediniospores, teliospores) on/in various media (rotten wood, soil, leaves) and associated with different plant diseases, especially forest diseases of tropical plantation trees

2. Testing the suitability of DNA from FTA card for different molecular techniques, including PCR, DNA sequencing and species-specific PCR.

In this thesis, chapter 2 describes the general methodology which is used in this research while chapter 3 describes the use of FTA card technology to obtain fungal DNA from three different types of fungal material i.e. fruit bodies, cultures and spore prints of larger basidiomycetes. Chapter 4 describes the use of FTA cards to obtain fungal DNA from several types of plant material such as leaves, roots and seed. Chapters 5 and 6 describe case studies in which FTA cards were used as an integral part of the diagnostic process in the forest environment. Chapter 7 is a general discussion about the potential of FTA card technology and what should be done to maximise its effectiveness in the collection of fungal DNA associated with plant and forest diseases.

CHAPTER 2

General Methodology

This chapter describes the methods that were used in this research and were common to all chapters. Several types of sample materials were tested in this thesis using FTA card technology. FTA card and reagents were supplied in a kit from Whatman Laboratories USA and the standard sampling procedures outlined in the instructions for most plant samples can be followed.

However, in order to extend the range of sample types associated with plant pathogens from which DNA can be obtained, a few modifications were made. These included grinding or mixing the plant material with an extraction buffer or sterile water before spotting onto the card.

Some of the substrates (e.g. soil and some plant materials) from which DNA extraction was attempted using FTA card are known to contain PCR inhibitors and so require specialised procedures to minimise these (Pandey *et al.*, 1996, Tsai and Olson, 1992). Soil is particularly notorious for PCR inhibition (Porteous and Armstrong 1991; Claassen *et al.* 1996; Yeates *et al.* 1997; Schneegurt *et al.* 2003).

FTA Card Sample Preparation

Samples of seeds, leaves, roots or soil were ground in a 1.5 mL microcentrifuge tube with a motorised micro-pestle or in a mortar and pestle

with the aid of liquid nitrogen. Ground samples were mixed with DNA extraction buffer, vortexed and incubated at room temperature (30-60 minutes) before pipetting of the supernatant onto the FTA card. Other types of sample such as fungal materials and putatively infected plant material were cut into small pieces and squashed directly onto the card. The FTA cards were air dried for a minimum of two hours in the case of water and homogenized samples, and one hour minimum for samples directly squashed on the cards.

After the various samples were applied to the FTA card, the card was placed on the cutting mat. Using a 2.0 mm Harris Micro Punch ™ Tool a disc from the centre of the dried sample area was removed and transferred to an appropriate PCR amplification tube (or 1.5 mL microfuge tube). To each tube, about 200 µL of FTA Purification reagent was added. The tubes then were capped, inverted twice and incubated for 4-5 minutes at room temperature. The FTA reagent was pipetted up and down twice then as much as possible was removed and discarded using a pipette to prevent loss of the disc during decanting. This step was repeated for a total of two FTA purification reagent washes.

To each tube, about 200 µL of TE buffer were added. After that, the tubes were capped and inverted twice then incubated for 4-5 minutes at room temperature. The TE buffer was pipetted up and down twice then as much as possible was removed by pipette and discarded, keeping the card punch in the tube. The steps were repeated for a total of two TE buffer washes. The card

discs were dried for one hour at room temperature or for 20 minutes at 56°C before storage at 4°C or -20°C.

Glassmilk DNA Extraction Method

The glassmilk DNA extraction method (Glen, M. *et al.* 2002) was used as a basic DNA extraction method for fungal and leaf samples. Fresh materials i.e. cultures (approximately 100 mg) were ground in a 1.5 mL microcentrifuge tube using a plastic pestle and a motorised pestle grinder (Pellet Pestle Kontes Motor) with addition of a little extraction buffer if necessary.

Dried materials i.e. pieces of leaves, fruitbodies and seeds (20 mg) were ground by hand using a mortar (50 mL volume), pestle and liquid nitrogen then transferred into a 1.5 mL microcentrifuge tube. Extraction buffer from Raeder and Broda (1985), was added (250 µL) and samples were incubated in a water bath (Grant Instrument Cambridge Ltd Type 20) at 65°C for 1 hour.

Samples were centrifuged (Sigma 1-13 B.Braun Biotech International) at 14000 rpm for 15 minutes. Into a 1.5 mL tube, 7 µL glass milk (Boyle and Lew 1995), 800 µL of 1 g/mL NaI (BDH Chemicals Australia) and 200 µL supernatant were added. The mixture was vortexed briefly (Yellowline TTS 2-Fisher Biotech Australia), incubated on ice for 15 minutes and shaken occasionally. The samples were centrifuged for 10 seconds, the supernatant discarded and pellets resuspended in 800 µL wash solution.

The samples were again centrifuged for 10 seconds, the supernatant discarded and pellets were resuspended in 800 µL 100% ethanol (Univar-Ajax Finechem Australia, Analytical Grade). The samples were centrifuged 10 seconds, supernatants were discarded and tubes were inverted to dry. The pellets were then resuspended in 25 µL TE buffer and incubated at 45°C for 10 minutes before centrifugation at 14000 rpm for 1-2 minutes. The supernatant was removed into a new clean 1.5 mL tube and an aliquot diluted 1/20 before PCR.

Powersoil DNA Extraction Method

A Powersoil™ DNA extraction kit (Mo Bio Laboratories Inc, Carlsbad, USA) was used for extracting DNA from soil samples. All the solutions (C1 to C6) were supplied by Mo Bio Laboratories Inc; each solution is under patent and its composition is not publicly available. Approximately 0.25 g of soil from each sample was added to a PowerBead tube and then gently vortexed for 1 minute.

Into those tubes 60 µL of solution C1 were added, the tube was inverted several times or vortexed briefly before it was secured horizontally on a flat-bed vortex pad with tape. The tubes were vortexed at maximum speed for 10 minutes then centrifuged at 10000 x *g* for 30 seconds at room temperature.

About 400-500 µL of supernatant were transferred into a clean 2 mL collection tube and 250 µL of solution C2 were added. Tubes were vortexed for 5 seconds before incubation at 4°C for 5 minutes followed by centrifugation at room temperature for 1 minute at 10000 x *g*.

Carefully avoiding the pellet, about 600 µL of supernatant were transferred into a clean 2 mL collection tube. Into those tubes 200 µL of solution C3 were added, the tubes vortexed briefly and incubated at 4°C for 5 minutes before centrifugation at room temperature for 1 minute at 10000 x *g*. Avoiding the pellet, about 750 µL of supernatant were transferred into a clean 2 mL collection tube and 1200 µL of C4 solution were added to the supernatant before the tubes were vortexed for 5 seconds.

The supernatant was transferred, 675 µL at a time, into a spin filter and centrifuged at 10000 x *g* for 1 minute at room temperature. The eluate was discarded. Total of three loads, approximately 675 µL each, for each sample was required. The filter was washed with 500 µL of solution C5 and centrifuged at room temperature for 30 seconds at 10000 x *g*. The eluate was discarded. The tubes were centrifuged again at room temperature for 1 minute at 10000 x *g* to dry the filters. The spin filter was moved into a clean 2 mL collection tube and 100 µL of solution C6 were added into the centre of the white filter membrane. The tubes then were centrifuged at room temperature for 30 seconds at 10000 x *g*, and the spin filter was discarded.

Filter Paper DNA Extraction

DNA extraction of spores trapped on Whatman filter paper followed the method described by Schweigkofler *et al.* (2004). This method was also used for the membrane of a millipore filter. Briefly, the filter papers were cut up and washed with 20 mL of hot (65°C) 4xTE buffer and resuspended by vortexing

(maximum speed) for 5 min. The suspensions were centrifuged at 1000 x *g* for 90 minutes to concentrate the spores. After the supernatant was removed, DNA was extracted from the pellet (100 µL). DNA from half of the pellets was extracted using the glassmilk DNA extraction method and the remaining pellets were squashed onto the FTA cards.

Polymerase Chain Reaction (PCR)

PCR amplification was performed in 50 µL volumes of reaction mix containing unless otherwise stated in relevant chapter; 5 µL of 10 X reaction buffer (BioLine), 2 mM $MgCl_2$ (BioLine), 0.2 mg/mL of Bovine Serum Albumin (Fisher Biotech Australia), 0.2 mM dNTPs (BioLine), 0.2 mM each primer, 0.05 U/µL Biotaq DNA Polymerase (BioLine), 10 µL of template DNA or one disk (2 mm^2) of clean FTA card sample and water (sterile water, Astra Zeneca, USA).

Primers used in this research included primers targeting the rDNA ITS; ITS1-F, CTTGGTCATTTAGAGGAAGTAA (Gardes and Bruns 1993) ITS3, GCATCGATGAAGAACGCAGC and ITS4, TCCTCCGCTTATTGATATGC (White *et al.* 1990). Primers targeting the elongation factor gene *tef1* were EF1, ATGGGTAAGGARGACAAGAC and EF2, GARGTACCAGTSATCATGTT (O'Donnell *et al.* 1998) and primers to amplify a portion of the Beta-tubulin 2 gene were Bt2a, GGTAACCAAATCGGTGCTGCTTTC and Bt2b, ACCCTCAGTGTA GTGACCCTTGGC (Glass and Donaldson 1995). Details of several species-specific primers are provided in the relevant chapters.

PCR was performed by a PTC-100™ Programmable Thermal Controller (MJ Research Inc USA). The programme used for the above primer sets consisted of 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds; 55°C for 30 seconds; 72°C for 30 seconds, followed by 72°C for 7 minutes and a hold at 14°C. Every set of PCR reactions included a negative control (no template DNA) and a positive control (a DNA sample that had previously been amplified by the primers in use) with the exception of some of the species-specific PCRs for *Phytophthora* spp., where positive control DNA was not available.

Gel Electrophoresis

The PCR products (5 µl of PCR products mixed with approx. 1 µL 6 X gel loading buffer) were electrophoresed in 1% or 2% agarose (Fisher Biotech, Australia) gels in TAE buffer at 10 V/cm (Bio Rad Power Pac 200) for approximately 30-60 minutes. Every gel included at least one lane containing 250 µg of a DNA size marker, bacterio-phage lambda DNA cut with the restriction enzymes EcoRI and Hind III (Fisher Biotech Australia).

The gels were stained with 0.5 µg/mL of Ethidium bromide (Mo Bio Laboratories, USA) solution in TAE buffer at room temperature for 20 minutes. DNA bands were visualised on a UV trans-illuminator (Vilber Lourmat, Paris) and images captured with BioCaptMW software (Vilber Lourmat, Paris).

DNA Sequencing

DNA sequencing was carried out by Macrogen (Macrogen Inc Korea, www.macrogen.com). Sequence editing was done using the ChromasPro programme (*www.technelysium.com.au/ChromasPro.htmL*). After editing, the sequence was saved in FASTA file format and analysed using Australian National Genomic Information Service (ANGIS) BioManager. Sequences with high similarity were retrieved from public databases (GenBank, EMBL, DDBJ) using BLAST software (Altschul *et al.* 1990) .

Recipes

Extraction buffer (Raeder and Broda 1985) contained 200 mM Tris-HCl (Sigma Chemicals, WA) pH 8.5, 250 mM NaCl (BDH Chemicals Australia), 25 mM di-sodium ethylene-di-amino-tetra-acetate (EDTA) (Univar, Asia Pacific) and 0.5% SDS (BDH Chemicals Australia).

Glassmilk was prepared according to (Boyle and Lew 1995) from silica (Sigma S-5631), phosphate buffered saline (PBS) and 3 M NaI (BDH Chemicals Australia).

NaI solution was made by dissolving 100 g NaI (BDH Chemicals Australia) in 80 mL sterile water (Baxter International, USA) and adding 1.5 g sodium sulphite (Standard Laboratories Pty.Ltd Australia). The solution was filtered through Whatman No.1 (Whatman Inc, UK), made up to 100 mL by adding water and stored at 4°C in an opaque bottle.

Wash solution consisted of 100 mM Tris-HCl (Sigma Chemicals, Western Australia) pH 7.5, 1 mM di-sodium EDTA (Univar, Belgium); 100 mM NaCl (BDH Chemicals Australia) in 50% ethanol (Ajax Finechem, Australia) and was stored at 4°C.

TE buffer (Sambrook and Russell 2001) contained 10 mM Tris (Sigma T-1378) pH 8.0 and 1mM di-sodium EDTA (Univar Asia Pacific).

50 X TAE buffer (Sambrook and Russell 2001) contained, per litre, 242 g Tris (Sigma Chemicals, WA), 57.1 mL glacial acetic acid (Ajax Finechem, Australia), 100 mL 0.5 M EDTA (Univar, Belgium) pH 8.0 and water.

The 6 x gel loading buffer (Sambrook and Russell 2001) contained 0.25% of Bromophenol blue (Sigma Chemicals, Western Australia) and 50% glycerol (BDH Chemicals, Australia) in water.

CHAPTER 3

Assessing FTA cards for DNA capture from different types of fungal material

INTRODUCTION

The diagnostic signs of fungal pathogens causing a particular plant disease will be associated with one or a number of identifiable components. In most cases, the most common element is the hyphal network (mycelium) which ramifies through the substrate from which the fungus is gaining its organic energy. However other structures, morphologically distinct to mycelium, are more commonly used to identify a pathogen. These structures are frequently associated with reproduction, survival and dispersal e.g. respectively, spores, somatic structures such as sclerotia and melanised cords (rhizomorphs).

A fungi reproduces asexually and/or sexually at some period during its existence. Asexual reproductive structures may simply be compartments which have separated from the thallus, or identifiable structures where spores are formed and then released. Spores (asexual and sexual) vary in size, shape, septation, colour, and wall thickness; they are produced directly on hyphae, on specialised hyphal branches, or on multicellular fruiting structures which range in size from approx. 0.2 mm to over 600 mm in diameter. (Alexopoulos *et al.* 1996)

The Fungi are now thought to contain several Phyla, including Chytrids, Zygomycota, Glomeromycota, Ascomycota and Basidiomycota. Many of the fungi that cause serious forest diseases fall within Ascomycota and Basidiomycota. Ascomycota constitute the largest taxonomic group within the Eumycota. When reproducing sexually, they produce non-motile spores in a distinctive type of microscopic cell called an "ascus". Some Ascomycota never appear to reproduce sexually and are referred to as "mitosporic ascomycota" because of the production of conidia, generally formed on the ends of specialized hyphae.

Basidiomycota produce meiospores called basidiospores on club-like stalks called basidia. This group which includes macrofungi such as mushrooms, puffballs, stinkhorns, bracket fungi, jelly fungi, boletes, chanterelles, earth stars and the major pathogens of many plants and trees – rusts and root rot fungi. Rust fungi are a group of basidiomycete fungi in the order Uredinales which produce several kinds of spores including aeciospores, basidiospores, urediniospores and teliospores. Urediniospores are the thinner-walled spores of some fungi that develop in the uredium on the surface of a leaf, shoot or fruit and erupt in masses. Teliospores are the thick-walled resting spores that develop in a telium, often turning black as they mature. Basidiomycete genera such as *Ganoderma*, *Phellinus* and the rhizomorph forming *Armillaria* include species which are serious root rot pathogens of many plantation tree species.

There are also many serious forest diseases caused by fungal-like organisms such as the water molds or Oomycota (e.g. species of *Pythium* (Nechwatal and Oßwald 2001; Moralejo *et al.* 2008), and *Phytophthora* (Aberton *et al.* 2001; Balci, Y. *et al.* 2008; Jung and Nechwatal 2008; Burgess *et al.* 2009). Damage significant to forestry cause by water molds may include seed decay, seedling damping off, root rots, stem lesions. Many of these fungal-like organisms produce sporangia which release motile zoospores.

Identification of fungi causing disease traditionally depends on examination of the reproductive structures such as those described above. If these structures are not present, costly delays in treatment or response may occur. Faster, more reliable identification may be assisted by DNA techniques, as all cell types of the one organism contain the same DNA and there is no requirement for morphological distinctiveness. Whatman International Ltd from Flinders Technology Associates (Moscoso *et al.* 2004) have patented FTA Card which provides a simple and rapid method for the room temperature collection, transport and storage of DNA. FTA card developed for capture of plant DNA directly from leaves has been successfully used for some fungal cultures, but has not been tested on a broader range of fungal cell types (Gitaitis *et al.* 2005; Borman *et al.* 2006; Suzuki *et al.* 2006).

The first step in the capture of DNA by the FTA card is the spotting or squashing the sample onto the FTA card, which contains chemicals that can lyse the cells and bind the DNA. Squashing materials onto the card involves

breaking the cell walls of the fungal material or plant material. Fungal cell wall and plant cell wall are slightly different. In plants, the strongest component of the complex cell wall is a polysaccharide called cellulose; microfibrils of cellulose are strengthened with hemicellulose, pectin and in many cases lignin, which are secreted by the protoplast to the outside of the cell membrane (Heredia *et al.* 1995). The normal size range for a plant cell is from 10-100 μm, the cell walls can range from 0.1 to 10 μm thick.

Oomycota (fungal-like organisms) typically possess cell walls of cellulose and glucans. In true fungi however the cell walls are constructed of chitin; this polysaccharide (composed of glucans, mannans, galactans, and heteropolysaccharides) is also present in insects and other arthropods. Physically, the fungal cell wall is a structure of interwoven microfibrils set in or covered by unstructured matrix substances. The wall structure is very active, shifting continuously during the stages of cell division, development and morphogenesis (Adams 2004). The cell walls tend to thicken as the cell grows. The thickness, as well as the composition and organization, of fungal cell walls can vary significantly.

The effective use of FTA cards could be influenced by the particular cell wall composition and structure of fungi. Structures with thickened cell walls could be difficult to crush without some kind of pre-treatment and contaminants introduced by the medium in which disease propagules are supported (e.g. soil, leaf tissue) could inhibit DNA extraction and analysis. This chapter describes

the testing of FTA card suitability for capturing DNA from various types of fungal structures, those frequently used for the identification of the causal agents of various forest diseases i.e. pure fungal cultures, macrofungal sporocarps and their spore prints, rust and mildew spores.

MATERIAL AND METHODS

Material

Pure fungal cultures; a wide collection of pure fungal cultures (Table 3.1) inclusive of *Oomycetes*, *Ascomycetes* and *Basidiomycetes* were made available for testing with FTA cards. These cultures were obtained from a variety of sources; a) the fungal culture collection lodged at the forest health laboratory at Hobart, CSIRO Sustainable Ecosystems; b) the fungal culture collection located at the Centre for Forestry Biotechnology and Tree Improvement (CFBTI), Forestry Research Development Agency laboratory at Jogjakarta, Indonesia; c) ongoing research projects at Hobart (including this study). All cultures originating as a result of ongoing projects in Hobart and used in this research have been lodged in the Hobart collection.

The origins of the cultures are referenced by a numerical superscript in Table 3.1 which relates to the following information as follows;

1. The cultures were provided by Dr. Genevieve Gates, who carried out a study, in Tasmania, of the macro-fungal flora present on rotting wood in the southern wet sclerophyll forest. These cultures were, in the majority, isolated from basidiomycetes identified with a species or tag name. An

asterisk indicates that the sporocarp from which the isolate originated was not given either a species or tag name.

2. The *Armillaria* culture was provided by Luci Agustini and had been recently isolated from dying *Cupressus* sp. in Cascade Gardens, Hobart, Tasmania.

3. Cultures in this category were isolated from *Pinus radiata* and were provided by Istiana Prihatini, currently undertaking a phylogenetic analysis of *Cyclaneusma*. Three of the cultures had been identified as *Cyclaneusma minus*.

4. The *Mycosphaerella* cultures were isolated from lesions on eucalypt leaves collected in Tasmania as part of a study about Mycosphaerella leaf disease and were subcultured from the Hobart fungal culture collection.

5. These basidiomycete cultures were tested by the author in the laboratory at CFBTI in Jogjakarta, Indonesia. The cultures had been isolated from sporocarps (see example of sporocarp and culture in Figure 3.1) that had been collected as part of an investigation into root rot disease in Indonesian plantations of *Acacia mangium*. The unknown basidiomycete was isolated from rotten wood and is being tested as a biocontrol for root rot disease.

6. These isolates (denoted also by a double asterisk) were isolated by the author in Jogjakarta from the roots and stems of dying *Acacia mangium* seedlings (see Chapter 5). The identities of cultures S, T and U were unconfirmed but had been isolated from a black stem lesion typical of foot rot caused by *Fusarium* spp.

7. The *Phytophthora* culture was isolated by the author for the case study detailed in Chapter 6.

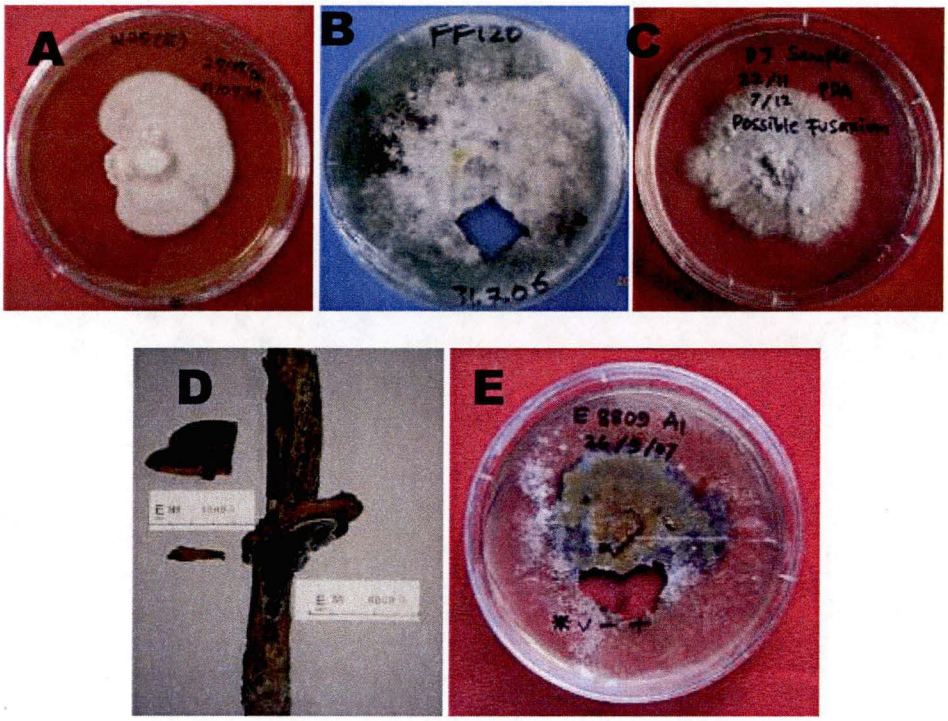


Figure 3.1 Examples of fungal pure cultures used to test FTA cards; (A) W25; (B) FF120; (C) from a black lesion on stem of *Acacia mangium* seedling (D) Culture (to the right) and the basidiomycete (*Phellinus* sp.) sporocarp (E 8809) from which it was isolated.

Table 3.1 Fungal cultures tested with FTA cards (age of culture is in weeks)

Code	Species/tag name	Fungal class	Age
FF-24[1]	White paint, *Aleurodiscus*?	*Basidiomycota*	18
FF-59[1]	*	? *Basidiomycota*	6
FF-76[1]	White polypore	*Basidiomycota*	16
FF-120[1]	White cords	? *Basidiomycota*	3
FF-144[1]	Tiny, cottony rods	? *Basidiomycota*	15
7 T 170 A2[5]	*Ganoderma.* aff. *australe*	*Basidiomycota*	17
8 T 169 A2[5]	*Ganoderma mastoporum*	*Basidiomycota*	17
8 T 201 A1[5]	*Ganoderma philippii*	*Basidiomycota*	13
Co 227[5]	*Unknown basidiomycete*	*Basidiomycota*	10
E 8809 A1[5]	*Phellinus* sp.	*Basidiomycota*	19
E 8812 A1[5]	*Ganoderma* sp.	*Basidiomycota*	19
E 8822 C1[5]	*Unknown basidiomycete*	*Basidiomycota*	19

Table 3.1 (Continued) Fungal cultures tested with FTA cards (age of culture is in weeks)

Code	Species/tag name	Fungal class	Age
E 8823 A2[5]	*Ganoderma* sp.	*Basidiomycota*	19
E 8828 C1[5]	*Formitopsis feei*	*Basidiomycota*	13
E 8831 A1[5]	*Ganoderma philippii*	*Basidiomycota*	17
E 8831 B1[5]	*Gymnopilus* sp.	*Basidiomycota*	17
E 8832 A1[5]	*Ganoderma philippii*	*Basidiomycota*	17
E 8832 B1[5]	*Ganoderma philpipii*	*Basidiomycota*	18
E 8842 C1[5]	*Ganoderma philippii*	*Basidiomycota*	17
E 8851 A1[5]	*Ganoderma* aff. *australe*	*Basidiomycota*	17
E 8852 B1[5]	*Amauroderma rugosum*	*Basidiomycota*	20
E 8861 C2[5]	*Ganoderma* sp.	*Basidiomycota*	19
FB 1 A2[5]	*Phlebia* sp.	*Basidiomycota*	13
FB 16 B1[5]	*Ganoderma philippii*	*Basidiomycota*	17
FB 17 A1[5]	*Ganoderma subresinosum*	*Basidiomycota*	17
FB 20 A2[5]	*Pycnoporus* sp.	*Basidiomycota*	16
FB 4 A1[5]	*Ganoderma philippii*	*Basidiomycota*	17
FB1 B2? [5]	*Antrodia* sp.	*Basidiomycota*	19
T 19 A1[5]	*Trametes* sp.	*Basidiomycota*	17
T 57 A1[5]	*Fomes* sp.	*Basidiomycota*	19
T 42 B1[5]	*Fomes* sp.	*Basidiomycota*	19
T 72 B1[5]	*Amauroderma rugosum*	*Basidiomycota*	13
T 75 A2[5]	*Fomes* sp.	*Basidiomycota*	17
2A-1[6]	**	? *Ascomycota*	7
2A-2[6]	**	? *Ascomycota*	7
2A-3[6]	**	? *Ascomycota*	7
2B-1[6]	**	? *Ascomycota*	7
2B-2[6]	**	? *Ascomycota*	7
2B-3[6]	**	? *Ascomycota*	7
2B-4[6]	**	? *Ascomycota*	7
P [6]	**	? *Ascomycota*	7
Q[6]	**	? *Ascomycota*	7
R[6]	**	? *Ascomycota*	7
S[6]	*possible Fusarium* sp.	*Ascomycota*	7
T[6]	*possible Fusarium* sp.	*Ascomycota*	7
U[6]	*possible Fusarium* sp.	*Ascomycota*	7
Isolate A[7]	*Phytophthora* sp.	*Oomycota*	2

Sporocarps (Figure 3.2); these basidiomycete sporocarps were collected as part of a study into the mycorrhizal biodiversity of *E. delegatensis* forest in the highlands of northwest and northeast Tasmania (Table 3.2), although not all of the sporocarps collected were those of mycorrhizal fungi. The majority were identified to species or genus level but not all of the sporocarps could be identified (those with an asterisk). These sporocarps have been stored as herbarium material.

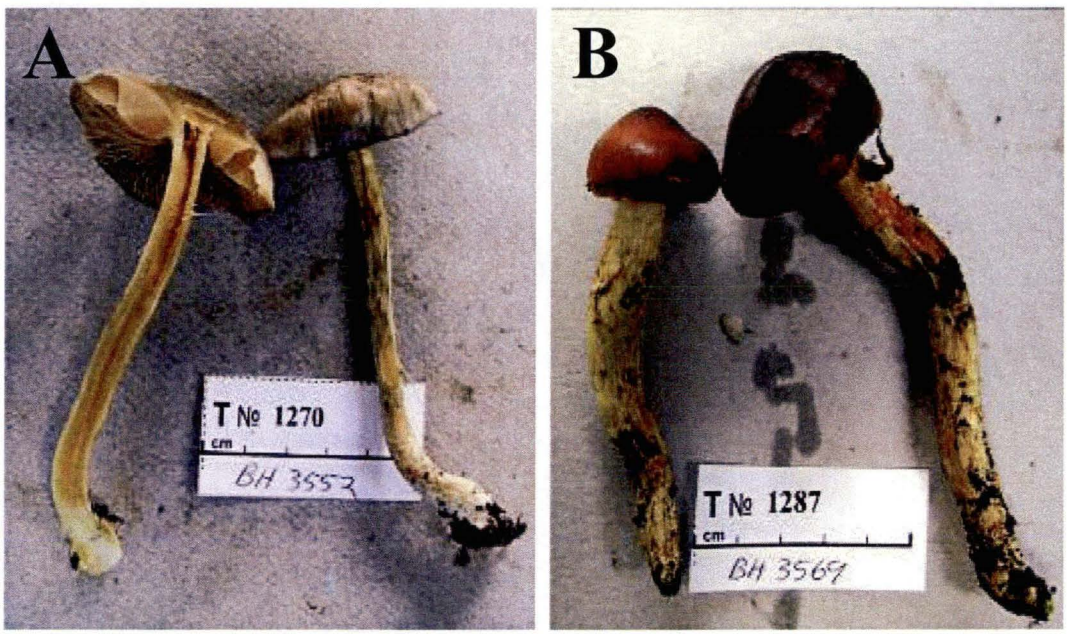


Figure 3.2 Sporocarps of mycorrhizal fungi (examples only) collected from northern Tasmania. (A) *Cortinarius* sp. (B) *Dermocybe* sp.

Table 3.2 Basidiomycete sporocarp material from Northern Tasmania

Code	Species	Code	Species
NE01	*	T 1259	*Hydnum umbilicatum*
NE02	*	T 1260	*Basidiomycete* sp. A
NE03	*	T 1261	*Cortinarius* sp. D
NE04	*	T 1262	*Lactarius* sp. B
T 1245	*	T 1263	*Laccaria* sp. E
T 1246	*Lactarius eucalypti*	T 1264	*Russula* sp. B
T 1247	*Lactarius eucalypti*	T 1265	*Cortinariaceae* sp. B

Table 3.2 (Continued) Basidiomycete sporocarp material from Northern Tasmania

Code	Species	Code	Species
T 1248	*Laccaria* sp. B	T 1266	*Lactarius eucalypti*
T 1249	*	T 1267	*
T 1250	*Lactarius eucalypti*	T 1268	*
T 1251	*Lactarius* sp. B	T 1269	*Boletaceae* sp. C
T 1252	*Descomyces* sp. A	T 1270	*Cortinarius* sp. E
T 1253	*	T 1271	*
T 1254	*	T 1272	*Laccaria* sp. E
T 1255	*Lycoperdon* sp. A	T 1273	*Cortinarius* sp. F
T 1256	*Laccaria* sp. C	T 1275	*Cortinarius* aff. *schlerophyllarum*
T 1257	*	T 1276	*Laccaria* sp. D
T 1258	*	T 1277	*Cortinarius* sp. C
T 1278	*Lactarius eucalypti*	T 1301	*Russula sp. A*
T 1279	*Thaxterogaster sp. A*	T 1358	*Galerina sp.*
T 1280	*Thaxterogaster sp.*	T 1359	*Mycena spp.*
T 1281	*Laccaria sp. A*	T 1360	*Hygrocybe astatogala*
T 1282	*Lactarius eucalypti*	T 1361	*Mycena subgalericulata*
T 1284	*Cortinarius sp. A*	T 1362	*Tremella fuciformis*
T 1286	*	T 1363	*Gymnopilus feruginosus*
T 1287	*Dermocybe sp. A*	T 1364	*Panellus longinquus*
T 1289	*Cortinarius sp. B*	T 1365	*Laccaria spp.*
T 1290	*	T 1366	*Ryvardenia campyla*
T 1291	*Cortinariaceae sp. A*	T 1367	*Cortinarius spp.*
T 1292	*	T 1368	*Clavariaceae*
T 1293	*Boletaceae sp. A*	T 1369	*Collybia eucalyptorum*
T 1295	*Lactarius sp. A*	T 1372	*
T 1297	*Boletaceae sp. B*	T 1373	*
T 1298	*Thaxterogaster sp. B*	T 1374	*
T 1299	*Lactarius eucalypti*	T 1375	*
T 1300	*Inocybe sp. A*	T 1376	*

Spore-prints (Figure 3.3); these were made from sporocarps of gilled basidiomycetes collected around the Thomas Crawford Trail on the University of Tasmania and from mushroom bought at the supermarket (*Agaricus*

bisporus; Table 3.3). Spore-print samples SP5 and SP6 (Table 3.3) were obtained from sporocarps that could not be formally identified.

Table 3.3 Spore-print material

Sample number	Species
SP1	*Cortinarius* sp.
SP2	*Cortinarius* sp.
SP3	*Cortinarius* sp.
SP4	*Agaricus bisporus*
SP5	Unidentified
SP6	Unidentified
SP7	*Cortinarius* sp.
SP8	*Cortinarius* sp.
SP9	*Psathyrella* sp.
SP10	*Psathyrella* sp.

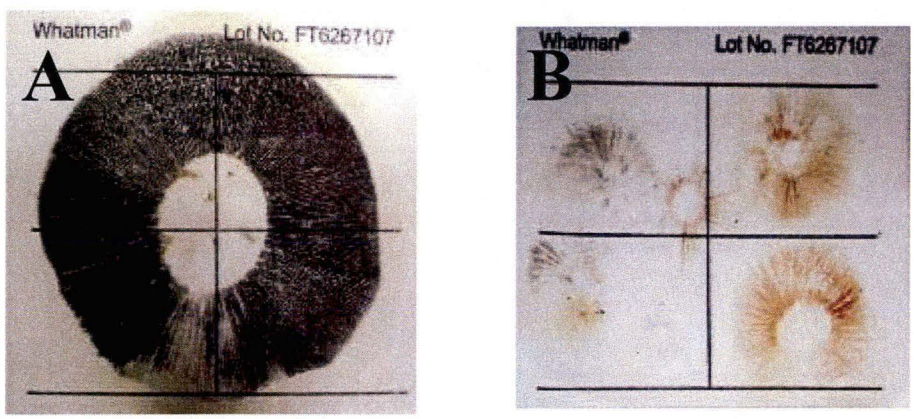


Figure 3.3 (A) Spore-print of *Agaricus bisporus* and (B) Spore-prints of *Cortinarius* sp.

Rust urediniospores (Figure 3.4 and 3.5) *and teliospores*; these were obtained from common garden plants infected with fungal pathogens that cause rust diseases i.e. geranium (*Pelargonium* sp.), blackberry (*Rubus* sp.), rose (*Rosa* sp.) and grass (*Arrenathereum elatium* var. *bulbosum*). Rust samples were not kept as herbarium specimens as they are common in gardens around Hobart

and therefore were considered as being readily available for reference if so required.

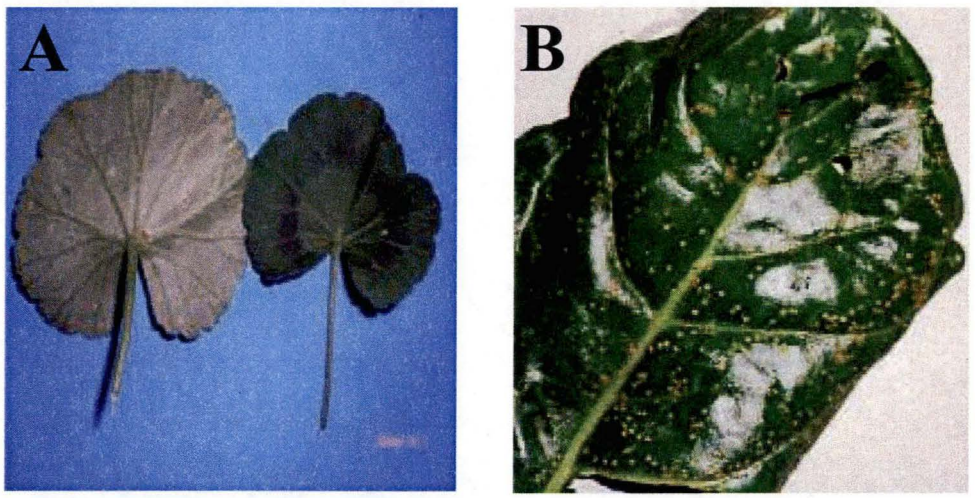


Figure 3.4 (A) Geranium leaf (*Pelargonium* sp.) and (B) Silverbeet leaf (*Beta vulgaris*) with rust (brown spots)

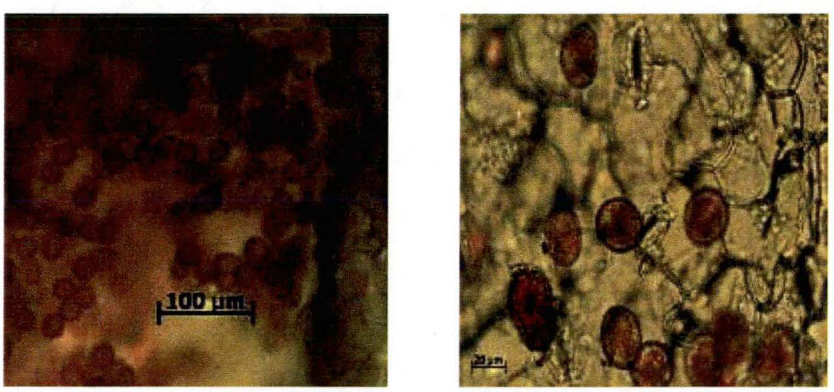


Figure 3.5 Silverbeet rust (*Uromyces betae*) urediniospores under microscope

Powdery mildew were also obtained from the leaves of two common garden plants; rose (*Rosa* sp.), and brussel sprouts (*Brassica oleracea*) (Table 3.4, Figure. 3.6). These mildew samples were collected and stored as herbarium specimens. Cleistothecia were not present on the specimens, only conidia.

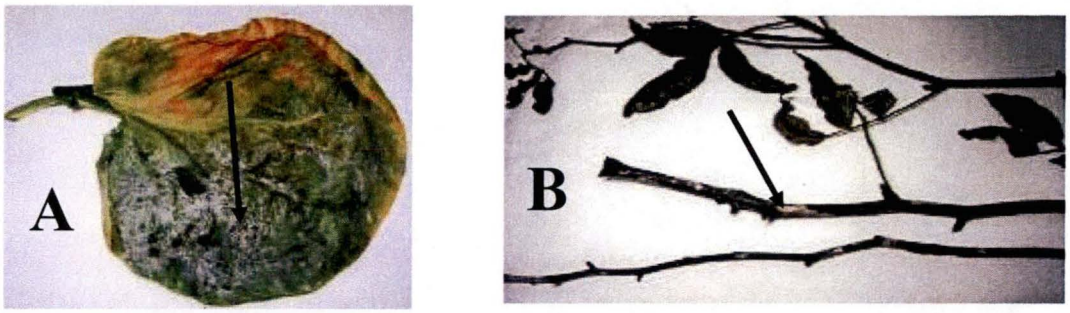


Figure 3.6 Mildew samples from (A) brussel sprout leaf (*Brassica oleracea*) and (B) rose leaves and stems (*Rosa* sp).

Table 3.4 Rust and mildew material

Sample number	Species
Ge1	Urediniospores on *Pelargonium* sp.
Ge2	Urediniospores on *Pelargonium* sp.
Bb1	Teliospores of *Phragmidium violaceum* from *Rubus* sp.
Bb2	Teliospores of *Phragmidium violaceum* from *Rubus* sp
Bb3	Teliospores of *Phragmidium violaceum* from *Rubus* sp
Bb4	Teliospores of *Phragmidium violaceum* from *Rubus* sp
Ro1	Urediniospores on *Rosa* sp.
Ro2	Urediniospores on *Rosa* sp.
Gr1	Urediniospores on *Arrenathereum elatium var. bulbosum*
Rm2	Conidia of powdery mildew on *Rosa* sp.
Bs1	Conidia of powdery mildew on *Brassica oleracea*

Methods

Pure fungal cultures were scraped and the material squashed onto the card as explained in Chapter 2. The softest part of a sporocarp was excised for squashing on the card. Spore-prints were obtained by placing the sporocarps, gill side down, onto the FTA card overnight and then were squashed. The spores from the fungi left a mark around the card (Figure 3.3). Spores from mildew and rust samples were scraped onto the card and then squashed.

After preparing all the FTA cards, the sample preparation and PCR amplification followed the methodology as described in chapter 2 with the use of the following primers for PCRs;

- a primer pair targetting the rDNA ITS included the fungal-specific primer ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3' (Gardes and Bruns 1993) and the universal primer ITS4 (5'- TCCTCCGCTTATTGATATGC-3', (White *et al.* 1990) were used for all culture samples;
- primers targetting the elongation factor gene; EF1 (ATGGGTAAGGARGACAAGAC) and EF2 (GARGTACCAGTSATCATGTT) (O'Donnell *et al.* 1998) and the Beta-tubulin gene; Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC) (Glass and Donaldson 1995) were used in PCR for cultures suspected to be *Fusarium* spp (Table 3.1; samples isolated from *Acacia mangium* seedlings i.e. 2A-1, 2A-2, 2A-3, 2B-1, 2B-2, 2B-3, 2B-4, P, Q, R, S, T, and U);
- Species-specific PCR for *Ganoderma philippii* used primers Gphil2F/Gphil6R and Gphil3F/Gphil4R (Glen, unpublished) for samples marked with superscript 5 (Table 3.1). The thermocycler programme for these primers was: 94°C for 3 minutes, then 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds followed by 72°C for 7 minutes and a 14°C hold. Triplicate samples were subjected to PCR.

RESULTS

The main results are summarised and presented in this section, with full results of all PCR reactions presented in Appendices.

PCR amplification of rDNA ITS from fungal cultures

DNA capture of cultures onto FTA card was successful for 62 of the samples taken from cultures as demonstrated by a positive PCR in at least one of three replicates using the fungal-specific primer combination ITS1-F/ITS4 (Appendix 3.1, Figures. 3.7 and 3.8). PCR amplification was unsuccessful for all three replicate PCRs for 20 samples. The samples that did not amplify (Appendix 3.1) included those from two out of three *Cyclaneusma* cultures, Tasmanian basidiomycete culture W163 (*Chondrostereum purpureum*) and three of the basidiomycete cultures of wood rotting fungi tested in Indonesia (T72B1, T75A2, and Co227). All 14 samples from cultures isolated from acacia nursery seedlings did not amplify in PCR. The details of results for all replicates can be seen in Appendix 3.1.

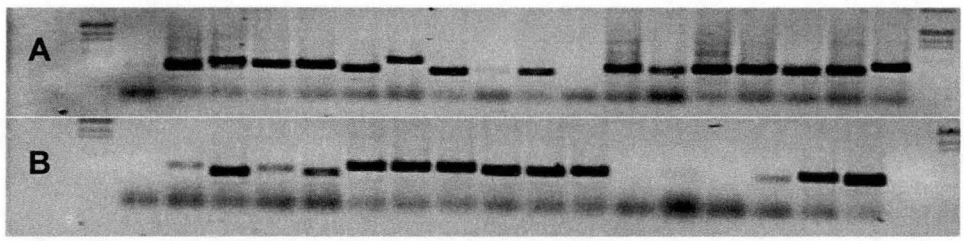


Figure 3.7 PCR amplification of rDNA ITS from fungal cultures applied to FTA cards using primers ITS1-F/ITS4.A. Lanes contain: 1 and 20, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, W190; 3, FF188; 4, FF59; 5, W25(iii); 6, *Phytophthora*; 7, T1394; 8, *Armillaria* Cas; 9, *Mycosphaerella* 80/1; 10, *Cyclaneusma* SN 815; 11, *Mycosphaerella* St. Marys; 12, *Cyclaneusma*; 13, FF214; 14, FF24; 15, FF120; 16, W225; 17, W276; 18, FF144; 19, positive control (FF269 from glassmilk DNA extraction method). B. Lanes contain: 1 and 20, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, W163; 3, W234; 4, M-U01; 5, FF76; 6, W344; 7, T1400B; 8, T1400A; 9, T1399; 10, M-U02; 11, FF264; 12, FF265; 13, IPC(32); 14, IPC10; 15, IPC20/1; 16, FF266; 17, FF268; 18, FF269; 20, negative control (water).

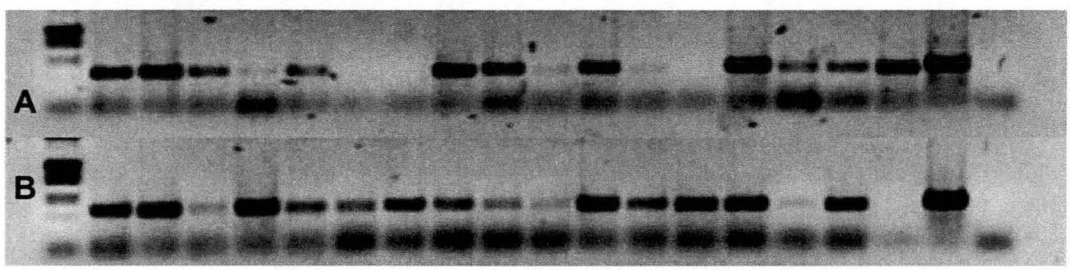


Figure 3.8 PCR amplification of the rDNA ITS from fungal cultures applied to FTA cards using primers ITS1-F/ITS4. A. Lanes contain: 1, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, E8831A1; 3, 7T170A2; 4, E8861C2; 5, 6T172A2; 6, T42B1; 7, E8809A1; 8, E 8852B1; 9, 8T201A1; 10, E8828C1; 11, E8832A1; 12, E8822C1; 13, E8832B1; 14, T72B1; 15, E8831B1; 16, 8T169A2; 17, FB4A1; 18, FB20A2; 19, positive control (FF 269 from glassmilk DNA extraction method); 20, negative control (water). B. Lanes contain: 1, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, 5T168A2; 3, 12T175B1; 4, FB1B2; 5, 5T160A2-1; 6, T19A1; 7, E8823A2; 8, E8851A1; 9, FB16B1; 10, FB17A1; 11, 6T200A2-1; 12, E8812A1; 13, FB1A2; 14, T57A1; 15, 10BT205; 16, Co227; 17, E8842C1; 18, T75A2, 19, positive control (FF269 from glassmilk DNA extraction method); 20, negative control (water).

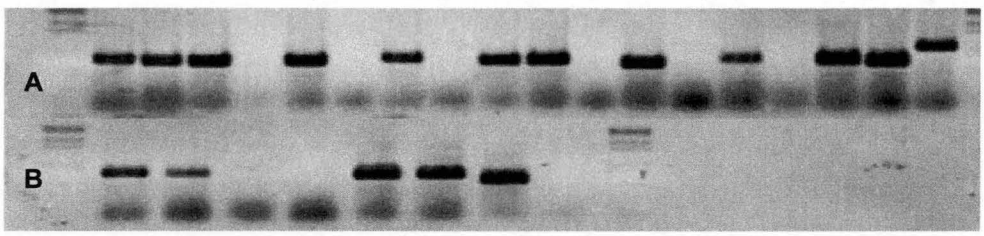


Figure 3.9 PCR amplification of the rDNA ITS from sporocarps applied to FTA cards using primers ITS1-F/ITS4. A. Lanes contain: 1 and 20, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, T1298; 3, T1297; 4, T1295; 5, T1301; 6, T1373; 7, T1374; 8, T1375; 9, T1376; 10, T1287; 11, T1292; 12, T1293; 13, T1290; 14, T1289; 15, T1277; 16, T1278; 17, T1281; 18, T1282; 19 ,T1284. B. Lanes contain: 1 and 10, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, T1279; 3, T1280; 4, T1300; 5, T1299; 6, T1291; 7, T1286; 8, positive control (FF 269 from glassmilk DNA extraction method); 9, negative control (water).

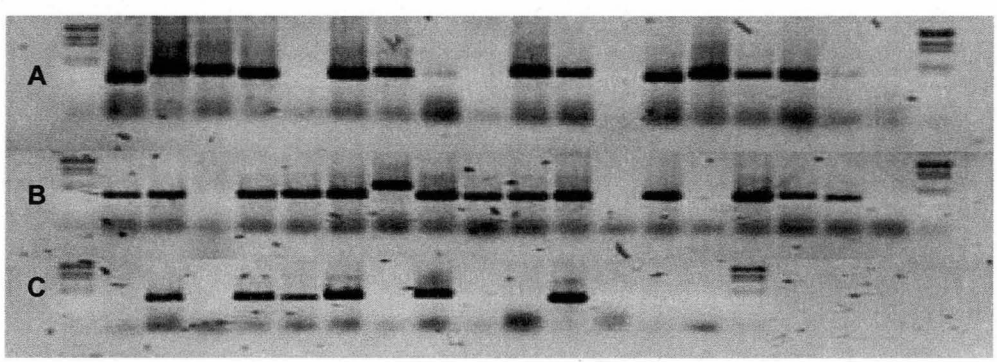


Figure 3.10 PCR amplification of the rDNA ITS from sporocarps applied to FTA cards, using primers ITS1-F/ITS4. A. Lanes contain: 1 and 20, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, T1362; 3, T1363; 4, T1354; 5, T1365; 6, T1358; 7, T1359; 8, T1360; 9, T1361; 10, T1366; 11, T1367; 12, T1368; 13, T1369; 14, T1257; 15, T1262; 16, T1264; 16, T1263; 17, T1265; 18, T1266. B. Lanes contain 1 and 20, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, T1267; 3, T1268; 4, T1269; 5, T1270; 6, T1272; 7, T1273; 8, T1254; 9, T1252; 10, T1246; 11, T1245; 12, T1259; 13, T1261; 14, T1260; 15, T1258; 16, T1251; 17, T1250; 18, T1255; 19, T1253. C. Lanes contain: 1 and 16, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, T1247; 3, T1249; 4, T1248; 5, T1256; 6, T1271; 7, T1276; 8, T1275; 9, T1372; 10, NE01; 11, NE02; 12, NE03; 13, NE04; 15 negative control (water).

PCR amplification of DNA from sporocarps

DNA capture of basidiomycete sporocarps onto FTA cards was successful for 65 out of 72 samples, as demonstrated by amplification in at least one of three replicate PCRs using fungal-specific primers, ITS1-F/ITS4 (Figures 3.9 and 3.10, Appendix 3.2). PCR was unsuccessful in all three replicates for the following 6 samples; T1253, T1258, T1268, T1271, T1372, T1374, and T1376. The results for all replicates for each sample can be seen in Appendix 3.2.

PCR amplification of DNA from basidiospores (spore-print)

DNA was successfully captured and amplified from basidiospores, with only one sample negative in all three replicates (Figure 3.11, appendix 3.3).

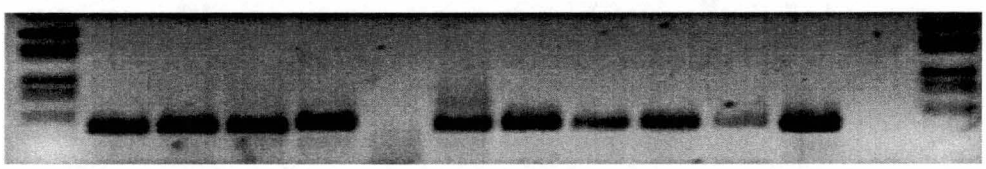


Figure 3.11 PCR amplification of the rDNA ITS from spore-prints applied to FTA cards using primers ITS1-F/ITS4. Lanes contain: 1 and 14, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, SP1; 3, SP2; 4, SP3; 5, SP4; 6, SP5; 7, SP6; 8, SP7; 9, SP8; 10, SP9; 11, SP10; 12, Positive control (FF269 from glassmilk DNA extraction method); 13, negative control (water).

PCR amplification of DNA from rust urediniospores and mildew conidia

PCR amplification was successful with only one of nine urediniospore samples (the *Arrenathereum elatium* var. *bulbosum* (grass) rust urediniospore sample, Table 3.5). Positive PCR amplification results were obtained for the two samples of mildew conidia (Table 3.5) but for only one out three replicates for each sample.

Table 3.5 PCR amplification using ITS1-F/ITS4 primers of DNA captured from rust urediniospores and mildew conidia

Sample code	Species	PCR amplification using primers ITS1-F/ITS4[1]		
		1	2	3
Ge1	Urediniospores on *Pelargonium* sp.	N	N	N
Ge2	Urediniospores on *Pelargonium* sp.	N	N	N
Bb1	Teliospores of *Phragmidium violaceum* from *Rubus* sp.	N	N	N
Bb2	Teliospores of *Phragmidium violaceum* from *Rubus* sp.	N	N	N
Bb3	Teliospores of *Phragmidium violaceum* from *Rubus* sp.	N	N	N
Bb4	Teliospores of *Phragmidium violaceum* from *Rubus* sp.	N	N	N
Ro1	Urediniospores on *Rosa* sp.	N	N	N
Ro2	Urediniospores on *Rosa* sp.	N	N	N
Gr3	Urediniospores on *Arrenathereum elatium* var. *bulbosum*	**P**	**P**	N
Rm2	Conidia of powdery mildew on *Rosa* sp.	N	N	**P**
Bs1	Conidia of powdery mildew on *Brassica oleracea*	N	N	**P**

[1]P indicates amplification of a fragment of approximately 600 bp; N indicates no amplification.

Age of culture and success in PCR amplification

When the success of PCR amplification was examined in relation to the age of the culture (Table 3.6) there did not seem to be a strong correlation of successful amplification and age of culture, except in the case of very old cultures (approximately 10 months). For cultures older than this, PCR amplification was consistently negative.

Table 3.6 Age of culture (in weeks) and success of PCR amplification using ITS1-F/ITS4 primer.

Code	Species or tag name	Culture Age (weeks)	PCR amplification
Isolate A	*Phytophthora* sp.	2 weeks	**P**
W-225	*Postia dissecta*	2 weeks	**P**
FF-144	Tiny, cottony rods	2 weeks	**P**
M-U01	Unknown	3 weeks	**P**
M-U02	Unknown	6 weeks	**P**
T-1394	*Postia subcaesia*	7 weeks	**P**
W-344	*Panellus ligulatus*	7 weeks	**P**
T-1400	*Ganoderma aff. australe*	7 weeks	**P**
T-1399	*Postia subcaesia*	7 weeks	**P**

[1]P indicates amplification of a fragment of approximately 600 bp; N indicates no amplification.

Table 3.6 (Continued) Age of culture (in weeks) and success of PCR amplification using ITS1-F/ITS4 primers.

Code	Species or tag name	Culture Age (weeks)	PCR amplification
2A-1	Unknown	7 weeks	N
2A-2	Unknown	7 weeks	N
2A-3	Unknown	7 weeks	N
2B-1	Unknown	7 weeks	N
2B-2	Unknown	7 weeks	N
2B-3	Unknown	7 weeks	N
2B-4	Unknown	7 weeks	N
P	Unknown	7 weeks	N
Q	Unknown	7 weeks	N
R	Unknown	7 weeks	N
S	Unknown	7 weeks	N
T	Unknown	7 weeks	N
U	Unknown	7 weeks	N
V	Unknown	7 weeks	N
FF-24	White paint, *Aleurodiscus*?	8 weeks	P
Co 227	Unknown basidiomycete	10 weeks	N
W-234	*Ryvardenia crustacea*	13 weeks	P
8 T 201 A1	*Ganoderma philippii*	13 weeks	P
E 8828 C1	*Formitopsis feei*	13 weeks	P
T 72 B1	*Amauroderma rugosum*	13 weeks	N
FB 1 A2	*Phlebia* sp.	13 weeks	P
5 T 160 A2-1	*Ganoderma* sp.	14 weeks	P
W-25 (iii)	*Gymnopilus tyallus*	15 weeks	P
Mycosphaerella 80/1	*Mycospherella* sp.	16 weeks	P
W-276	*Trametes hirsuta*	16 weeks	P
FF-264	Greyish snow	16 weeks	P
IPC (32)?	Unknown	16 weeks	P
FF-266	Unknown	16 weeks	P
F-269	Unknown	16 weeks	P
FB 20 A2	*Pycnoporus* sp.	16 weeks	P
FF-76	White polypore	17 weeks	P
E 8831 A1	*Ganoderma philippii*	17 weeks	P
7 T 170 A2	*Ganoderma.* aff. *australe*	17 weeks	P
6 T 172 A2	*Phanerochaete* sp.	17 weeks	N
T 42 B1	*Fomes* sp.	17 weeks	P
E 8832 A1	*Ganoderma philippii*	17 weeks	P
E 8831 B1	*Gymnopilus* sp.	17 weeks	P
8 T 169 A2	*Ganoderma mastoporum*	17 weeks	P
FB 4 A1	*Ganoderma philippii*	17 weeks	P
5 T 168 A2	*Ganoderma mastoporum*	17 weeks	P
12 T 175 B1	*Phlebia* sp.	17 weeks	P

[1]P indicates amplification of a fragment of approximately 600 bp; N indicates no amplification.

Table 3.6 (Continued) Age of culture (in weeks) and success of PCR amplification using ITS1-F/ITS4 primers.

Code	Species or tag name	Culture Age (weeks)	PCR amplification
T 19 A1	*Trametes* sp.	17 weeks	P
E 8851 A1	*Ganoderma* aff. *australe*	17 weeks	P
FB 16 B1	*Ganoderma philippii*	17 weeks	P
FB 17 A1	*Ganoderma subresinosum*	17 weeks	P
6 T 200 A2-1	*Phlebia* sp.	17 weeks	P
10 B T 205	*Ganoderma* sp.	17 weeks	P
E 8842 C1	*Ganoderma philipii*	17 weeks	P
T 75 A2	*Fomes* sp.	17 weeks	N
Mycosphaerella St. Mays	*Mycospherella sp.*	18 weeks	P
FF-214	Creamy flat fungi	18 weeks	P
FF-265	Pink snow	18 weeks	P
FF-268	Cream polypore	18 weeks	P
E 8832 B1	*Ganoderma philpipii*	18 weeks	N
FF-120	White cords	19 weeks	P
E 8861 C2	*Ganoderma* sp.	19 weeks	P
E 8809 A1	*Phellinus* sp.	19 weeks	P
E 8822 C1	*Unknown basidiomycete*	19 weeks	P
FB1 B2?	*Antrodia* sp.	19 weeks	P
E 8823 A2	*Ganoderma* sp.	19 weeks	P
E 8812 A1	*Ganoderma* sp.	19 weeks	P
T 57 A1	*Fomes* sp.	19 weeks	P
E 8852 B1	*Amauroderma rugosum*	20 weeks	N
IPC 10	Unknown	39 weeks	P
IPC 20/1	Unknown	42 weeks	P
Armillaria Cas	*Armillaria luteobubalina*	43 weeks	P
Cyclaneusma SN 815	*Cyclaneusma* sp.	43 weeks	N
Cyclaneusma	*Cyclaneusma* sp.	43 weeks	N
FF-188	*Hypoxylon crocopeplum*	91 weeks	N
W-163	*Chondrostereum purpureum*	99 weeks	N
W-190	*Crepidotus* sp.	107 weeks	N
FF-59	Unknown	107 weeks	N

[1]P indicates amplification of a fragment of approximately 600 bp; N indicates no amplification.

PCR amplification of DNA from cultures isolated from acacia nursery seedlings, using primers EF1/EF2 and Bt2a/Bt2b

PCR using primer pairs EF1/EF2 and Bt2a/Bt2b were done in triplicate (Appendix 3.4). Table 3.7 shows a compilation of PCR results using primers EF1/EF2 and Bt2a/Bt2b for all culture samples isolated from *A. mangium* seedlings in the nursery that were showing symptoms of root and foot rot. DNA from all but two cultures was amplified using Bt2a/Bt2b primers. All PCR products were sequenced.

Table 3.7 PCR amplification using beta-tubulin and elongation factor primers and DNA captured from cultures isolated from acacia nursery seedlings applied to FTA cards

Code	Description	PCR amplification[1]	
		Bt2a/Bt2b	EF1/EF2
2A-1	Black lesion (brown myc)	P	N
2A-2	Black lesion (brown myc)	P	N
2A-3	Black lesion (brown myc)	P	N
2B-1	Black lesion (dark green myc)	P	N
2B-2	Black lesion (dark green myc)	N	N
2B-3	Black lesion (dark green myc)	P	N
2B-4	Black lesion (dark green myc)	P	N
P	Black lesion	P	N
Q	DJ sample 1A	P	N
R	DJ sample 1B	P	N
S	Possible *Fusarium*	N	P
T	Possible *Fusarium*	P	P
U	Possible *Fusarium*	P	P

[1] P indicates PCR amplification of a fragment of approximately 550 bp (primers Bt2a/Bt2b) or 400 bp (primers EF1/EF2), N indicates no PCR amplification.

PCR amplification using *Ganoderma philippii* species-specific primers

Thirty-four Basidiomycete cultures (samples with superscript 5, see Table 3.1) were subjected to PCR using primers specific for *Ganoderma philippii* (Figures 3.12 and 3.13). Only *G. philippii* cultures were amplified with

Gphil2F/Gphil6R, but three other samples were also positive with Gphil3F/Gphil4R (Table 3.8). The complete results are shown in Appendix 3.5.

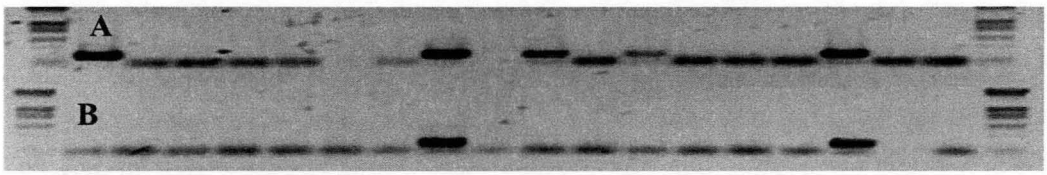


Figure 3.12 PCR amplification of the rDNA ITS from cultures applied to FTA cards using species-specific primers Gphil2F/Gphil6R. A. Lanes contain: 1 and 20, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, E8831A1; 3, 7T170A2; 4, E8861C2; 5, 6T172A2; 6, T42B1; 7, E8809A1; 8, E 8852B1; 9, 8T201A1; 10, E8828C1; 11, E8832A1; 12, E8822C1; 13, E8832B1; 14, T72B1; 15, E8831B1; 16, 8T169A2; 17, FB4A1; 18, FB20A2; 19 negative control (water). B. Lanes contain: 1 and 20, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, 5T168A2; 3, 12T175B1; 4, FB1B2; 5, 5T160A2-1; 6, T19A1; 7, E8823A2; 8, E8851A1; 9, B16B1; 10, FB17A1; 11, 6T200A2-1; 12, E8812A1; 13, FB1A2; 14, T57A1; 15, 10BT205; 16, C0227; 17, E8842C1; 18, T75A2; 19 negative control (water).

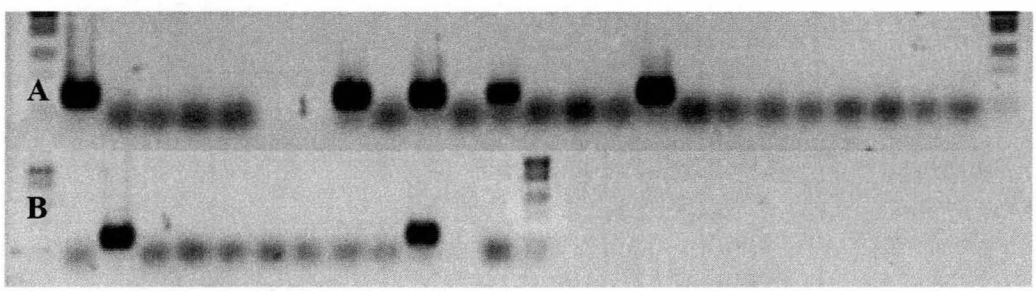


Figure 3.13 PCR amplification of the rDNA ITS from cultures applied to FTA cards, using species-specific primers Gphil 3F/Gphil4R. A. Lanes contain: 1 and 26, DNA size marker (lambda DNA cut with EcoR1 and HindIII); 2, E8831A1; 3, 7T170A2; 4, E8861C2; 5, 6T172A2; 6, T42B1; 7, E8809A1; 8, E8852B1; 9, 8T201A1; 10, E8828C1; 11, E8832A1; 12, E8822C1; 13, E8832B1; 14, T72B1; 15, E8831B1; 16, 8T169A2; 17, FB4A1; 18, FB20A2; 19, 5T168A2; 20, 12T175B1; 21, FB1B2; 22, 5T160A2-1; 23, T19A1; 24, E8823A2; 25, negative control (water). B. Lanes contain: 1 and 14, DNA size marker (lambda DNA cut with EcoR1 and HindIII);2, E8851A1; 3, B16B1; 4, FB17A1; 5, 6T200A2-1; 6, E8812A1; 7, FB1A2; 8, T57A1; 9, 10BT205; 10, C0227; 11, E8842C1; 12, T75A2; 13, negative control (water).

Table 3.8 PCR amplification using *G. philippii* species-specific primers of DNA captured from fungal cultures on FTA cards.

Code	Name	FTA Amplification[1]	
		Gphil2F/ Gphil6R	Gphil 3F/ Gphil4R
E 8831 A1	*Ganoderma philippii*	**P**	**P**
10 B T 205	*Ganoderma* sp.	N	N
12 T 175 B1	*Phlebia* sp.	N	**P**
5 T 160 A2-1	*Ganoderma* sp.	N	N
5 T 168 A2	*Ganoderma mastoporum*	N	N
6 T 172 A2	*Phanerochaete* sp.	N	N
6 T 200 A2-1	*Phlebia* sp.	N	N
7 T 170 A2	*Ganoderma.* aff. *australe*	N	N
8 T 169 A2	*Ganoderma mastoporum*	N	**P**
8 T 201 A1	*Ganoderma philippii*	**P**	**P**
Co 227	unknown basidiomycete	N	N
E 8809 A1	*Phellinus* sp.	N	N
E 8812 A1	*Ganoderma* sp.	N	N
E 8822 C1	unknown basidiomycete	N	N
E 8823 A2	*Ganoderma* sp.	N	N
E 8828 C1	*Formitopsis feei*	N	N
E 8831 B1	*Gymnopilus* sp.	N	N
E 8832 A1	*Ganoderma philippii*	**P**	**P**
E 8832 B1	*Ganoderma philippii*	**P**	**P**
E 8842 C1	*Ganoderma philippii*	**P**	**P**
E 8851 A1	*Ganoderma* aff. *australe*	N	N
E 8852 B1	*Amauroderma rugosum*	N	N
E 8861 C2	*Ganoderma* sp.	N	N
FB 1 A2	*Phlebia* sp.	N	N
FB 16 B1	*Ganoderma philippii*	**P**	**P**
FB 17 A1	*Ganoderma subresinosum*	N	N
FB 20 A2	*Pycnoporus* sp.	N	N
FB 4 A1	*Ganoderma philippii*	**P**	**P**
FB1 B2?	*Antrodia* sp.	N	N
T 19 A1	*Trametes* sp.	N	N
T 42 B1	*Fomes* sp.	N	N
T 57 A1	*Fomes* sp.	N	N
T 72 B1	*Amauroderma rugosum*	N	**P**
T 75 A2	*Fomes* sp.	N	N

[1] P indicates amplification of fragment of 450 bp for primers Gphil2F/Gphil6R and 230 bp for primers Gphil3F/Gphil4R, N indicates no PCR amplification

Sequencing of PCR products and identification based on sequence similarity

Public DNA databases (GenBank, EMBL and DDBJ) were searched using BLAST (Altschul *et al.* 1990) (Table 3.9). Samples in bold are those from which a DNA fragment was amplified that produced a sequence consistent with the morphological identification. BLAST search results are summarised in Appendix 3.6.

Table 3.9 Comparison of morphological identification and BLAST Search results

Code	Morphological identification	Primers	Identification based on BLAST results
SP1	Spore-print of *Cortinarius* sp.	ITS1-F/ITS4	*Cortinarius* sp.
SP4	Spore-prints of *Agaricus bisporus*	ITS1-F/ITS4	*Agaricus* sp.
SP9	Spore-prints of *Psathyrella* sp.	ITS1-F/ITS4	*Psathyrella* sp.
Gr1	Grass rust	ITS1-F/ITS4	*Eudarluca aff. caricis*
FF59	unknown	ITS1-F/ITS4	*Hypholoma fasciculare*
W25 (iii)	*Gymnopilus tyallus*	ITS1-F/ITS4	*Ganoderma* sp.
Cu7	*Armillaria* Cas	ITS1-F/ITS4	*Armillaria luteobubalina*
Cu8	*Mycosphaerella* sp.	ITS1-F/ITS4	*Mycosphaerella cryptica*
Cu10	*Mycosphaerella* sp.	ITS1-F/ITS4	*Mycosphaerella nubilosa*
FF214	Creamy flat fungi	ITS1-F/ITS4	*Pichia* sp.
FF 120	white cords fungi	ITS1-F/ITS4	*Verticillium* sp.
W225	*Postia dissecta*	ITS1-F/ITS4	*Verticillium* sp.
W276	*Trametes hirsuta*	ITS1-F/ITS4	*Trametes hirsuta*
FF144	tiny, cottony rods	ITS1-F/ITS4	*Hypholoma aff. fasciculare*
Cu21	Unknown	ITS1-F/ITS4	Xylariaceae sp.
FF76	white polypore	ITS1-F/ITS4	*Basidiomycota* sp.
W344	*Panellus ligulatus*	ITS1-F/ITS4	*Hypholoma* sp.
T1400	*Ganoderma australe*	ITS1-F/ITS4	*Ganoderma* sp.
T1399	*Postia pelliculosa*	ITS1-F/ITS4	*Postia* sp.
Isolate A	Possible Phytophthora	ITS1-F/ITS4	Zygomycete sp.
U	Possible Fusarium	EF	*Fusarium oxysporum*
2A-2	Possible Fusarium	Bt	*Phoma* sp.
2B-2	Possible Fusarium	Bt	*Phoma* sp.
P	Possible Fusarium	Bt	*Fusarium oxysporum*

Table 3.9 (Continued) Comparison of morphological identification and BLAST Search results

Code	Morphological identification	Primers	Identification based on BLAST results
S	Possible Fusarium	Bt	*Fusarium oxysporum*
U	Possible Fusarium	Bt	*Fusarium oxysporum*
T	Possible Fusarium	Bt	*Fusarium oxysporum*

DISCUSSION

PCR amplification of the rDNA ITS using primers ITS1-F/ITS4 from fungal material applied to FTA cards

It can be seen from the results that most of the fungal DNA from cultures and sporocarps can bind easily onto FTA card. In the case of cultures, PCR amplification using ITS1-F/ITS4 primers appeared marginally more successful with young cultures which were easier to squash; the mycelium still fresh and soft, with high moisture content. The cell walls of young cultures are also not thickened, are actively dividing mycelium and most likely to have a higher DNA content to promote better DNA capture. Older cultures such as W190 (*Crepidotus* sp.) age 107 weeks, FF188 (*Hypoxylon crocopeplum*) age 91 weeks, FF59 (age 107 weeks) and W163 (*Chondrostereum purpureum*) age 99 weeks were manually difficult to squash onto the card, possibly due to thickened cell walls, and PCR amplification was not successful.

Nearly all the rust urediniospores applied to FTA card gave negative PCR amplification results using ITS1-F/ITS4 primers and there was also low

success rate for mildew conidia. It is probable that the rust spores (urediniospores) with thicker and melanised cell walls were more resistant to adequate cell disruption, so that the DNA cannot be bound onto the FTA card. *Agaricus bisporus*, *Cortinarius*, *Psathyrella* basidiospores and mildew conidia may be easier to squash as they were not highly thickened or melanised and PCR amplification of the rDNA ITS using the same primers ITS1-F/ITS4 was positive with these spores. The method of grinding material with buffer and application of the buffer onto the card should be tried for more resistant spores. In addition fungal spores may require harsher treatments such as sonication (Kennedy *et al.* 2000; Thines *et al.* 2004; Nirmala *et al.* 2006).

The number of spores that were captured on the FTA cards by allowing spore-prints to form overnight was far greater than the number applied to the card by scraping spores off lesions. Poor amplification of mildew and rust spore samples squashed onto FTA cards might therefore be explained by the capture of a low amount of fungal DNA /or insufficient moisture from squashing a low number of spores to allow adequate penetration of the card.

There must be adequate penetration of the sample material to the back of the FTA card. A specimen leaves marks on the card when the material is squashed onto the card, and these marks must be visible on the back of the card to ensure that the DNA has been carried through the card. Most of cultures, sporocarps and spore-print samples left marks on the back of the card but this level of penetration was not observed with the rust and mildew spore samples. Often

the mark disappears as the FTA card dries and it is important to look at the back of the card, draw the mark in pencil, so samples can be taken from that area.

Does spore size influence the capture of DNA on FTA cards? Basidiospores of *Agaricus bisporus* are approximately 5-6 x 4-5 µm (Evered *et al.* 2000). Those of different *Cortinarius* species are fairly variable in size but the range is approximately 10-15 x 5-10 µm (Froslev *et al.* 2006). Basidiospores of the subgenus *Psathyrella* are approximately 10-12 x13-16 µm (Antonin and Urban 2008).

We did not identify the species of rust but the mean urediniospores size for several species of *Puccinia* is larger than that for basidiospores and reported as 25 x 20.6 um (Anikster *et al.* 2005). Teliospores of *Phragmidium violaceum* can be up to 75 x 40 µm (Laundon and Rainbow 1969). The conidia of fungal species which cause powdery mildew are even larger than rust spores e.g. tomato powdery mildew (*Erysiphe orontii*) varying between 22-45 x 12-20 µm (Whipps *et al.* 1998) The smaller spores making up the spores-prints of *Agaricus bisporus*, *Cortinarius* and *Psathyrella* species, when examined under the microscope, were physically trapped on the FTA card matrix. This raises an important issue in that the FTA card may have trapped spores rather than DNA. Spore DNA could have been released from the spores in the PCR amplification process. It would be inadvisable to transport FTA cards with spore material if

this could pose a biosecurity risk. Spore viability after application to cards and different storage periods should be stringently tested.

Other reasons that may contribute significantly to poor amplification of DNA captured from spores (or any other material) are primer mismatch and PCR inhibition. There could be many reasons for PCR inhibition such as the presence of inhibitory substances in thickened spore walls. Understanding the role of inhibitors in the substrate (plant or fungal) could be improved by conducting further tests incorporating an IAC (Glen *et al.* 2007). If PCR inhibition is shown to occur, it may be possible to develop additional steps to remove inhibitors from the FTA card before PCR, thus improving the success rate of PCR amplification. PCR inhibition can be reduced by adding a reagent such as BSA (bovine serum albumin) as was used in this study or increasing the amount of polymerase.

The dilution of the DNA sample sometimes improves PCR amplification because inhibitors are diluted as well as the DNA (O'Brien 2008). A dilution effect can be achieved with FTA card by using a smaller punch or increasing the volume of the PCR reaction. Other steps to overcome PCR inhibition or low target DNA could include the use of nested PCR or selection of primers to amplify a smaller fragment.

DNA captured from rust spores may not be efficiently amplified with the primers selected here (Langrell *et al.* 2008) and alternative primers could be

tested as discussed in the following section in respect to the PCR amplification of DNA bound to FTA cards from fungal cultures.

PCR amplification with elongation factor and beta-tubulin primers

The symptoms shown by the *Acacia mangium* seedlings in the nursery were characteristic of a foot rot disease often caused by species of *Fusarium* (i.e. black lesions on the stem 2-3 cm above soil level). The spores of certain cultures isolated from these black lesions were banana shaped spores typical of *Fusarium*.

Amplification of the ITS region using the primer pair ITS1-F/ITS4 was negative when the DNA of these cultures was captured by FTA cards. All samples suspected to be *Fusarium* were positive with primers targetting either the elongation factor or beta-tubulin regions despite the lower copy number of these gene regions.

The lack of amplification of the ITS region is therefore unlikely to be due to PCR inhibition and is more likely to be due to primer mismatch. Though the primer pair used here are considered to be universal fungal primers, failure of amplification in some fungal species may be caused by single nucleotide mismatches close to the 3' end of the primer region (Glen, Morag *et al.* 2001). Use of alternative primers, including substitution of ITS1-F with one of the universal primers, ITS1 or ITS5, would be an appropriate remedy when working with DNA from pure cultures, but was not attempted in this study.

Amplification of the ß-tubulin and elongation factor 1-α regions was successful and sequences of these regions are more informative for discriminating *Fusarium* spp. than the rDNA ITS (Yang *et al.* 2009)

Species-specific amplification using Ganoderma philipii primer sets

The two different pairs of *Ganoderma philippii* specific primers successfully amplified all *G. philippii* isolates applied to FTA cards. Several other species applied to FTA cards also gave positive results using the specific primers e.g. *G. aff. australe* (7T170A2), *Formitopsis feei* (E8828C1), *G. mastoporum* (8T169A2 and 5T168A2) and *Phlebia* sp. (12T175B1 and 6T200A2-1). However, when the specific primers were used with DNA that was extracted from those non *G. philippii* using the glassmilk extraction method PCR amplification was negative (Glen and Widyatmoko, unpublished).

The most likely explanation of unexpected positive results when the *G. philippii* specific primers are used is cross contamination when *G. philippii* cultures and cultures of other species are applied to the same card (one card has spaces for 4 different samples). The occurrence of such contamination could be checked by sequencing the unexpected or false positive PCR products to determine the source of the amplicon, a *G. philippii* sequence indicating cross-contamination. Therefore when doing a species-specific PCR avoid having four different samples on one card to circumvent cross contamination.

Identification of isolates using DNA sequences

DNA sequence success is related to the starting material squashed onto the FTA card. With a good starting material, e.g. a clean culture or sporocarp, and the correct matching of primers, fungal DNA was easily amplified and a legible DNA sequence obtained. When primers other than ITS1-F/ITS4 were used, DNA sequences from the young and clean cultures isolated from black lesions on root collars of *Acacia mangium* seedlings were identified as *Phoma* sp., *Cylindrocladium/Calonectria* sp. and *Fusarium oxysporum*. All these cultures belong to fungal pathogens capable of causing root and foot rot in *Acacia mangium* seedlings (see Chapter 5). Several cultures isolated from sporocarps or other materials were successfully identified by their sequences although they were not identified as expected and were contaminant e.g. ascomycete contaminants had been unknowingly isolated from Basidiomycete sporocarps and a putative *Phytophthora* sp. was shown to be a contaminant.

Good sequence results were obtained from spore-print samples, allowing identification of the fungal DNA as *Cortinarius* sp., *Agaricus bisporus*, and *Psathyrella* sp. The successful amplification of fungal DNA from spore-prints using FTA card indicates the potential for further applications such as environmental spore-trapping. A hyperparasite of rust was identified from the rust leaf sample (*Eudarluca* aff. *caricis*) but the rust fungal pathogen was not itself identified. The single sample of positive PCR amplification with grass rust spores gave very poor sequencing results. The illegible chromatogram may have been the result of a mixed template, possibly caused by co-amplification

of more than one fungal species. Poor amplification of rust fungi with primers ITS1-F/ITS4 may have contributed to this result and as previously discussed the choice of primers with higher specificity for rust fungi would possibly improve PCR amplification and sequence quality as well as the efficiency of DNA extraction.

Poor sequence quality of DNA amplified from host material is a problem not confined to FTA cards. The presence of multiple fungal DNA templates can be accommodated by cloning PCR products; however this is a time-consuming process. If the target species is known or suspected, the use of primers with narrower specificities may also assist in overcoming the problem of multiple templates. The use of species-specific primers may also avoid the need for DNA sequencing if primer specificity has been adequately tested.

CONCLUSION

Overall, DNA from fungal materials such as cultures, agaricoid sporocarps and their basidiospores can be reliably captured on FTA cards designed for plant DNA if sufficient material is available to be squashed and adequate penetration of the card is achieved. There is no requirement for complicated DNA extraction procedures or laboratory facilities making it easier for field sampling and also reducing any biosecurity risk associated with transport of viable cultures. Age of fungal cultures only explained unsuccessful amplification in respect to very old cultures. Material such as the more resistant urediniospores and teliospores may require additional procedures for efficient disruption and

release of DNA such as grinding of samples in buffer prior to FTA card application but primer mismatch is likely to offer a better explanation for lack of PCR amplification. The final application of the captured DNA should be considered and care must be taken to avoid cross-contamination of samples where species-specific primers will be used.

CHAPTER 4

Assessing FTA card for DNA capture from different types of plant material

INTRODUCTION

Fungi do not posses stems, roots or leaves, nor have they developed a vascular system. Fungal plant pathogens live by infecting living plants as parasites, often destroying otherwise healthy plants. Some fungal plant pathogens may also be able to obtain nutrients from dead organic matter and act as saprobes; other fungal pathogens are obligate parasites.

The fungal mycelium penetrates the host plants forming a mycelial network within the host plant which efficiently exploits it for nutrients. Different fungal pathogens are specialised to attack different parts of plants including, seed, roots, stems and leaves.

Identification of many forest diseases may be facilitated by DNA analyses (Hamelin *et al.* 1996; Hoff *et al.* 2004; Hunter *et al.* 2004; Maxwell *et al.* 2005). A rapid sampling and DNA extraction procedure would be especially useful in many investigations of forest diseases as many forest plantations are remote from laboratory facilities. FTA card has been designed for extracting plant DNA from leaf tissue and in the previous chapter was tested for extracting DNA from fungal tissue. It has not been tested to determine whether it can be used successfully to obtain fungal pathogen DNA directly from plant

material, especially from harder, woody material such as stems and roots. Some leaf types, e.g. eucalypt leaves or pine needles; may also present problems because of their tough, sclerophyllous nature or the presence of PCR inhibitors (Tibbits *et al.* 2006; Glen, M. *et al.* 2007; Schwelm *et al.* 2009).

This chapter examines the suitability of FTA card technology for capturing the DNA of fungal pathogens from different plant materials such as seed, root and leaves. Plant materials were tested that were symptomatic or asymptomatic. The FTA cards were used to obtain the DNA directly by squashing the infected plant material (leaves and root) directly onto the cards. Adaptations, including grinding material in buffer before spotting onto FTA card, were tested on harder material including seeds and roots. Knowledge of the limitations of FTA card technology will be very useful for collection of fungal pathogen DNA directly from infected plant material since it can speed up the identification steps by avoiding some laboratory procedures such as culturing and standard DNA extraction methods.

MATERIAL AND METHOD

Materials

The plant materials used in this research are seeds, leaves, bark and root (Table 4.1). Seeds obtained from Australian Tree Seed Centre were *Acacia mangium* seed, *Pinus radiata* seed and *Eucalyptus camaldulensis* seed (Figure 4.1). Leaf samples with mildew infections were obtained from common garden plants; Brussel sprout (*Brassica oleracea*) (Chapter 3: Figure 3.6 A), rose (*Rosa* sp.)

leaves (Chapter 3: Figure 3.6 B), bean (*Phaseolus vulgaris*), pear (*Pyrus communis*) (Figure 4.2), forget-me-not (*Myosotis arvensis*). Eucalypt leaves with spots characteristic of *Mycosphaerella* spp. (Figure. 4.3 A) and *Aulographina eucalypti* (Figure 4.3 B) infection were collected from native forest; and herbarium specimens of *Pinus radiata* needles infected with *Cyclaneusma* spp. were tested. Bark samples (Figure 4.4 A) were taken from a peach tree (*Prunus persica)* that had been killed by *Armillaria* root rot and the lesions were cut from the roots of *Eucalyptus nitens* trees (Figure 4.4 B) that had been inoculated with *Armillaria luteobubalina*. DNA extracted by the glassmilk method from the same specimens was also tested as a comparison. Several blades of the grass *Arrhenatherium elatius* var. *bulbosum* infected with undetermined rust were included, as was a leaf of *Beta vulgaris* (silverbeet) infected with *Uromyces betae.* DNA from cultures of *Mycosphaerella cryptica* and *Mycosphaerella nubilosa* were supplied by Dr Morag Glen for use as positive controls in species-specific nested PCRs for those species.

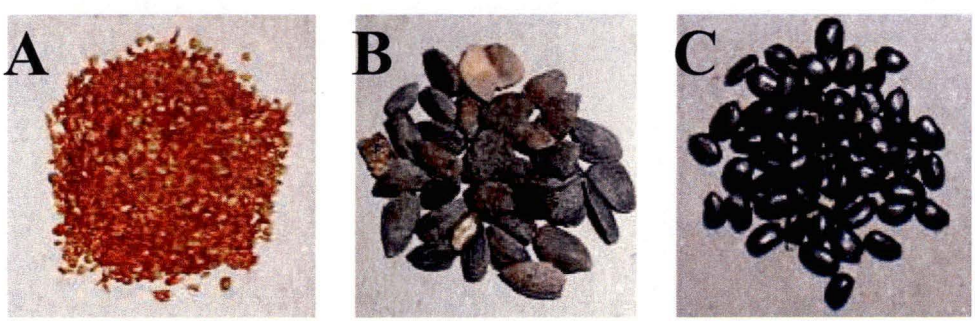


Figure 4.1 Seed sample pictures; A. *Eucalyptus camaldulensis* var. *camaldulensis* ; B. *Pinus radiata*; C. *Acacia mangium*

Table 4.1 DNA samples from plant material

Code	Samples	DNA Extraction Methods
Sd1	*Pinus radiata* seed	Seed ground in DNA extraction buffer[1]
Sd2	*Pinus radiata* seed	Sterile water eluate applied to FTA card
Sd3	*Pinus radiata* seed	Glassmilk DNA extraction
Sd4	*Eucalyptus camaldulensis* seed	Seed ground in DNA extraction buffer
Sd5	*Eucalyptus camaldulensis* seed	Sterile water eluate applied to FTA card
Sd6	*Eucalyptus camaldulensis* seed	Glassmilk DNA extraction
Sd7	*Acacia mangium* seed	Seed ground in DNA extraction buffer
Sd8	*Acacia mangium* seed	Sterile water eluate applied to FTA card
Sd9	*Acacia mangium* seed	Glassmilk DNA extraction
Mb1	*Phaseolus vulgaris* leaf with mildew	Leaf squash onto FTA card
Mb2	*Phaseolus vulgaris* leaf with mildew	Leaf squash onto FTA card
Mb3	*Phaseolus vulgaris* leaf with mildew	Leaf squash onto FTA card
Mb4	*Phaseolus vulgaris* leaf with mildew	Leaf squash onto FTA card
Mp1	*Pyrus communis* leaf with mildew	Leaf squash onto FTA card
Mp2	*Pyrus communis* leaf with mildew	Leaf squash onto FTA card
Mp3	*Pyrus communis* leaf with mildew	Leaf squash onto FTA card
Mp4	*Pyrus communis* leaf with mildew	Leaf squash onto FTA card
Rm3	*Rosa* sp. leaf with mildew	Leaf ground in DNA extraction buffer
C12	*Rosa* sp. leaf with mildew	Glassmilk DNA extraction
Rm4	*Rosa* sp. leaf with mildew	Leaf
Fm1	*Myosotis* sp. leaf with mildew	Leaf squash onto FTA card
Fm2	*Myosotis* sp. leaf with mildew	Leaf ground in DNA extraction buffer
C11	*Myosotis* sp. leaf with mildew	Glassmilk DNA extraction
Bs2	*Brassica oleraceae* leaf with mildew	Direct squash onto FTA card
Bs3	*Brassica oleraceae* leaf with mildew	Leaf ground in DNA extraction buffer
C14	*Brassica oleraceae* leaf with mildew	Glassmilk DNA extraction
Euc1	Eucalyptus leaf A (*Aulographina eucalypti* infection)	Leaf squash onto FTA card
Euc2	Eucalyptus leaf A	Leaf ground in DNA extraction buffer
C5	Eucalyptus leaf A	Glassmilk DNA extraction
Euc3	Eucalyptus leaf B (*Mycosphaerella* sp. infection)	Leaf squash onto FTA card
Euc4	Eucalyptus leaf B	Leaf ground in DNA extraction buffer
C6	Eucalyptus leaf B	Glassmilk DNA extraction
Euc5	Eucalyptus leaf C (*Mycosphaerella* sp. infection)	Leaf squash onto FTA card
Euc6	Eucalyptus leaf C	Leaf ground in DNA extraction buffer
C7	Eucalyptus leaf C	Glassmilk DNA extraction
Euc7	Eucalyptus leaf D (*Mycosphaerella* sp. infection)	Leaf squash onto FTA card

Table 4.1 (Continued) DNA samples from plant material

Code	Samples	DNA Extraction Methods
Euc8	Eucalyptus leaf D	Leaf ground in DNA extraction buffer
C8	Eucalyptus leaf D	Glassmilk DNA extraction
Euc9	Eucalyptus leaf E (*Mycosphaerella* sp. infection)	Leaf squash onto FTA card
Euc10	Eucalyptus leaf E	Leaf ground in DNA extraction buffer
C9	Eucalyptus leaf E	Glassmilk DNA extraction
Euc11	Eucalyptus leaf F (*Mycosphaerella* sp. infection)	Leaf squash onto FTA card
Euc12	Eucalyptus leaf F	Leaf ground in DNA extraction buffer
C10	Eucalyptus leaf F	Glassmilk DNA extraction
Pn1	Pine needle G2617	Dried needle ground in DNA extraction buffer
C15	Pine needle G2617	Glassmilk DNA extraction
Pn2	Pine needle G2682	Dried needle ground in DNA extraction buffer
C16	Pine needle G2682	Glassmilk DNA extraction
Pn3	Pine needle G2685	Dried needle ground in DNA extraction buffer
C13	Pine needle G2685	Glassmilk DNA extraction
Pn4	Pine needle G2683	Dried needle ground in DNA extraction buffer
C4	Pine needle G2683	Glassmilk DNA extraction
Pn5	Pine needle G2686	Dried needle ground in DNA extraction buffer
C2	Pine needle G2686	Glassmilk DNA extraction
Bt1	Mycelium from under bark of *Prunus persica*	Direct squash onto FTA card
Bt2	Mycelium from under bark of *Prunus P. persica*	Direct squash onto FTA card
Bt3	Mycelium from under bark of *Prunus persica*	Direct squash onto FTA card
Bt4	Mycelium from under bark of *Prunus persica*	Direct squash onto FTA card
Bt5	Mycelium from under bark of *Prunus persica*	Mycelium ground in DNA extraction buffer
C1	Mycelium from under bark of *P. persica*	Glassmilk DNA extraction
Lr1	*Eucalyptus nitens* root 13	Root ground in DNA extraction buffer
Lr2	*Eucalyptus nitens* root 23	Root ground in DNA extraction buffer
Lr3	*Eucalyptus nitens* root 25	Root ground in DNA extraction buffer
Lr4	*Eucalyptus nitens* root 27	Root ground in DNA extraction buffer
Gr1	Rust (undet.) on grass (*Arrhenatherum elatius* var. *bulbosum)*	Leaf squash onto FTA card
Gr2	Rust (undet.) on grass (*Arrhenatherum elatius* var. *bulbosum)*	Leaf squash onto FTA card
Gr4	Rust (undet.) on grass (*Arrhenatherum elatius* var. *bulbosum)*	Leaf squash onto FTA card
Gr5	Rust (undet.) on grass (*Arrhenatherum elatius* var. *bulbosum)*	Leaf squash onto FTA card
Sb1	Rust (*Uromyces betae*) on *Beta vulgaris*	Leaf squash onto FTA card

[1]Samples ground in DNA extraction buffer were then applied to the FTA card.

Figure 4.2 Mildewed *Pyrus communis* leaf with white powdery appearance (right arrow) and necrotic lesions (left arrow)

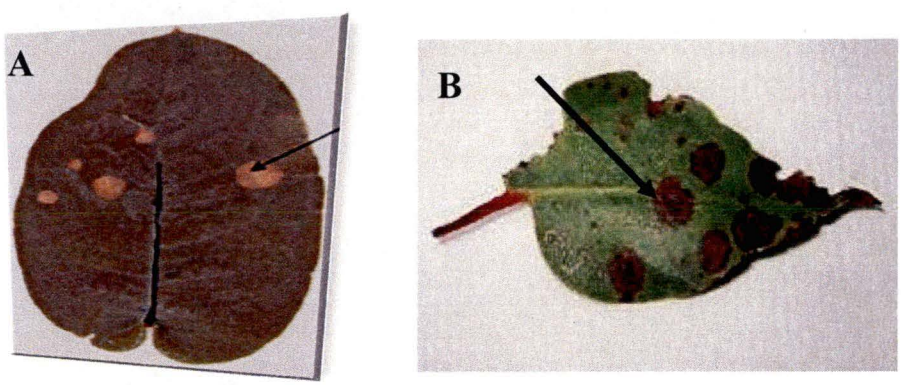


Figure 4.3 A. *Eucalyptus globulus* leaf with lesions caused by *Mycosphaerella* sp. B. *E. obliqua* leaf with lesions caused by *Aulographina eucalypti*

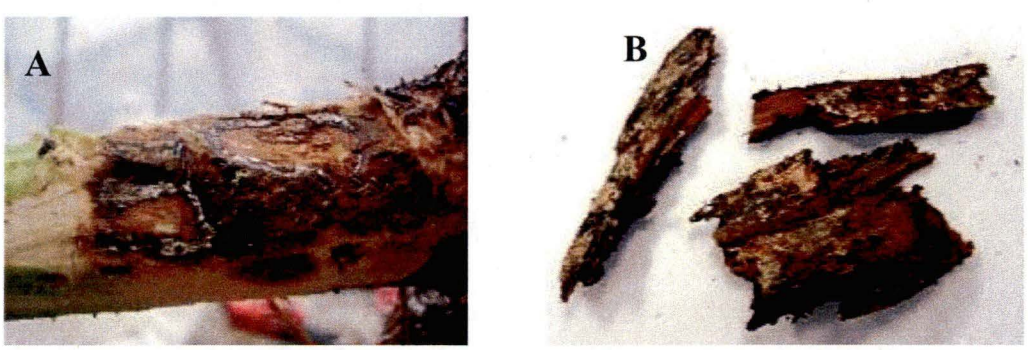


Figure 4.4 A. *Eucalyptus nitens* root 13 showing white mycelium in tissue; B. Bark of *Prunus persica*, showing white mycelium of *Armillaria luteobubalina*

Methods

Five seeds were ground in a mortar and pestle, mixed with 400 µL of extraction buffer in a 1.5 mL micro-centrifuge tube and vortexed. A 200 µL aliquot was removed using a pipette and spotted onto the FTA card while the rest was processed further using the glassmilk DNA extraction method (Chapter 2). The remainder of the un-ground seeds were washed with 1 mL of sterile water and shaken occasionally during incubation at room temperature for 5 minutes. All the liquid was removed to a new sterile tube. About 200 µL of each sample were spotted onto the FTA card. The card was allowed to dry at room temperature (approximately 30-40 minutes) and another 200 µL of the eluate was spotted again onto the same card. This was repeated about 5 times until the entire sample was applied to the card.

Infected leaves, and mycelium picked from the underside of bark samples or scraped from the surface of leaves, were directly squashed onto the cards. Duplicate samples and additional samples for which direct squashing was deemed to be impractical, e.g. root samples and dried pine needles, were ground and mixed with extraction buffer before application onto the FTA card. These samples were ground in a mortar and pestle, or a 1.5 mL microcentrifuge tube with a micro-pestle and 500 µL of DNA extraction buffer was added, mixed and incubated at room temperature for 1 hour. A 200 µL aliquot was removed and applied to FTA card. DNA was extracted from the rest of the ground sample using the glassmilk extraction method (Chapter 2). All the cards were dried at room temperature. PCR using ITS primers were performed in

triplicate as described in Chapter 2. Nested PCR using primers specific for *Mycosphaerella cryptica* and *M. nubilosa* were conducted according to Glen *et al.*, (2007)

RESULTS

Fungal DNA was successfully amplified from direct squashes onto FTA card of all of the different leaf types tested (Table 4.2). Pine needles were not tested with direct squashes, as dried herbarium material was used. Fungal DNA was successfully amplified from all 3 replicates in 11 of the 22 samples that were squashed directly onto FTA card, including most of the mildewed leaf samples (Figure 4.5), one of the *Eucalyptus* leaves and 2 of the 4 samples of *Armillaria* mycelium picked from under the bark of the *Prunus persica* (Appendix 4.1). In contrast, PCR using primers ITS1-F/ITS4 and ITS3/ITS4 did not amplify DNA from any of the seed samples (Appendix 4.1). Table 4.2 shows a proportion of positive PCRs using fungal specific (F) or general (G) primers. Triplicate PCRs using the fungal-specific primer pair ITS1-F/ITS4 and duplicates using the general primer pair ITS3/ITS4 were performed on each FTA card sample. Duplicate PCRs using ITS1-F/ITS4 were performed on DNA extracted by the glassmilk method. Details of all PCR results are provided in Appendix 4.1.

Table 4.2 Proportions of positive PCRs using fungal-specific (F) or general (G) primers.

Type of material	No. of samples	Direct squash onto FTA card		Ground in buffer then applied to FTA		Glass milk
		F	G	F	G	F
Eucalyptus leaves infected with *Mycosphaerella* spp. or *Aulographina eucalypti*	6	9/18	2/12	4/18	7/12	6/12
Pinus radiata needles infected with *Cyclaneusma* spp.	5	NT[1]	NT	7/15	4/10	4/10
Mildewed *Phaseolus vulgaris* leaves	4	7/12	7/8	NT	NT	NT
Mildewed *Pyrus communis* leaves	4	12/12	8/8	NT	NT	NT
Mildewed *Rosa* sp. leaf	1	3/3	2/2	1/3	0/2	0/2
Mildewed *Myosotis arvensis* leaf	1	2/3	1/2	2/3	1/2	0/2
Mildewed *Brassica oleracea* leaf	1	1/3	1/2	2/3	1/2	1/2
Root of *Eucalyptus nitens* infected with *Armillaria luteobubalina*	4	NT	NT	3/12	5/8	4/4
Armillaria mycelium from under bark of *Prunus persica*	4	8/12	3/8	0/3	1/2	0/2
Armillaria mycelium from under bark of *Prunus persica*	4	8/12	3/8	0/3	1/2	0/2
Grass (*Arrhenatherum elatius* var. *bulbosum*) infected with rust (undet.)	4	0/12	NT	NT	NT	NT
Beta vulgaris infected with *Uromyces betae*	1	0/3	NT	NT	NT	NT

[1]NT indicates the sample was not tested.

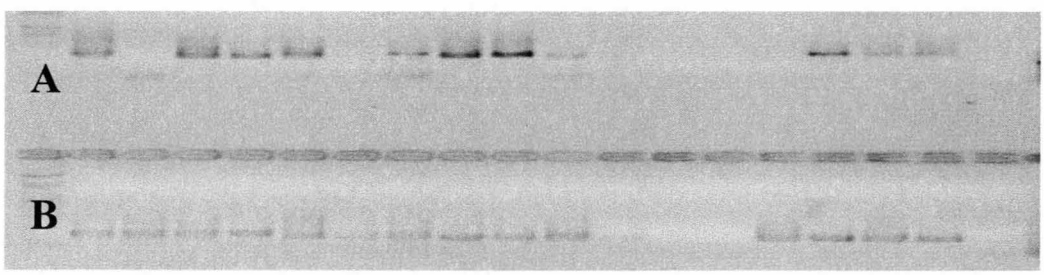


Figure 4.5 PCR amplifications from mildewed leaf samples using primer pairs ITS1-F/ITS4 (A) and ITS3/ITS4 (B). Lanes contain: 1, DNA size marker – lambda DNA cut with EcoRI and HindIII; 2, Mb1; 3, Mb2; 4, Mb3; 5, Mb4; 6, Mp1; 7, Mp2; 8, Mp3; 9, Mp4; 10, Rm1; 11, Rm2; 12, Rm3; 13, Rm4; 14, Fm1; 15, Fm2; 16, Bs1; 17, Bs2; 18, Bs3; 19, negative control (no template DNA). All samples were squashes of infected leaf material onto FTA card, except for Rm1 and Bs1, which were squashes of conidia scraped from leaf surfaces (see chapter 3 for details), and Rm2, Rm3, Fm2 and Bs3, which were ground in extraction buffer then applied to the FTA card.

Species-specific and nested PCR

Nested PCR using primers specific for *Mycosphaerella cryptica* and *M. nubilosa* was performed on the eucalyptus leaf samples. First-round PCR was with the primers ITS1-F/ITS4 as described in Chapter 2, and 5 µl of a 1/5 dilution was the template for second-round PCR with the primer combinations McrypF/McrypR and MnubF/MnubR (Glen *et al.* 2007). Only the positive control DNA (from an isolate of *M. nubilosa*) was amplified with MnubF/MnubR (Table 4.3), but several positive results were obtained from leaf samples with the McrypF/McrypR primer pair (Table 4.3, Figure 4.6).

Table 4.3 Species-specific nested PCR results.

Sample Code	Description	DNA Extraction Method	PCR Results[1]	
			McrypF/ McrypR	MnubF/ MnubR
Euc1	Eucalyptus leaf A	Leaf squash onto FTA card	N	N
Euc2	Eucalyptus leaf A	Leaf ground in DNA extraction buffer	N	N
C5	Eucalyptus leaf A	Glassmilk DNA extraction	N	N
Euc3	Eucalyptus leaf B	Leaf squash onto FTA card	N	N
Euc4	Eucalyptus leaf B	Leaf ground in DNA extraction buffer	N	N
C6	Eucalyptus leaf B	Glassmilk DNA extraction	N	N
Euc5	Eucalyptus leaf C	Leaf squash onto FTA card	N	N
Euc6	Eucalyptus leaf C	Leaf ground in DNA extraction buffer	**P**	N
C7	Eucalyptus leaf C	Glassmilk DNA extraction	N	N
Euc7	Eucalyptus leaf D	Leaf squash onto FTA card	**P**	N
Euc8	Eucalyptus leaf D	Leaf ground in DNA extraction buffer	N	N
C8	Eucalyptus leaf D	Glassmilk DNA extraction	N	N
Euc9	Eucalyptus leaf E	Leaf squash onto FTA card	**P**	N
Euc10	Eucalyptus leaf E	Leaf ground in DNA extraction buffer	**P**	N
C9	Eucalyptus leaf E	Glassmilk DNA extraction	N	N
Euc11	Eucalyptus leaf F	Leaf squash onto FTA card	N	N
Euc12	Eucalyptus leaf F	Leaf ground in DNA extraction buffer	**P**	N
C10	Eucalyptus leaf F	Glassmilk DNA extraction	**P**	N
T2	*Mycosphaerella cryptica*	DNA provided	**P**	NT
T5	*Mycosphaerella nubilosa*	DNA provided	NT	**P**

[1]P indicates the amplification of a fragment of approx. 330 bp for primers McrypF/McrypR and approx. 400 bp for primers MnubF/MnubR; N indicates no amplification; NT – not tested

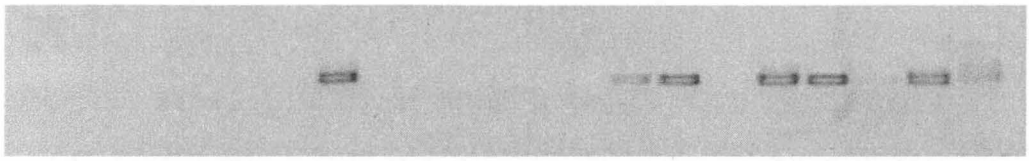


Figure 4.6 Nested PCR amplifications from Eucalyptus leaf samples applied to FTA card, using primers McrypF/McrypR. Lanes contain: 1, DNA marker, Lambda DNA cut with EcoRI and HindIII; 2, C5; 3, C6; 4, C7; 5, C8; 6, C9; 7, C10; 8Euc1; 9, Euc2; 10, Euc3; 11, Euc4; 12, Euc5; 13, Euc6; 14, Euc7; 15, Euc8; 16, Euc9; 17, Euc10; 18, Euc11; 19, Euc12; 20, positive control (DNA from an isolate of M. cryptica).

DISCUSSION

Fungal DNA from leaf, bark and root samples was successfully amplified using the primers ITS-1F/ITS4. Several negative results were probably due to the low amount of the target fungal pathogen DNA bound onto FTA card and PCR inhibition (these issues were discussed in Chapter 3 in relation to the capture of DNA from spores). Nested PCR was shown in this Chapter to be useful when there is low target DNA which is not visible from a single PCR. The use specific primers as in the nested PCRs for *Mycosphaerella* spp., there is a greater likelihood of amplifying a single fungal template that can be verified by sequencing without cloning. Some of the samples that did not give a product from ITS-1F/ITS4 did amplify with ITS3/ITS4 but the PCR product may be plant and/or fungal DNA (the selection of appropriate primers has been discussed in Chapter 3).

There was no fungal DNA amplified from the seed samples tested in this chapter. The seed samples were obtained from Australian Tree Seed Centre; so

it is possible that the seeds were already treated in order to avoid any development of fungal propagules and any fungal DNA that had been present was degraded. Testing of seed with known fungal contamination is recommended.

Although methodology to capture fungal pathogen DNA on FTA cards from infected plant tissue needs improvement, positive results show that fungal pathogen DNA could be obtained with relative ease directly from the plant material when working in the field by squashing the fungal infected plant material onto the FTA card. When FTA cards are used for the first time with a certain type of diseased tissue, the amount and precise point of tissue collection (such as at the edge of a lesion) can be determined by carrying out a series of squashes. This will assist with any problems involved in sampling e.g. a non-uniform distribution of fungal cells within the plant material.

CONCLUSION

Based on the results obtained from this research, it can be concluded that FTA card will successfully bind fungal pathogen DNA directly from plant material such as leaves, roots and bark. Further optimisation of sampling and application methods is required.

CHAPTER 5

Case Study: Forestry nursery diseases

INTRODUCTION

Healthy seedling stock is an essential prerequisite for the success of forest plantations. Nurseries in South-East Asia are gradually upgrading their facilities so that the physical structures, procedures to generate and protect seedlings are changing. These changes are being driven by a rapid increase in the size of plantation estates and the need to adopt new technologies, especially clonal forestry, for the maximisation of profit. Recently forest industrial plantation companies in Indonesia, Malaysia and Thailand have established large nurseries with clonal plants generated from tissue culture.

A forestry nursery is similar to an agricultural nursery and shares the need to give a plant the best start in life. However there are some significant differences in forestry, including the remote location of many of the SE Asia nurseries which may disadvantage the capacity of nursery managers to adopt good practices. Old *et al* (2000) emphasises that location and how this impinges on practices that can be feasibly adopted by a nursery manager is an all important factor in the production of healthy and vigorous seedlings. Vital requirements needed in nursery operation are light-textured, fertile soil, the use of a balanced potting medium with moderate pH, an open area, an adequate water supply free of disease-causing propagules and the effective management

of insect pests and diseases. Seedlings cannot be transported long distances to the field unless suitable vehicles are provided that do not allow plants to suffer by wind or heat damage.

Bloomberg (1985) cites factors which influence the incidence and severity of nursery diseases as nursery location and distance to each other, the tree species grown, the stock type (seed, mother hedge, tissue culture), genotype and age class of seedlings. In the tropical regions of SE Asian forest, forest nursery plants can be especially vulnerable to disease due to the damp humid conditions that prevail and are favourable to the dispersal and survival of disease propagules as well as the development of disease. Technological changes in seedling production in respect to stock type will also have a major impact on nursery management and the management of pests and diseases.

Disease occurrence resulting in high seedling mortality and economic loss in nurseries is most often due to poor management practices which result in unhygienic conditions favourable to increased incidence and severity of disease. Once a disease appears, it will spread rapidly under conditions of high soil moisture and close proximity of young susceptible plants. Disease surveys in nurseries show that seedlings are commonly infected by soil-borne fungal diseases like damping off, collar rot and wilt (Mohanan *et al.* 2005). Many species, often unidentified of nursery pathogens such as species of *Fusarium, Pythium, Phytophthora, Rhizoctonia, Phoma* and *Cylindrocladium* are widespread and frequently associated with nursery diseases in sub tropical and

tropical areas. They can cause various types of diseases (damping-off, root rots, foliage blights and shoot diebacks) at different growth phases of seedlings (Ramsden *et al.* 2002; Rai and Mamatha 2005).

Damping-off is recognised as one of the most severe diseases in SE Asian nurseries (Old, K. M. *et al.* 2003). The damping-off organisms may decay the seed or kill the seedlings before they emerge from the soil (pre-emergence damping-off); or the seedlings are affected after they appear above the ground (post-emergence damping-off) (Eng 1983; Ahmad 1987; Hon 1995). Damping–off fungi may attack the root tips, hypocotyls and young stems of many host species. Since most damping off fungi are not host specific there are reports of *Acacia* spp. being damped off by many different species of *Fusarium., Phytophthora, Pythium, Rhizoctonia, Botrytis* and *Cylindrocladium* (Alexopoulos *et al.* 1996; Lee 1999; Old, Kenneth M. *et al.* 2000).

Initial airborne contamination of seedlings by pathogenic fungi will occur in even the best-managed nursery in the sub tropical and tropical environment. After an initial contamination event the regular watering and high humidity required for rapid early growth of seedlings; cuttings or tissue-cultured plantlets provides ideal conditions for fungal proliferation. The nursery manager's skills and experience are needed to maintain the balance between these conflicting influences, and to identify the time in standard procedure when any application of fungicide is warranted as a preventative or curative. Other hygiene measures include ensuring that water supplies are free from

water-borne pathogens, and eradication of sources of airborne spores, for example by efficient disposal of diseased or otherwise discarded plant material.

Damping off pathogens are able survive and disperse in soil, compost and any other type of nursery potting media. There is the requirement to ensure that sources of soil media are as clean and disease free as possible, and that recycled containers are sterilised with steam. Whether conventional nursery or container nursery, if proper management practices such as appropriate seedling density, shade, water regime and an acceptable level of hygiene are followed and good quality seed is used, disease incidence is minimised (Old, Kenneth M. *et al.* 2000).

The ability to recognise and identify different forest tree nursery diseases e.g. whether caused by a fungus or bacterial, or by a single or many organisms, is very important to the nursery manager and will enable the adoption of the correct management strategy, whether this is based on the improvement of hygiene, the use of particular types of fungicide or both. Detection of the source of infection is also critical to management of nursery diseases especially those based on hygiene e.g. is the source of infection in the water, potting soil or from a nearby nursery

Many nurseries in SE Asia are remotely located in respect to diagnostic laboratories which may impede or delay the identification the diseases. The use of FTA cards could speed up identification by allowing the nursery manager to

easily sample diseased material which can be quickly sent to a diagnostic laboratory accompanied by digital pictures of symptoms. The aim of this chapter is to test the effectiveness of FTA cards in an operational situation when the pathogens were unknown although evidently causing damping off, root and stem rot symptoms. The nursery manager requested that the pathogen(s) involved be identified. The main concern of the nursery manager was that fungicides being applied by the nursery were not reducing disease levels and he wanted guidance to select fungicides which would better target the organisms identified.

MATERIAL AND METHODS

Material

Samples of infected root seedling (Figure. 5.1), seeds and soil (Appendix 5.1) were collected from a nursery in SE-Asia growing *Acacia mangium* from seed. This nursery had been experiencing severe losses when the plants were placed outside after being under cover. Up to 50 % of the plants were being lost between the ages of 6-12 weeks. Young seedlings (up to 8-9 weeks old) showed typical symptoms of damping off or root rot (e.g. reduced growth, wilting, discoloration and death) which may be associated with one or a variety of fungal pathogens (e.g. *Fusarium, Phytophthora, Pythium, Rhizoctonia, Botrytis* and *Cylindrocladium*).

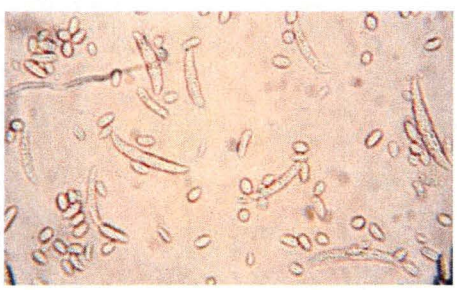
Figure 5.1 A healthy root seedling (right) and an unhealthy root seedling (left)

Older seedlings (more than 8 weeks) as well as showing symptoms of root rot showed symptoms typical of foot rot (i.e. a black stem lesion about 2 cm above the ground) (Figure 5.2 A). In this case, the stem is infected by propagules of a fungus such as species of *Fusarium, Cylindrocladium* or *Phoma* (Gretenkort and Helsper 1993; Shivanna 2005; Frenkel *et al.* 2007) being splashed up to the stem from the medium in which the seedlings are grown, infecting the stem and causing death by a girdling lesion. Some of these lesions were collected and incubated on moist tissue paper. After a couple of days the fungal mycelium was observed growing on the lesions.

A

C

B



D

Figure 5.2 A. Black lesions (indicated by red circles) on seedling stems. B and C. Fungal growth on black lesion after incubation. D. Spores typical of *Fusarium* sp.

The lesions were incubated on wet cotton paper (Figure 5.2 B) and examined under a stereo microscope and cultures on PDA medium were made directly from stem tissue and from evident fungal structures and (Figure 5.2 C and D). This resulted in 13 different isolates of fungi in culture (e.g. Figure 5.3). Several cultures isolated from black lesions were identified morphologically as species of *Fusarium* (Figure 5.2 D) or *Cylindrocladium*.

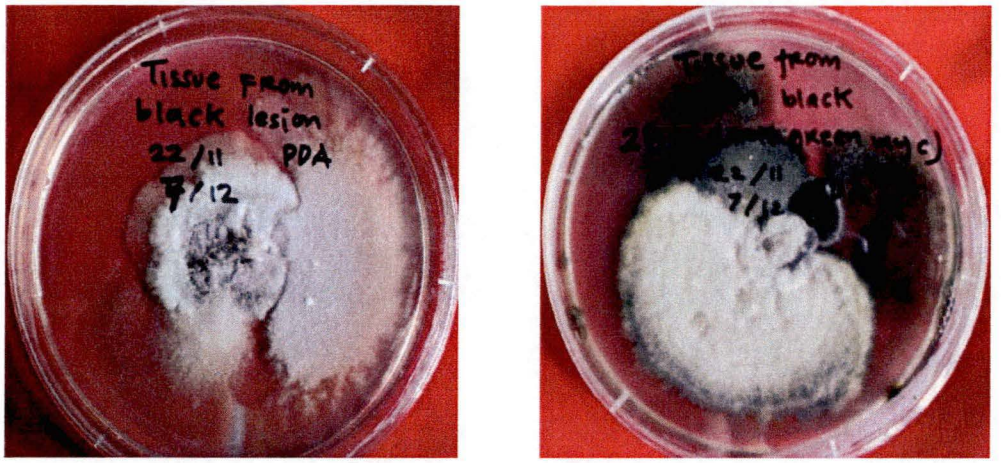


Figure 5.3 Cultures isolated from black lesions on the stems of *Acacia mangium* seedlings

Since all the seedlings were grown from seed, the nursery manager provided seed as he wished to know if the disease was originating from the seed he was using. DNA was extracted from seed or washings from seed of four families of *Acacia mangium* (GP SPA 2071; CM 0774; CM 0773 and CM 0580) (Figure 5.4).

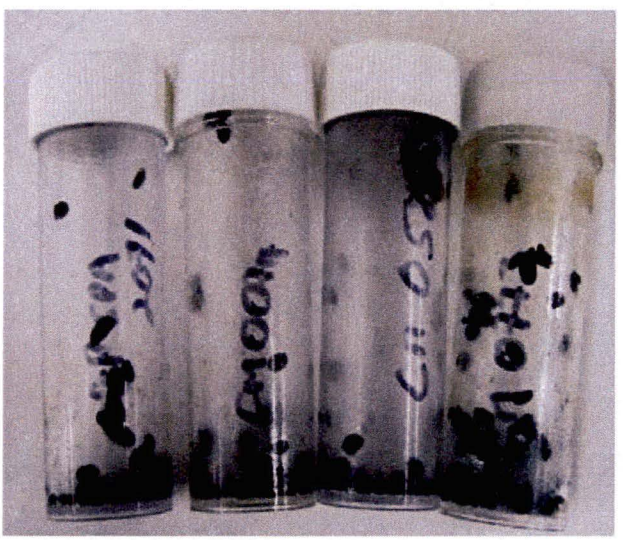


Figure 5.4 *Acacia mangium* seeds from four families. From left to right: GP SPA 2071, CM 0074, CM 0580, and CM 0773

The roots from thirty-five seedlings of *Acacia mangium* seedlings were tested (see Appendix 5.1 for details of code for each sample). The seedlings samples were categorised as follows:

Healthy

3 seedlings sown on 29/9/07 (8 weeks old) and which were apparently healthy (e.g. seedlings in Figure 5.4 A).

4 seedlings sown on 21/09/07 (9 weeks old) and which were apparently healthy

Symptomatic

7 seedlings sown on the 11/9/07 (10 weeks old) and which were symptomatic but not visibly dying, growth has been reduced compared to older seedlings (e.g. seedlings in Figure 5.4 B)

Dying

12 seedlings sown on 12/10/07 (6 weeks old) and which were clearly diseased with some dying

6 seedlings sown on 29/9/07 (8 weeks old) and which were clearly diseased with some dying (e.g. seedling in Figure 5.4 C)

Stem with black lesion

1 seedling sown on 21/9/07 (9 weeks old) and which had a black stem lesion (e.g. seedling in Figure 5.4 D).

2 seedlings sown on 11/9/07 (10 weeks old) and which had black lesions on their stems.

A Healthy seedling (8 weeks)

B Symptomatic seedlings but not dying (9 weeks old)

C Diseased and dying seedlings (6 weeks)

D Seedling (9 weeks) with black stem lesion

Figure 5.4 A-D Healthy and diseased *Acacia mangium* seedlings.

Twelve soil samples were collected from the soil around seedlings (e.g Figure 5.5). The soil samples were categorised by the age and disease symptoms of the seedlings. There were four seedling ages (4, 6, 8 and 10 weeks) and three

categories of disease for each of these seedling ages. The disease categories were; healthy (asymptomatic seedlings with strong growth), symptomatic (disease symptoms and slightly reduced growth) and dying (advanced symptoms including reduced growth, wilting and discoloration).

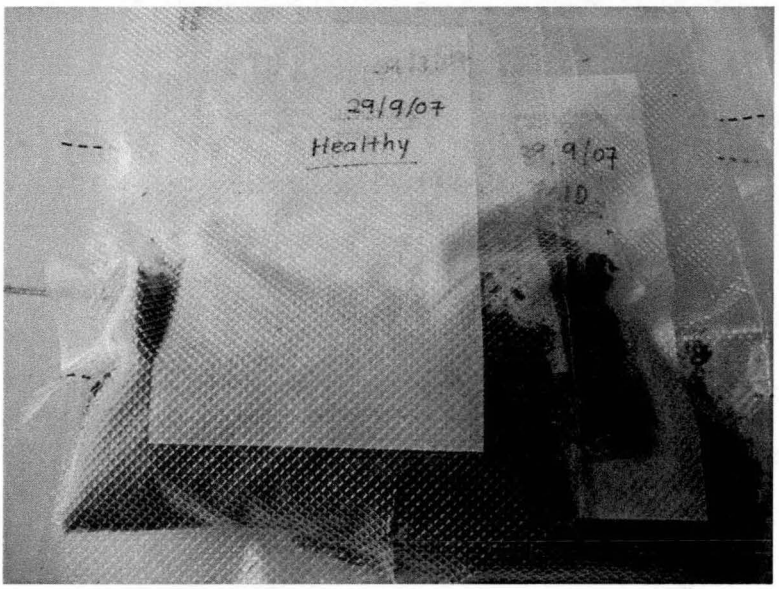


Figure 5.5 Soil collected from 8 week-old asymptomatic and diseased seedlings.

Methods

Mycelium from cultures was scraped from the surface of petri dishes and squashed onto FTA card. Duplicate mycelial samples were put into 1.5 mL tubes and DNA extracted by the glassmilk method (see Chapter 2).

Five seeds from each seed sample were ground in 500 µL of DNA extraction buffer (Raeder and Broda 1985) using a mortar and pestle, vortexed and incubated at room temperature for one hour. A 250 µL aliquot was spotted onto FTA cards while the remaining 250 µL were processed using the glassmilk

DNA extraction method (see Chapter 2). The rest of the seeds were washed by shaking in 1 mL of sterile water in 20 mL tubes for 10 minutes. The water was removed by pipette and dripped onto FTA card, which was dried then treated according to standard methods (see Chapter 2).

Seedlings were cut about 2 cm above the stem collar and whole root systems were ground in a mortar and pestle. Approximately 20 mg of ground root were put into 1.5 mL tubes and ground again with a micropestle in 500 µL of DNA extraction buffer, vortexed and incubated at room temperature for one hour. A 250 µL aliquot was spotted onto FTA card and DNA extracted from the remainder using the glassmilk DNA extraction method (see Chapter 2).

Another 20 mg subsample of ground root was further ground with 500 µL of sterile water, vortexed and incubated at room temperature for one hour then spotted onto FTA card. Only cultures from the black lesions were tested on FTA cards, the tissue from black lesions was not squashed onto cards or used for molecular analyses, only the roots from seedlings with black lesions on the stems.

Soil samples, approximately 20 mg, were put into 1.5 mL tubes and ground with a micropestle in 500 µL of DNA extraction buffer, vortexed and incubated at room temperature for one hour. A 250 µL aliquot was spotted onto FTA card and DNA extracted from the remainder using glassmilk DNA extraction methods. Another 20 mg subsample of soil was ground with 500 µL of sterile

water, vortexed and incubated at room temperature for one hour then spotted onto FTA card. DNA was also extracted from soil samples using a Powersoil DNA extraction kit (see Chapter 2). A 50 µL aliquot of the DNA extracted using the Powersoil kit was applied to FTA card while the remaining 50 µL were directly used for PCR reactions.

Samples of DNA on FTA card were dried at room temperature and processed using standard FTA card sample preparation techniques (see Chapter 2). DNA samples from the glassmilk DNA extraction method were diluted into several series; 1/20, 1/30, 1/40, 1/50, 160, 1/80, 1/100 and 1/300 before PCR.

All DNA samples from FTA cards, glassmilk DNA extraction and Powersoil kit extraction were amplified by PCR using ITS primers (ITS1-F/ITS4 and ITS3/ITS4, (White *et al.* 1990; Gardes and Bruns 1993). DNA from putative *Fusarium* cultures was also amplified using primers targeting the elongation factor (EF1/EF2, O'Donnel *et al.*,(1998) and Beta-tubulin regions (Bt2a/ Bt2b, Glass and Donaldson (1995)). The primer sequences are given in Chapters 2 and 3. All samples were tested in triplicate.

For some samples that consistently gave negative PCR results, PCR inhibition was tested by addition of an internal amplification control (IAC) plasmid, p35B-04 (Glen, M. *et al.* 2007). Plasmid was added to a final concentration of 1 fg/µL, all other reagents and conditions were unaltered.

RESULTS

Cultures isolated from black lesions on the stems of Acacia mangium seedlings

PCR amplification using primers ITS1-F/ITS4 was negative for all cultures isolated from black stem lesions on *Acacia mangium* seedlings. PCR amplification using elongation factor and beta-tubulin primers was more successful, and sequencing of PCR products facilitated identification to genus level. Among 13 samples from both glassmilk DNA extraction and FTA card direct squash method; there were 3 samples positively amplified using EF1-EF2 primers (cultures S, T and U) and 11 samples positively amplified using Bt 2a-Bt 2b primers (cultures 2A-1, 2A-2, 2A-3, 2B-1, 2B-3, 2B-4, P, Q, R, T, and U). The complete PCR amplification results can be seen in Appendix 5.2. Sequence identifications are given in see Chapter 3 and Table 5.5.

Table 5.1 PCR amplification of cultures isolated from *Acacia mangium* seedlings using ITS (ITS1-F/ITS4), Bt2 (Bt2a/Bt2b) and EF (EF1/EF2) primers.

Code	Details	PCR Amplification[1]					
		FTA Card			Glassmilk DNA Extraction		
		ITS	Bt2	EF	ITS	Bt2	EF
2A-1	Brown mycelium*	N	**P**	N	NT	**P**	**P**
2A-2	Black lesion (brown myc)	N	**P**	N	NT	**P**	**P**
2A-3	Black lesion (brown myc)	N	**P**	N	NT	**P**	**P**
2B-1	Black lesion (dark green myc)	N	**P**	N	NT	**P**	N

[1] P indicates PCR amplification of a fragment of approximately 550 bp (primers Bt2a/Bt2b) and 400 bp (primers EF1/EF2); N indicates no amplification.
Glass-milk extracted DNA was not tested with ITS primers as the amplification of the ß-tubulin and elongation factor 1-α regions was successful in most cases, and more informative for *Fusarium* spp.
* = *culture isolated from black stem lesion*

Table 5.1 (Continued) PCR amplification of cultures isolated from *Acacia mangium* seedlings using ITS (ITS1-F/ITS4), Bt2 (Bt2a/Bt2b) and EF (EF1/EF2) primers.

Code	Details	PCR Amplification[1]					
		FTA Card			Glassmilk DNA Extraction		
		ITS	Bt2	EF	ITS	Bt2	EF
2B-2	Black lesion (dark green myc)	N	N	N	NT	N	N
2B-3	Black lesion (dark green myc)	N	**P**	N	NT	**P**	N
2B-4	Black lesion (dark green myc)	N	**P**	N	NT	**P**	N
P	Black lesion	N	**P**	N	NT	**P**	N
Q	Possible Fusarium 1A	N	**P**	N	NT	**P**	N
R	Possible Fusarium 1B	N	**P**	N	NT	**P**	N
S	Possible Fusarium	N	N	**P**	NT	**P**	**P**
T	Possible Fusarium	N	**P**	**P**	NT	N	**P**
U	Possible Fusarium	N	**P**	**P**	NT	**P**	**P**

[1] P indicates PCR amplification of a fragment of approximately 550 bp (primers Bt2a/Bt2b) and 400 bp (primers EF1/EF2); N indicates no amplification.
Glass-milk extracted DNA was not tested with ITS primers as the amplification of the ß-tubulin and elongation factor 1-α regions was successful in most cases, and more informative for *Fusarium* spp.
* = *culture isolated from black stem lesion*

Acacia mangium seed

PCR amplification for all seed samples whatever the DNA extraction method was negative using fungal primers ITS1-F/ITS4 (Table 5.2). Primers ITS3/ITS4 amplifies a shorter DNA fragment and PCR products were obtained with these primers (Table 5.2); with sterile water seed eluate applied to FTA card samples and with one seed sample ground in DNA extraction buffer before application to the FTA card.

Table 5.2 Results of PCR amplification using primers ITS1-F/ITS4 and ITS3/ITS4 from DNA obtained by different extraction methods from *Acacia mangium* seed.

Samples	DNA Extraction Method[1]	PCR Amplification[2]					
		ITS1-F/ITS4			ITS3/ITS4		
		1	2	3	1	2	3
GP SPA 2071	1	N	N	N	N	N	N
	2	N	N	N	**P**	**P**	**P**
	3	N	N	N	**P**	**P**	**P**
CM 0580	1	N	N	N	N	N	N
	2	N	N	N	N	N	N
	3	N	N	N	**P**	**P**	**P**
CM 0773	1	N	N	N	N	N	N
	2	N	N	N	N	N	N
	3	N	N	N	**P**	**P**	**P**
CM 0774	1	N	N	N	N	N	N
	2	N	N	N	N	N	N
	3	N	N	N	**P**	**P**	**P**

[1]Extraction methods were:
1) Glassmilk DNA extraction from ground seeds;
2) Seeds were ground and incubated in DNA extraction buffer that was then applied to FTA card;
3) Seeds were washed in sterile water that was then applied to FTA card.
[2]P indicates PCR amplification of a fragment approx. 500-600 bp for the primers ITS1-F/ITS4 and approx. 300 bp for the primers ITS3/ITS4, N indicates no amplification.

Acacia mangium roots of diseased and healthy Acacia mangium seedlings

PCR amplification using fungal primer pairs ITS1-F/ITS4 and ITS3/ITS4 was negative for all FTA card samples on which extracts from ground root tissue had been applied (Appendix 5.3). Amplification was obtained from conventional glassmilk purified DNA extracts for 7 of the 35 root samples (Appendix 5.3) for primers ITS1-F/ITS4.

Results from a PCR with 8 root samples, each sample subjected to two methods of extraction prior to FTA card application, and including an IAC

plasmid are given in Table 5.3. In 3 out of 16 PCRs, the amplification of both a fungal product and IAC product indicated that the DNA bound to the card was sufficient for amplification and there was no inhibition of the PCR. Negative PCR results with the inclusion of the IAC indicated PCR inhibition in 6 out 16 samples, particularly those that had been ground in DNA extraction buffer prior to FTA card application. The IAC plasmid was amplified in 9 out of 16 samples indicating that there was no DNA or only a very small quantity but that there no PCR inhibition.

Table 5.3 PCR amplification using ITS1-F/ITS4 primers with IAC plasmid from 8 root samples ground in water or DNA extraction buffer before applying to FTA card.

Root samples	DNA[1]	PCR Amplifications[2] ITS-1F/ITS4+IAC plasmid			PCR inhibition or DNA concentration?
		1	2	3	
R2-10 week-old *A. mangium* seedling with black stem lesion	1	N	N	N	Inhibition
	2	N	N	N	Inhibition
R4-10 week-old *A.mangium* symptomatic but not dying, seedlings	1	PC	PC	PC	Sufficient DNA and no inhibition
	2	C	C	C	No or very low fungal DNA and no inhibition
R5-10 week-old *A.mangium* symptomatic but not dying, seedling	1	N	N	N	Inhibition
	2	C	C	C	No or very low fungal DNA and no inhibition
R6-10 week-old *A.mangium* symptomatic but not dying, seedling	1	PC	PC	PC	Sufficient DNA and no inhibition
	2	C	C	C	No or very low fungal DNA and no inhibition
R8-10 week-old *A.mangium* symptomatic but not dying, seedling	1	N	N	N	Inhibition
	2	C	C	C	No or very low fungal DNA and no inhibition
R10-9 week-old *A. mangium* seedling with black stem lesion	1	C	C	C	No or very low fungal DNA and no inhibition
	2	PC	PC	PC	Sufficient DNA and no inhibition

[1]Extraction methods were:
1) Roots ground and incubated in DNA extraction buffer that was then applied to FTA card.
2) Roots ground and incubated in sterile water that was then applied to FTA card.
[2]1, 2 and 3 are results of three replicates; P indicates PCR amplification of a fragment approx. 500-600 bp, N indicates no amplification, C indicates amplification of IAC (approx. 1100 bp).

Table 5.3 (Continued) PCR amplification using ITS1-F/ITS4 primers with IAC plasmid from 8 root samples ground in water or DNA extraction buffer before applying to FTA card.

Root samples	DNA [1]	PCR Amplifications[2] ITS-1F/ITS4+IAC plasmid			PCR inhibition or DNA concentration?
		1	2	3	
	2	PC	PC	PC	Sufficient DNA and no inhibition
R15-8 week-old diseased and dying *A. mangium* seedling	1	C	C	C	No or very low fungal DNA and no inhibition
	2	N	N	N	Inhibition
R20-8 week-old diseased and dying *A. mangium* seedling	1	N	N	N	Inhibition
	2	C	C	C	No or very low fungal DNA and no inhibition

[1]Extraction methods were:
1) Roots ground and incubated in DNA extraction buffer that was then applied to FTA card.
2) Roots ground and incubated in sterile water that was then applied to FTA card.
[2]1, 2 and 3 are results of three replicates; P indicates PCR amplification of a fragment approx. 500-600 bp, N indicates no amplification, C indicates amplification of IAC (approx. 1100 bp).

The complete PCR amplification results for all the seedling root samples, each DNA extraction method tested, with primer pairs ITS1-F/ITS4 and ITS3/ITS4 and the inclusion of IAC are given in Appendix 5.3.

Soil from Acacia mangium seedlings

Of the five different DNA extraction methods tested, only the Powersoil kit method produced DNA that was amplifiable. DNA was not amplified from FTA card samples to which DNA extracted by the Powersoil method had been applied. Both primer pairs ITS1-F/ITS4 and ITS3/ITS4 amplified the same 8 soil samples and gave negative PCR results with the same 4 soil samples (Table 5.4). PCR was negative when the IAC plasmid was included in the PCR with 8 soil samples that had been ground in the glassmilk DNA extraction

buffer and sterile water, incubated in room temperature before spotting onto FTA cards (Appendix 5.4), indicating that there inhibition of PCR.

Table 5.4 PCR amplification using primer pairs ITS1-F/ITS4 and ITS3/ITS4 of soil DNA samples extracted with the Powersoil extraction method

Sample code	Age and health status of seedlings growing in soil sample	PCR Amplifications[1]					
		ITS1-F/ITS 4			ITS3/ITS4		
		1	2	3	1	2	3
S4-H	4 weeks-healthy	P	P	P	P	P	P
S4-S	4 weeks-symptomatic	P	P	P	P	P	P
S4-D	4 weeks-dying	P	P	P	P	P	P
S6-H	6 weeks-healthy	N	N	N	N	N	N
S6-S	6 weeks-symptomatic	P	P	P	P	P	P
S6-D	6 weeks-dying	N	N	N	N	N	N
S8-H	8 weeks-healthy	P	P	P	P	P	P
S8-S	8 weeks-symptomatic	P	P	P	P	P	P
S8-D	8 weeks-dying	P	P	P	P	P	P
S10-H	10 weeks-healthy	P	P	P	P	P	P
S10-S	10 weeks-symptomatic	N	N	N	N	N	N
S10-D	10 weeks-dying	N	N	N	N	N	N

[1]1, 2 and 3 are results of three replicates; P indicates amplification of a fragment approx. 500-600 bp, N indicates no amplification.

Sequencing of PCR amplicons from DNA of seed, soil, root and cultures

DNA amplicons were sequenced, identifying cultures isolated from stem lesions in *A. mangium* seedlings as *Phoma* sp, *Fusarium oxysporum* and *Cylindrocladium/Calonectria* sp. (see Chapter 3). PCR amplicons from seed, root and soil samples were also sequenced, however most of the chromatograms from those samples were illegible, most likely due to the presence of multiple templates. One root samples, R10, had clear, legible chromatograms, allowing the identification of *Fusarium oxysporum* from these samples.

Table 5.5 DNA sequencing results and fungal identification (ID) based on DNA database search results (see appendix 5.1 for more detailed sample descriptions)

Samples	Region	DNA[1]	ID	Samples	Region	DNA[1]	ID
Seeds				***Roots from seedlings***			
GP SPA 2071	ITS	P	*n.a.*	R5-Symptomatic	ITS	P	*n.a.*
CM 0580	ITS	P	*n.a.*	R6-Symptomatic	ITS	P	*n.a.*
CM 0773	ITS	P	*n.a.*	R7-Symptomatic	ITS	P	*n.a.*
CM 0774	ITS	P	*n.a.*	R8-Symptomatic	ITS	P	*n.a.*.
Soil from seedlings				R9-Symptomatic	ITS	P	*n.a.*
4 wks healthy	ITS	P	*n.a.*	R10-Black stem lesion	ITS	G	*F. oxysporum*
4 wks symptomatic	ITS	P	*n.a.*	R15-Dying	ITS	P	*n.a.*
4 wks dying	ITS	P	*n.a.*	R20-Dying	ITS	P	*n.a.*
6 wks healthy	ITS	P	*n.a.*	***Cultures from black stem lesion***			
6 wks symptomatic	ITS	P	*n.a.*	2A-2	Bt T	G	*Phoma* sp
6 wks dying	ITS	P	*n.a.*	2A-3	Bt T	G	*Phoma* sp
8 wks healthy	ITS	P	*n.a.*	2B-1	Bt T	G	*Phoma* sp
8 wks symptomatic	ITS	P	*n.a.*	2B-2	Bt T	G	*Phoma* sp
8 wks dying	ITS	P	*n.a.*	2B-3	Bt T	G	*Phoma* sp
10 wks healthy	ITS	P	*n.a.*	2B-4	Bt T	G	*Phoma* sp
10 wks symptomatic	ITS	P	*n.a.*	P	Bt T	G	*F. oxysporum*
10 wks dying	ITS	P	*n.a.*	Q	Bt T	G	*Cylindrocladium / Calonectria*
Roots from seedlings				R	Bt T	G	*Cylindrocladium / Calonectria*
R2-Black lesion	ITS	P	*n.a.*	S	EF	G	*F. oxysporum*
R3-Symptomatic	ITS	P	*n.a.*	T	EF	G	*F. oxysporum*
R4-Symptomatic	ITS	P	*n.a.*	U	EF	G	*F. oxysporum*

[1]P= poor quality sequence; G=good quality sequence. Region: ITS=Internal Transcribe. Spacer; Bt T=Beta Tubulin; EF=Elongation factor. ID: *F. oxysporum* = *Fusarium oxysporum*

DISCUSSION

Seed samples and FTA cards

Positive PCR products with primers ITS3/ITS4 were obtained in all cases when seed water washings were applied to the FTA cards and in one instance when

seed was ground, left in buffer and then applied to the card. Both types of DNA extraction methods may extract DNA from several microbial organisms in or on the seed and sequences from amplicons were very poor quality and unusable indicating multiple templates.

Since DNA was amplified using primers ITS3/ITS4 and not the fungal specific primers ITS1-F/ITS4 there are no assurances that the DNA amplified is not that of organisms other than fungi such as bacteria and insects. The methodology could be improved by using other fungal primers which will more efficiently amplify a smaller product (e.g. primers beta tubulin (Bt2a/Bt2b) and elongation factor (EF1/EF2)). DNA was not amplified using the control method (traditional glassmilk DNA extraction which includes grinding the seed) for both primer sets (ITS1-F/ITS4 and ITS3/ITS4). The low rate of success when the seed is ground may be explained by high seed lipid content which may inhibit the amplification of fungal DNA (Yarosh and Megorskaya (1975) in Abd-Elsalam *et al* (2007)). Additionally if fungal DNA is contained inside seed cells (Zhang *et al.* 1999) the grinding process needs to be very effective to release the fungal DNA from the seed cells.

Root samples and FTA cards

For a small number (approximately 20 %) of the root samples, only the extracts obtained with the control DNA glassmilk extraction method contained amplifiable DNA in PCR with fungal primers ITS1-F/ITS4. It was shown by

including plasmid 35B-4 in the PCR reaction as an IAC that PCR was inhibited or fungal DNA was extracted in minute quantities or not all.

It is interesting that a possible vascular wilt pathogen (*Fusarium oxysporum*) was identified from diseased root tissue but must not be assumed that this pathogen was actually causing the death or damping-off of seedlings. Many fungi are found in soil and roots without causing disease even if they are pathogenic. *Fusarium* is a common, widespread fungal genus found in soil and it is an abundant and active saprophyte in soil and organic matter, with some specific species and forms that are plant pathogenic (Smith *et al.* 1988). As expected fungal primers ITS1-F/ITS4 amplified DNA from roots whatever the status of seedling i.e. healthy or dying. It is not surprising that *F. oxysporum* was identified from root tissue. Old *et al* (2000) reported that *Fusarium* spp. are known as fungal pathogens responsible for the visible damping-off in *Acacia mangium* and other *Acacia* spp in Indonesia, Malaysia and India.

The use of the beta-tubulin and EF primers in the PCR of root samples might have given more informative results. These primer pairs successfully amplified DNA from fungal cultures when primers ITS1-F/ITS4 gave negative results and the amplicons resulted in legible sequences.

Soil samples and FTA cards

Soil cannot be easily squashed onto FTA card and would probably damage the card. DNA extraction using FTA cards needed an additional step such as

mixing the soil samples with extraction buffer or mixing the soil samples with sterile water before spotting the eluate onto FTA cards. No amplicons however were obtained when carrying out PCR with pre-treated soil extracts dotted on to FTA cards.

The only DNA extraction method that obtained fungal DNA from soil was a kit specifically recommended for soil – the "Powersoil" method. Even when DNA from the "Powersoil" extractions was spotted onto FTA cards, it was not amplified using ITS primers and the inclusion of the IAC in the PCR indicated that the negative results could be attributed to PCR inhibition. Obtaining DNA from soil is notoriously difficult because of the soil impurities in the DNA samples (Smalla *et al.* 1993). Porteous and Armstrong (1991), Ernst *et al* (1996), Schneegurt *et al* (2003), Zhou *et al* (1996) and Yeates *et al* (1998) report that organic humic compounds in the and certain metal ions in soil act as inhibitors for Taq DNA Polymerase.

As for root tissue, there are a wide range of different microorganisms present in soil and many possible inhibitory substances that can pass into the DNA extraction. As for root samples, the beta-tubulin and EF primers which successfully amplified DNA from fungal cultures when primers ITS1-F/ITS4 gave negative results were not used for soil samples and it is recognised that these primers might have given rise to amplicons which could have been sequenced.

Identification of cultures isolated from black stem lesions on Acacia mangium stems

Cultures isolated from black stem lesions were used to test FTA cards (Chapter 3) and to identify the pathogen causing this foot rot disease symptom in nursery seedlings of *Acacia mangium* seedlings and to investigate whether the fungal pathogen causing foot rot disease was the same as that causing root rot in younger seedlings. Several pathogens were identified from cultures isolated from black stem lesions such as *Phoma* sp., *F. oxysporum* and *Cylindrocladium/Calonectria* sp. while pathogen identified from root samples was *F. oxysporum.*

Phoma black stem caused by *P. macdonaldii* in various crops such as sunflower (Carson 1991; Roustaee *et al.* 2000; Debaeke and Peres 2003; Darvishzadeh and Sarrafi 2007) and lucerne and *P. medicaginis* var. *pinodella* (Ali *et al* (1982); Frenkel *et al* (2007) is characterized by large, jet-black lesions on the stem, up to five centimetres (2 inches) in length. This soil-borne fungus survives in debris and is spread by splashing rain and insects. Lesions can be several inches in length. Eventually leaves above the lesion wilt and die.

Cylindrocladium species are fungal plant pathogens capable of causing black stem lesions in forest seedlings. *Cylindrocladium* species are major nursery pathogens in tropical eucalypt forest nurseries causing seedling damping off and root rot e.g *C. clavatum* (Blum *et al* (1992); an unidentified *Cylindrocladium* species in China (Zhou *et al* (2008); *C. scoparium* causes

seedlings to damp off as well as severe leaf blight in older trees (Old *et al* (2003). Given the severity of the damping off, root and foot rot disease in the nursery (up to 50 % of the plants were being lost between the ages of 6-12 weeks) it is possible that all the pathogens detected from root and fungal cultures were involved in the epidemic.

Each of the fungal pathogens identified can cause damping off, root rot and foot rot diseases depending on the age of the seedling e.g. *Fusarium* sp. can cause damping off in the youngest seedlings but as these age soil containing disease propagules gets splashed up the stem and a stem lesion results. There are many new species of *Fusarium*, *Phoma* and *Cylindrocladium* in SE Asia a fact which probably explains why the latter two fungal pathogens could only be identified to genus level from BLAST searches. There had been an unusually wet dry season and inoculum levels had probably carried over and built up from one rainy season to another.

FTA Card as a DNA sampling method for fungal pathogens in nurseries

Several previous studies using FTA card for field sampling have been done and this method works well in maize for plant and viral DNA (Danson *et al.* 2006; Danson *et al.* 2006; Owor *et al.* 2007). Most of these were in maize, which has softer leaves with high water content, facilitating squashing and DNA capture. In Chapter 4 amplifiable fungal DNA was also captured by squashing infected material onto FTA cards. However, from the current work in the chapter, it is

clear that some difficulties still need to be overcome in order to use FTA cards with complex and tougher material such as seed, root and soil samples from which it is difficult to extract clean DNA. The use of IAC plasmid in PCR is an important tool to determine to determine if negative PCR is due limited DNA or inhibitory compounds.

With certain samples in this study PCR amplification failure could be attributed to insufficient fungal DNA or a non-uniform distribution of fungal DNA in the FTA card. A non-uniform distribution of fungal DNA in material such as roots, soil and seed is highly likely and preliminary grinding of this material in water or buffer would be expected to result in more uniform distribution of fungal DNA on the FTA card. Alternatively, elution of the DNA from a larger sample of FTA card might also mitigate the problem. Preliminary grinding of root or soil material in buffer or water would also assist in penetration of the FTA card by the DNA, which is necessary for reliable DNA binding, as discussed in Chapter 4.

The most frequent problem however that occurred in this chapter was PCR inhibition with complex material such as roots and soil. Protocols to remove impurities either prior or post FTA application is an obvious requirement including the simplest approach of sample dilution. The DNA sequences obtained in this research were mostly of poor quality and unusable; probably because the target DNA for sequencing had multiple templates, another feature of analysing material such as soil and roots that contain multiple

microorganisms. The use of different fungal primers (e.g. beta-tubulin and EF primers were not used for root and soil samples) might solve this problem. If the target fungal pathogen is known and sufficiently well characterised species specific primers can be constructed. Cloning would also allow the discrimination of multiple fungal sequences, but this is considerably more work and cloning is expensive and needs advanced skills.

FTA cards could provide a rapid and simple method for DNA capture that could be carried out by semi-skilled nursery workers for sending to a diagnostic laboratory; fungal or infected plant material will quickly degrade and become contaminated by other microorganisms in a tropical country whereas the use of FTA cards would avoid this problem. However complex material such as investigated in this study presents several problems which may restrict their use with FTA cards.

What do the results mean for nursery manager?

The nursery manager was informed of the identity of fungal pathogens from cultures and roots and that the epidemic could not be attributed to a single pathogen but was more likely the result of inclement weather conditions favourable to a suite of fungal pathogens capable of causing the disease symptoms and mortalities observed.

Koch's Postulates is the accepted method to prove that a pathogen is the causal agent of a particular diseases. However it is not always possible to carry out

Koch's Postulates or traditional techniques at remote location with limited facilities. Also the manager in this case study was under extreme pressure due to the high percentage of plants killed and could not have waited for Koch's Postulates to be carried out. The manager changed the pesticide regime as quickly as possible in an attempt to halt seedling losses in the nursery and from the infomraiton given to him selected fungicides that specifically target *Fusarium* and *Cylindrocladium*. The manager also realised that these types of pathogens are most often associated with poor hygiene.

The FTA card technique did not provide a definite diagnosis, however, it indicated that more than one pathogen involved associated with poor hygiene. Improving hygiene is always a good start; since the dry season had been unusually humid there had been a build up of inoculum in weedy drains and other debris present in the nursery. Weeds, blocked drains and debris were cleared. The application of *Trichoderma* as a biological control of the pathogen by adding the *Trichoderma* into the potting media has also significantly increased the survival of the plants.

It was also suggested that in the future the manager should trial the FTA cards using the following protocol: infected material of concern is incubated on damp paper in a covered glass dish; any evident fungal growth observed under the binocular microscope is then applied to FTA card using the standard squashing procedure; the card is sent to a diagnostic laboratory for processing. Although the fungus or fungi that appear after incubation may not be the causal

agent, unless a specialist is present on site, other opportunities for achieving a correct diagnosis can be limited.

CONCLUSION

Based on the results obtained from this research, it can be concluded that DNA sampling using FTA cards for complex and tough substrates such as seeds, root and soils needs careful consideration and development of protocols either prior or post FTA card application that can eliminate the problems posed by inhibitory compounds. This includes the use of more appropriate or specific PCR primers to target the fungal pathogen so that multiple DNA templates do not prevent sequencing. However if both the limitations and advantages of using FTA cards are weighed then they can be used to increase the ease and accuracy of disease diagnostics e.g. by asking a nursery manager to obtain reasonably clean fungal material to apply to the card by incubating the infected material for a couple of days.

CHAPTER 6

Case Study: The potential for FTA Card to hasten detection and identification of *Phytophthora* spp. from water

INTRODUCTION

Phytophthora have a diploid life cycle and are differentiated from fungi on the basis of their cellulosic cell walls. The life cycle of *Phytophthora* spp. is usually dependent on the presence of free water in soil. *Phytophthora* has flagellated zoospores that are easily dispersed through water such as storm or drainage water (Ristaino and Gumpertz 2000) and thick-walled resting spores (chlamydospores) for survival through unfavourable conditions. *Phytophthora* has two different types of spores — chlamydospores and oospores — which can stay alive for extended periods of time in soil or dead plant material. When the conditions are again favourable for the spores, they will grow and attack new host plants.

All species of the genus *Phytophthora* are destructive pathogens, causing rots of roots, crown, stems, leaves and fruits of agriculturally important and ornamental plants (Tyler (2002). Symptoms of *Phytophthora* diseases include damping-off in seedlings, feeder root necrosis in perennials, stunting, soft, wet decay and colour changes in stele and cortex. *Phytophthora* as a root rot pathogen in woody plants grows through the root system and the stem of a plant. The first symptom of a plant infected by *Phytophthora* as a root rot

pathogen is wilting and yellowing of the foliage. The foliage then dries out and the young feeder roots darken. *Phytophthora* are considered to be significant pathogens in woody plants causing fine root diseases, collar rot or bleeding cankers and foliar infections (Brasier, C. *et al.* 2004; Duran *et al.* 2008).

Over the last decade studies of diseases caused by *Phytophthora* spp. have revealed many new species and distributions in woody plants (Maseko *et al.* 2007; Abad *et al.* 2008; Duran *et al.* 2008; Jung and Nechwatal 2008; Moralejo *et al.* 2008; Burgess *et al.* 2009; Duran *et al.* 2009; Hansen, E. 2009; Hansen, E. M. *et al.* 2009; Jung and Burgess 2009; Scott *et al.* 2009).

These new *Phytophthora* species are associated with a variety of ecological niches e.g. native forest, woodland and heath and with different disease symptoms e.g.

- *P. pinifolia* causes a new and severe disease of *Pinus radiata.* It appeared three years ago in the Arauco province of Chile and subsequently spread to other areas. The disease is typified by needle infections, exudation of resin at the bases of the needle brachyblasts and, in younger trees, necrotic lesions in the cambium, which eventually girdle the branches.
- *P. kernoviae*, another previously undescribed species was found in 2003 in Cornwall, the United Kingdom (Brasier *et al*, (2005) causing bleeding stem lesions and foliar necrosis principally in rhododendron, wild *Vaccinium myrtillus* (Beales *et al.* 2009) and beech trees (*Fagus*

sylvatica) although other tree species are susceptible. The scale of the initial outbreak was of such significant concern that in December 2004; a Statutory Instrument was brought in to force to help contain the disease.

- *P. frigida* and *P. alticola* are two new *Phytophthora* species responsible for collar and root rot disease outbreaks in cold tolerant plantation eucalypts in South Africa (Maseko *et al.* 2007).
- *P. europaea*, *P. gallica*, *P. plurivora*, *P. pseudosyringae*, *P. psychrophila*, *P. quercina*, and *P. uliginosa* are seven new species of *Phytophthora* found in the course of studies in Europe on oak decline and in N. America on multiple woody hosts. (Jung *et al.*, (1999), (2002), (2003), and Jung and Nechwatal (2008).
- *P. ramorum* was first discovered in California in 1995 when large numbers of tanoaks (*Lithocarpus densiflorus*) died, and was described as a new species of *Phytophthora* in 2000. This *Phytophthora* is responsible for the rapid death of a range of oaks (*Quercus* and *Lithocarpus* spp.) (Goheen *et al.* 2002; Brasier, C. *et al.* 2004; Rizzo *et al.* 2005; Frankel 2008); and may be hosted by many other forest tree or woody species e.g. in *Viburnum tinus* and rhododendrons it causes severe aerial dieback, stem base discoloration and partial root decay (Werres *et al.* 2001; Lane *et al.* 2003)

According to Hansen (2008), three *Phytophthora* species that have been found in a variety of forests around the world illustrate the range of impacts and

economic damage i.e. *Phytophthora ramorum* (as discussed above), *Phytophthora lateralis*, and *Phytophthora cinnamomi*. *Phytophthora lateralis* causes root diseases in western North America on cedar, *Chamaecyparis lawsoniana* (A. Murr.) Parl. *Phytophthora cinnamomi* has spread globally and has been found in declining oak plantations in the U.S.A. (Balci, Y *et al.* 2007), Europe (Jung *et al.* 2002), France (Marçais *et al.* 2004), Mexico (Tainter *et al.* 2000), California (Garbelotto *et al.* 2006), Portugal (Moreira and Martins 2005) and Italy (Vettraino *et al.* 2002). It is also found in temperate pine forest in North Scotland (Chavarriaga *et al.* 2007), and Fraser Fir forests (Benson *et al.* 2006) in the United States. It is a major problem in Australia (Aberton *et al.*, (2001); Hüberli *et al.*, (2002) and Cahill *et al.*, (2008). The fungi were probably introduced into Australia through European settlement, and have now spread to affect hundreds of thousands of hectares of native vegetation, especially in Western Australia, Victoria, Tasmania, South Australia and coastal Queensland.

Knowledge of the distribution of *Phytophthora* spp. is important for effective land management however, identifying the presence of *Phytophthora* in plant tissue can be difficult. The fungus may also be present as resistant propagules in soil or spread through waterways, and detection of these requires baiting of the affected water or soil (Bush *et al.* 2003). This involves growing the fungus into several types of baits and then plating the baits onto selective media to stimulate sporulation. Baiting is less commonly used to detect *Phytophthora*

species in infected host tissues but is more appropriate for isolation from soil and water samples and thus remains a common and appropriate approach.

A soil sample is placed in a container, flooded with water and baits are added, half sunken in the water or floating on the surface. After leaving for a period of time the baits (e.g. root or plants material) are washed and lesions are excised and cultured onto *Phytophthora* selective media amended with rifampicin and ampicillin (Gevens *et al.* 2007) for morphological identification methods or molecular DNA tests. There are several selective media to grow isolates of *Phytophthora* spp i.e. P10, PARPH-V8 and CMA (Tsao 1970; Jeffers and Martin 1986; Ferguson and Jeffers 1999).

Plant materials such as seedlings of *Eucalyptus sieberi*, the roots of freshly germinated Lupin seedlings (*Lupinus* spp), unripe pears, apples, lemon (*Citrus limon* (L.) Burm. f.) and Rhododendron (*Rhododendron* spp.) leaves are effective baits for recovery of *Phytophthora* spp. commonly found in soil and water over a range of conditions (Bush *et al.* 2003; Reid 2006). Eden *et al* (2000) baited *Phytophthora cinnamomi* from the soil using Blue lupin (*Lupinus angustifolius*) because this baiting system can be easily be prepared in 2-3 days and results in less contamination on the plates of selective medium than do pine or cedar needles. The seedlings of *Quercus* spp. have been used to bait several *Phytophthora* spp. such as *Phytophthora quercetorum* from soil associated with the decline of Oak trees (Nechwatal *et al.* 2001; Balcì and Halmschlager 2003; Balci, Y *et al.* 2007; Balci, Y. *et al.* 2008). Apples and

young leaves of *Quercus robur* were effective as baits for *Phytophthora cinnamomi* in northern temperate pine forests (Chavarriaga *et al.* 2007).

There is increasing interest in isolating *Phytophthora* spp. from water (Ghimire *et al.* 2006; Hong *et al.* 2008; Britt and Hansen 2009; Ghimire *et al.* 2009; Steddom 2009). This isolation technique includes baiting or filtering, centrifugation of water samples, plating the baits on to selective media for detection or the use of molecular tools to detect *Phytophthora* directly from baits, filters or pellets after centrifugation.

Rhododendron leaves are popular for *Phytophthora* baiting from water. Bush *et al* (2003) isolated several species of *Phytophthora* from *Rhododendron catawbiense* discs (5 mm in diameter) in plastic mesh bags attached to floats on the surface of a water cycling irrigation reservoir. Hwang *et al* (2007) effectively used leaves of *Rhododendron maximum* in a mesh bait bag to bait several *Phytophthora* spp. from five streams in three watersheds in Pisgah National Forest in western North Carolina, while Orlikowski *et al* (2007) found that *P. citricola* was preferentially baited by rhododendron leaves (about 70% of all *Phytophthora* isolates obtained from rhododendron leaf baits floated in water were *P. citricola*). Lupin baiting is also effective. Polashock *et al* (2005) obtained both *P. cinnamomi* and another *Phytophthora* sp. from diseased plant tissues and irrigation water. Several fruits have been successfully employed as baits. Reid (2006) stated that unripe pears or apples, secured in mesh bags and floated in the water for a week are useful for testing water sources such as

dams or streams for the existence of plant pathogens such as *Phytophthora* species. Pear and cucumber baits were floated in water to bait *P. capsici* (Gevens *et al.* 2007). Pear bait has also been used to detect *P.ramorum* propagules in streams which run through forest areas with sudden oak death in Santa Cruz County, CA from 2001 to 2007 (Tjosvold *et al.* 2008).

There has been an exponential increase over the last decade in the application of molecular analytical techniques to the detection of *Phytophthora* species; these methods offer improved sensitivity, specificity, analysis time, and the potential for high throughput applications. DNA sequencing or species-specific PCR is often required for the morphological identification of *Phytophthora* isolates, the latter being a process which may require considerable taxonomic knowledge of this genus. To support the identification of new *Phytophthora* isolates via comparison of their sequences at one or more loci with the corresponding sequences derived from the isolates archived in the *Phytophthora* Database, (Park *et al.* 2008) have generated and deposited sequence data from more than 1500 isolates representing the known diversity in the genus. The speed and accuracy of detection can be enhanced if PCR and/or DNA sequencing can be performed on DNA extracted directly from baits, rather than relying entirely on the yield of cultures (Sechler *et al.* 2009). In addition, there is potential for direct DNA capture from the bait material using FTA card or using the card itself as a "bait".

Phytophthora genus specific primers for a common first amplification would reduce the number of required amplifications, reducing costs of the analysis and, to some extent, the risks of false positives. Cooke *et al.* (2000) designed a primer to amplify the ITS1 and ITS2 regions from all members of the Peronosporales in combination with the universal primer ITS4. Schena *et al.* (2006) published a pair of *Phytophthora*-specific primers amplifying a fragment of the ras-related protein gene as well as a suite of species-specific primers targetting the same region. O' Brien (2008) developed PCR primers for the specific detection of *Phytophthora cinnamomi* from a RAPD fragment. Species-specific primers have been developed for *P. pinifolia* (Duran *et al.* 2009) since it is important to be able to dentify large numbers of cultures accurately and efficiently, for both quarantine work and biological research on this important new pathogen.

This chapter describes the testing of FTA card as bait for fungal DNA in streams and the use of FTA card to capture fungal DNA directly from baits comprised of various plant materials. FTA card has not previously been applied to detection of *Phytophthora* spp. or to capture of DNA directly from water. If successful, the DNA can be analysing using several specific PCR protocols for pathogen identification.

MATERIAL AND METHODS

Material

Materials specific to this chapter include baits; i. e. leaves from *Fragaria* sp. (strawberry), *Rhododendron* sp., *Citrus limon* (lemon), *Camellia* sp., *Saintpaulia* sp. (African violet), *Eucalyptus* sp., needles of *Cupressus sempervirens* (pencil pine) and *Pseudotsuga menziesii* (Douglas Fir), petals of *Rosa* sp., sprouted seeds of *Phaseolus mungo* (Mungbean) and *Medicago sativa* (Alfalfa), and fruits of *Malus* sp. (apple) and *Persea americana* (avocado).

Several isolates of *Phytophthora* spp. (Table 6.1, Figure 6.1), were obtained from Pine Lake, Tasmania for use as controls. A 2 L sample of water was also taken from the South Esk River in northern Tasmania.

Table 6.1 List of isolates of *Phytophthora* spp.

Code	Identity
PC	*Phytophthora cinnamomi*
NY 001	*Phytophthora cryptogea*
Hansen 133	*Phytophthora drechsleri*
Hansen 139	*Phytophthora drechsleri*
Hansen 266	*Phytophthora drechsleri*
P 501	*Phytophthora gonapodyides*
S 5	*Phytophthora gonapodyides*
S 42	*Phytophthora gonapodyides*

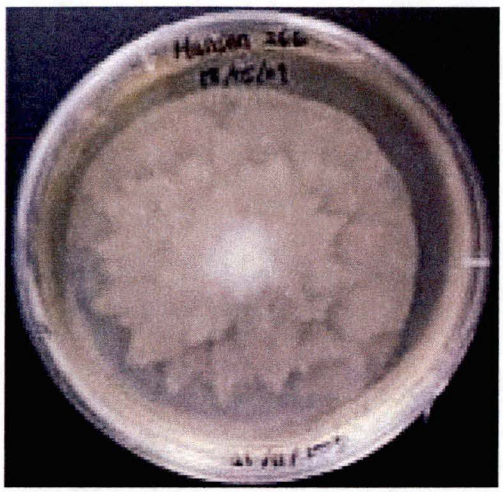


Figure 6.1 Phytophthora culture Hansen 266 (*Phytophthora drechsleri*)

Methods

Baits were prepared by sewing them into separate compartments of a mesh bag which was then left in the South Esk River for a week. Several *Phytophthora* spp. have previously been isolated from this stream (Wardlaw, pers. comm).

A piece of FTA card was also stitched into one of the compartments. After a week, the bag was retrieved and part of each bait plated onto media selective for *Phytophthora* spp. i.e. P10 and PARPH agar.

After 3 days incubation, fungal mycelium was sub-cultured onto V8-PARPH and Corn Meal Agar (The recipes of all selective media are given in Appendix 6.1). A portion of each bait was also squashed directly onto the FTA card and DNA was extracted from another portion using the glassmilk method (Chapter 2). FTA card sample preparation was as described in Chapter 2.

From one water sample (1 L), one mL of water was dripped onto FTA card, 100 µL at a time, allowing the card to dry between applications. The remainder of the water sample was placed into a 2 L glass beaker with a magnetic stirrer and a new piece of FTA card; and stirred for 2 hours.

The FTA card was removed and dried at room temperature and the water divided among 12 of 80 mL centrifuge bottles and centrifuged for 10 minutes at 14000 rpm (Eppendorf Centrifuge 5804 Germany). Most of the supernatant was decanted and the last 1 mL (approximately) was pipetted into 1.5 mL tubes which were centrifuged again at 14000 rpm (Eppendorf Centrifuge 5804 Germany) for 10 minutes.

All of the supernatants were discarded and half of the pellets were removed and squashed onto the FTA card. DNA was extracted from the remaining pellets using the glassmilk method.

The other 1 L water sample was divided into two 500 mL glass beakers. The first 500 mL were filtered using Whatman No.1 filter paper and a Buchner funnel. The second subsample was filtered through a millipore filter (Millex-GP Filter Unit 0.22 µm pore size) using a syringe (Terumo Syringe 20 cc/mL).

DNA was extracted from the Whatman filter paper and millipore membrane as described by Schweigkofler *et al.* (2004). Briefly, the filter papers were

chopped up, washed with 20 mL of hot (65°C) 4 x TE buffer and vortexed (maximum speed) for 5 min. to resuspend any spores present.

The suspensions were centrifuged at 1000 x *g* for 90 minutes to concentrate the spores. After the supernatant was removed, DNA was extracted from the pellet (100 µL). DNA from half of the pellets was extracted using the glassmilk DNA extraction method and the remaining pellets were squashed onto the FTA cards.

PCR was conducted in triplicates for all DNA samples both from glassmilk DNA extraction and FTA cards. In addition to the primers described in Chapter 2, PCR with the primers ITS1 (TCCGTAGGTGAACCTGCGG, White *et al.*,1990) and ITS2 (GCTGCGTTCTTCAT CGATGC, White *et al.*,1990), also targeting the rDNA ITS region was performed using the same reagent concentrations and thermocycler programme as for ITS1-F/ITS4 (Chapter 2).

Several pairs of species-specific or genus-specific primers were also used by O'Brien (2008), Schena *et al.* (2008) and (2006). Primers and their target species are shown in Table 6.2. The concentrations of the PCR reagents were the same as ITS PCR, while the amplification conditions were as follows: initial denaturation at 95°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 30 s; and a final extension at 72°C for 10 min (Schena, L. *et al.* 2008).

Table 6.2 List of Phytophthora species- and genus-specific primers (O'Brien (2008), Schena *et al.* 2008 and 2006)

Target species	Primer codes	Sequences (5'-3')
Phytophthora spp.	YPh1F	CGACCATKGGTGTGGACTTT
	YPh2R	ACGTTCTCMCAGGCGTATCT
Phytophthora cactorum	YCac1F	CCATACAAAATTCTGCGCTAGG
	YCac2R	AGACACACAAGTGGACCGTTAG
Phytophthora cinnamomi	YCin3F	GTCCTATTCGCCTGTTGGAA
	YCin4R	GGTTTTCTCTACATAACCATCCTATAA
Phytophthora citricola	YCit1F	TCCAACTTAGTAAGAGTGCTGGA
	YCit2R	CAACAGAAATCCTGAAGTACTGTATCA
Phytophthora kernoviae	YPtc1F	AGCTTCTGGGAAGGGCTATG
	YPtc2R	TCATGTGGTGGCAGATAGTTG
Phytophthora megasperma	YMeg1F	TCTGCTCTTCCGACTTGGTC
	YMeg2R	TGGCATTAGTTAGTTTCGTCCA
Phytophthora cinnamomi	LPC2	GTCCACACCTAACCCAGAGAT
	RPC3	CGTGTATGAGGAAGCGTAGG

RESULTS

Cultures isolated from baits

The two fruits that were used as baits, apple and avocado, were eaten by stream fauna. From the 11 remaining baits, 10 potential *Phytophthora* isolates were obtained (Table 6.3). No isolates were obtained from the *Eucalptus* leaf baits. All isolates were subcultured onto CMA and V8 agar, with a sample squashed onto FTA card and DNA extracted from another sample by the glassmilk DNA extraction method.

Table 6.3 PCR amplification, using primers targeting the rDNA ITS region, from *Phytophthora* isolates and fungi isolated from stream baits.

Code	Species (if known)	PCR Amplifications[1]			
		Glassmilk DNA extraction		FTA direct squash	
		ITS1-F ITS4	ITS1 ITS2	ITS1-F ITS4	ITS1 ITS2
PC	*Phytophthora cinnamomi*	**P**	**P**	**P**	**P**
NY 001	*Phytophthora cryptogea*	**P**	**P**	N	**P**
Hansen 133	*Phytophthora drechsleri*	N	**P**	N	N
Hansen 139	*Phytophthora drechsleri*	**P**	**P**	**P**	**P**
Hansen 266	*Phytophthora drechsleri*	N	**P**	N	N
P 501	*Phytophthora gonapodyides*	N	N	N	N
S 5	*Phytophthora gonapodyides*	N	**P**	N	N
S 42	*Phytophthora gonapodyides*	N	N	N	**P**
IAF	Isolate from *Saintpaulia* sp. leaf bait	N	N	N	**P**
ICO	Isolate from *Pseudotsuga menziesii* bait	**P**	**P**	N	**P**
IAL	Isolate from *Medicago sativa* bait	**P**	**P**	N	**P**
ILE	Isolate from *Citrus limon* leaf bait	**P**	**P**	N	**P**
IPE	Isolate from *Cupressus sempervirens* bait	**P**	**P**	N	N
IRH	Isolate from *Rhododendron* leaf bait	**P**	**P**	N	**P**
IRO	Isolate from *Rosa* petal bait	**P**	**P**	N	N
IST	Isolate from *Fragaria* leaf bait	**P**	**P**	N	N
IMB	Isolate from *Phaseolus mungo* bait	N	N	**P**	**P**
ICA	Isolate from *Camellia* sp. leaf bait	N	**P**	N	N

[1] P indicates amplification of a fragment of approximately 600 bp (primers ITS1-F/ITS4) or 300 bp (primers ITS1/ITS2) for at least one of the three replicate samples, N indicates no amplification in any of the three replicates.

All culture samples were tested by PCR of the rDNA ITS, using primer pairs ITS1-F/ITS4 and ITS1/ITS2. PCR results from extracted DNA and from direct squashes onto the FTA card were mostly positive (Table 6.3), though amplification efficiency was variable from FTA card (Figure 6.2). Complete results including all replicates are in Appendix 6.2.

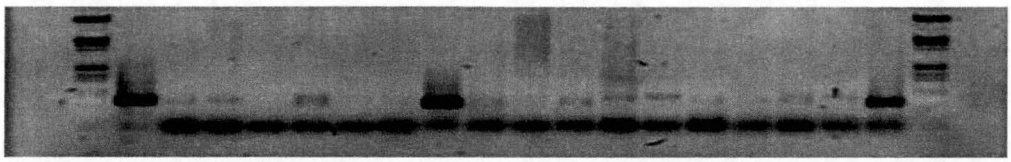


Figure 6.2 PCR amplification of DNA from FTA card direct squashes of *Phytophthora* cultures and isolates from baits, using primers ITS-1F/ITS 4. Lanes contain: 1 and 20, DNA size marker, lambda DNA cut with Eco RI and Hind III; 2, positive control (isolate A, chapter 3); 3, S42; 4, Hansen 139; 5, Hansen 133; 6, Hansen 266; 7, S5; 8, NY001; 9, PC; 10, IAF; 11, ICO; 12, IAL; 13, ILE; 14, P501; 15, IPE; 16, IRH; 17, IRO; 18, IST and 19, IMB.

PCR from baits

Fungal DNA was amplified directly from DNA extracted from 6 of the bait samples, and from direct squashes onto FTA card for only 3 of the baits (Table 6.4). The softer baits, mungbean sprouts (*Phaseolus mungo*) followed by African violet leaf (*Saintpaulia* sp.) gave the strongest product. Fungal DNA was also amplified from FTA card that had been left in the stream with the baits (Figure 6.3).

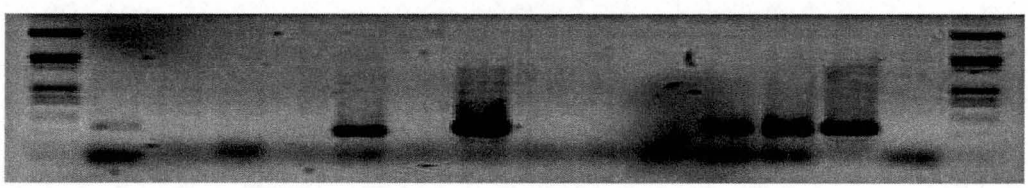


Figure 6.3 PCR amplification using primers ITS1-F/ITS4 from direct squashes of bait samples onto FTA card, and from FTA card that had been left in the stream as a bait. Lanes contain: 1 and 16, DNA size marker, lambda DNA cut with Eco RI and Hind III; 2, ICA (isolate from *Camellia* leaf); 3, *Pseudotsuga menziesii* needle; 4, *Fragaria* sp. leaf; 5, *Cupressus sempervirens* needle; 6, *Medicago sativa* sprout; 7, *Rhododendron* sp. leaf; 8, *Phaseolus mungo* sprout; 9, *Citrus limon* leaf; 10, *Camellia* sp. leaf; 11, *Rosa* sp. petal; 12, *Saintpaulia* sp. leaf; 13, FTA card (direct baiting), 14, positive control (DNA extract from culture FF-269, chapter 3); 15, negative control (no DNA template).

Table 6.4 PCR amplification from baits using primers targeting the rDNA ITS region

Code	Sample name	PCR Amplifications[1]			
		Glassmilk DNA extraction		FTA direct squash	
		ITS1F-ITS4	ITS1-ITS2	ITS1F-ITS4	ITS1-ITS2
CF	*Pseudotsuga menziesii* needle	**P**	**P**	N	N
SL	*Fragaria* sp. leaf	**P**	**P**	N	N
PL	*Cupressus sempervirens* needle	N	**P**	N	N
AL	*Medicago sativa* sprouts	N	**P**	**P**	N
RL	*Rhododendron* leaf	N	N	N	N
MB	*Phaseolus mungo* sprout	**P**	**P**	**P**	**P**
LL	*Citrus limon* leaf	**P**	**P**	N	N
CL	*Camellia* sp. leaf	N	N	N	N
RP	*Rosa* sp. petal	**P**	**P**	N	N
AV	*Saintpaulia* sp. leaf	**P**	**P**	**P**	**P**
EL	*Eucalyptus* sp. leaf	NT	NT	N	N

[1] P indicates amplification of a fragment of approximately 600 bp (primers ITS1-F/ITS4) or 300 bp (primers ITS1/ITS2) for at least one of the three replicate samples, N indicates no amplification in any of the three replicates, NT indicates the samples were not tested using the related primers.

PCR with genus- and species-specific primers

DNA from seven of the eight known *Phytophthora* isolates was amplified using the genus-specific primer pair YPh1F/YPh2R primers both from DNA extracts and FTA card samples (Table 6.5) and six of those were also amplified with the YMeg1F/YMeg2R primer pair. Only one of the bait isolates, ICO, gave a PCR product from these primer sets (Table 6.5). PCR with all other species-specific primer pairs gave negative results for all these isolates (Appendix 6.3).

Table 6.5 PCR amplifications of isolates using genus- and species-specific primers

Code	Sample Name	YPh1F/YPh2R		YMeg1F/YMeg2R	
		DNA[1]	FTA[2]	DNA	FTA
PC	*Phytophthora cinnamomi*	N	N	N	N
NY 001	*Phytophthora cryptogea*	**P**	**P**	**P**	**P**
Hansen 133	*Phytophthora drechsleri*	**P**	**P**	**P**	**P**
Hansen 139	*Phytophthora drechsleri*	**P**	**P**	**P**	**P**
Hansen 266	*Phytophthora drechsleri*	**P**	**P**	**P**	**P**
P 501	*Phytophthora gonapodyides*	**P**	**P**	N	N
S 5	*Phytophthora gonapodyides*	**P**	**P**	**P**	**P**
S 42	*Phytophthora gonapodyides*	**P**	**P**	**P**	**P**
IAF	Isolate from *Saintpaulia* sp. leaf bait	N	N	N	N
ICO	Isolate from *Pseudotsuga menziesii* bait	N	**P**	**P**	N
IAL	Isolate from *Medicago sativa* bait	N	N	N	N
ILE	Isolate from *Citrus limon* leaf bait	N	N	N	N
IPE	Isolate from *Cupressus sempervirens* bait	N	N	N	N
IRH	Isolate from *Rhododendron* leaf bait	N	N	N	N
IRO	Isolate from *Rosa* petal bait	N	N	N	N
IST	Isolated from *Fragaria* sp. leaf bait	N	N	N	N
IMB	Isolate from *Phaseolus mungo* bait	N	N	N	N
ICA	Isolate from *Camellia* sp. leaf bait	N	N	N	N

[1]DNA extracted by the glassmilk method, [2]Mycelium squashed onto FTA card. P indicates amplification of a fragment of 470 bp for primers Yph1F/Yph2R and 196 bp for primers Ymeg1F/Ymeg2R, N indicates no amplification.

DNA extracted directly from 4 of the 10 baits was successfully amplified with *Phytophthora*-specific primers YPh1F/YPh2R and from 2 of the baits using the primers YMeg1F/YMeg2R (Table 6.6). Amplification from baits squashed onto FTA card was less successful (Table 6.6). From the other *Phytophthora* species-specific primers tested, the only successful amplification was from DNA extracted by the glassmilk method from the Rose petal bait using primers YPtc1F/YPtc2R (Appendix 6.4).

Table 6.6 PCR amplification using genus- and species-specific primers of DNA from baits.

Code	Bait	Yph1F/Yph2R		YMeg1F/YMeg2R	
		DNA[1]	FTA[2]	DNA	FTA
CF	*Pseudotsuga menziesii* needle	**P**	N	N	N
SL	*Fragaria* sp. leaf	**P**	**P**	N	N
PL	*Cupressus sempervirens* needle	**P**	N	N	N
AL	*Medicago sativa* sprout	N	N	N	N
RL	*Rhododendron* sp. leaf	N	N	N	N
MB	*Phaseolus mungo* sprout	N	N	N	N
LL	*Citrus limon* leaf	N	N	N	N
CL	*Camellia* sp. leaf	N	N	N	N
RP	*Rosa* sp. petal	N	N	**P**	N
AV	*Saintpaulia* sp. leaf	**P**	N	**P**	N
EL	*Eucalyptus* sp. leaf	N	N	N	N

[1]DNA extracted by the glassmilk method, [2]Mycelium squashed onto FTA card. P indicates amplification of a fragment of 470 bp for primers Yph1F/Yph2R and 196 bp for primers Ymeg1F/Ymeg2R, N indicates no amplification.

DNA extracted directly from water

PCR results using the ITS1-F/ITS4 primers were negative for all DNA samples from water; except from FTA card left in the stream for a week (Table 6.7). However, several positives results were obtained from PCR with primers ITS1/ITS2 from water samples applied to FTA cards. Those positives results came from FTA card samples that had been stirred in water, 1 mL water samples dripped onto the FTA card, and pellets from centrifuged water samples. Amplification of DNA from filter washings was unsuccessful. Complete results are provided in Appendix 6.5

Table 6.7 PCR amplification results from water samples using primers targeting the rDNA ITS region

Sample Preparation Method	PCR Amplifications[1]	
	ITS1-F/ITS4	1TS1/2
FTA card left in stream with baits	**P**	N
Water (1 mL) dripped onto FTA card	N	**P**
FTA card stirred in 1L water	N	**P**
Pellets from centrifuged water squashed onto FTA card	N	**P**
Whatman filter paper eluate, pelleted and squashed onto FTA card	N	N
Whatman filter paper eluate, pelleted and DNA extracted by glassmilk method	N	N
Millipore filter eluate, pelleted and squashed onto FTA card	N	N
Millipore filter eluate, pelleted and DNA extracted by glassmilk method	N	N

[1] P indicates amplification of a fragment of approximately 600 bp (primers ITS1-F/ITS4) or 300 bp (primers ITS1/ITS2) for at least one of the three replicate samples, N indicates no amplification in any of the three replicates.

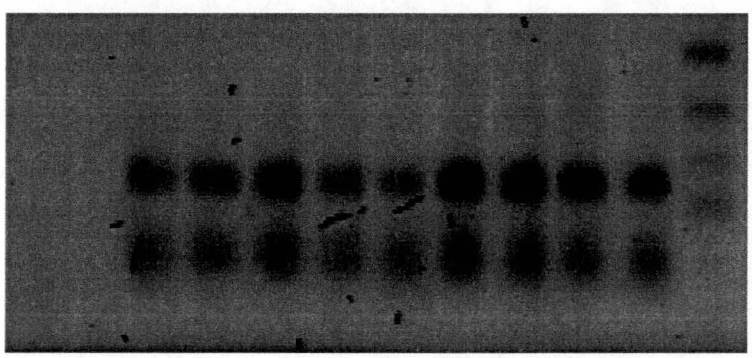


Figure 6.4 PCR amplification of water samples using YMeg1F/YMeg2R primers. Lanes contain: 1-3, FTA card in stirred water; 4-7, water dripped onto FTA card; 8-11 pellets from centrifuged water squashed onto FTA card.

Using species-specific primers, amplification was only successful using the YMeg1F/YMeg2R primers (Table 6.8, Figure 6.4). All the water samples on FTA cards which were positive using ITS1/ITS2 were also positive in species-specific PCR using YMeg1F/YMeg2R. In addition, glassmilk DNA extracts of filter membranes and filter papers were amplified with these primers. Complete results are provided in Appendix 6.5.

Table 6.8 PCR amplification of DNA from water samples using genus- and species-specific primers.

Sample Preparation Method	PCR Amplifications[1]	
	YPh1F-YPh2R	YMeg1F-YMeg2R
FTA card left in stream with baits	N	N
Water dripped onto FTA card	N	**P**
FTA card stirred in water	N	**P**
Pellets from centrifuged water applied to FTA card	N	**P**
Whatman filter paper eluate, pelleted and squashed onto FTA card	N	N
Whatman filter paper eluate, pelleted and DNA extracted by glassmilk method	N	**P**
Millipore filter eluate, pelleted and squashed onto FTA card	N	N
Millipore filter eluate, pelleted and DNA extracted by glassmilk method	N	**P**

[1] P indicates amplification of a fragment of approximately 470 bp (primers YPh1F/YPh2R) or 196 bp (primers YMeg1F/YMeg2R) for at least one of the three replicate samples, N indicates no amplification in any of the three replicates.

DISCUSSION

Though *Phytophthora* spp. are no longer considered to be fungi and current phylogenies place the Oomyceta in the Stramenopile phylum (Tyler *et al.* 2006), amplification with the primer pair ITS1-F/ITS4 was successful for some isolates of *Phytophthora* species. Designed to amplify fungal DNA in preference to plant DNA, the complete range of species for which this primer pair will amplify the rDNA ITS is unknown (Gardes and Bruns 1993).

Several PCRs gave positive results with fungal primers ITS1-F/ITS4 for DNA captured directly on the FTA card from baits. As might be expected DNA extracted from baits with the more traditional glassmilk extraction method gave a larger number of positive PCRs with fungal primers ITS1-F/ITS4. As the PCR products were nor sequenced, it is uncertain whether fungal or oomycete

DNA was amplified. Primers ITS1/ITS2 gave a higher proportion of positive results than primers ITS1-F/ITS4 from DNA captured directly onto FTA cards but it cannot be assumed that the DNA amplified with primers ITS1/ITS2 was fungal or oomycete DNA. As this primer pair amplifies a much shorter fragment than the ITS1-F/ITS4 primers, amplification is more efficient and this may be critical when target DNA is at low concentrations. However the range of target species is much greater and DNA from plants and/or insects may also be amplified so this primer pair is best suited to amplification of DNA from pure cultures.

Application of contaminated stream water to FTA card and subsequent amplification using the primers Ymeg1F/Ymeg2R indicates that capture of oomycete DNA was successful. Lack of species specificity for these primers under the conditions used here precludes complete certainty on this point, though DNA sequencing of the product would be informative. Lack of amplification using the *Phytophthora*-specific primer pair Yph1F/Yph2R for the same samples may have been due to low template concentration as the Yph1F/Yph2R product is more than twice as long as the Ymeg1F/Ymeg2R product, as discussed above for the ITS primers.

A puzzling result was the amplification of some samples (extracted DNA and/or FTA samples) with fungal primers ITS1-F/ITS4 but not with the general primers ITS1/ITS2. Stochastic effects may account for this discrepancy in FTA samples but should not have so great an effect for DNA in solution.

In future trials an IAC plasmid will be used to test for PCR inhibition or low DNA captured on the FTA card. In addition the optimisation and use of species-specific PCRs in our laboratory could improve amplification results with DNA captured on FTA cards; the amplification of isolates known to be *Phytophthora cryptogea*, *drechslerii* and *gonapodyides* with primers designed to be specific for *P. megasperma* indicates a need to further optimise PCR conditions for these primers or to use different primers. Specificity was likely compromised by use of a $MgCl_2$ concentration higher than that stipulated in the published protocol (Schena, L. *et al.* 2008).

Although the PCR amplification of DNA captured on FTA cards from baits or water was unreliable using primers designed for amplifying fungal DNA, the successful amplification of DNA from FTA card that had been left in the stream for a week is a new and exciting result, indicating that FTA matrix does not deteriorate after prolonged submersion in water. Further optimisation is clearly required but this could be developed into a valuable monitoring technique; the fungal species "baited" by the card needs to be identified using cloning and sequencing methodology or species-specific PCR.

CONCLUSION

The use of FTA card in *Phytophthora* baiting to either capture DNA from squashing leaf baits or as a direct bait substrate requires more research and

validation in order to use the FTA card. However this initial research is encouraging and the FTA card will be further tested in *Phytophthora* research in Tasmania.

CHAPTER 7

General Discussion

This project is an investigation into the use of FTA cards for storing DNA for later analysis by PCR. The intention is to be able to use cards for collection of DNA from diseased tissue samples in native forest and forest plantations, and for the DNA to be preserved for later analysis by PCR.

The research investigated the extraction of DNA from different samples types. DNA was harvested merely by squashing the tissues onto the FTA cards. The analysis was carried out by transferring a piece of the card to a PCR reaction. In most cases the results obtained with the FTA cards were compared with the results obtained from DNA extracted from the same sample by other means.

As one might expect the results were not consistent but depended on the material used. FTA cards captured amplifiable DNA and PCR gave positive results with fungal cultures, agaricoid sporocarps and spore-prints. Although the FTA cards appeared to capture DNA when spore prints were made directly onto the cards, PCR results were poor when rust and mildew spores were applied to cards. There could have been several reasons preventing DNA capture and subsequent PCR amplification especially with rust spores: inhibitory substances released from melanised rust spores; spores resistant to squashing; low DNA release; and not enough spore material applied to the

card. Fungal spores may require harsher treatments such as sonication (Kennedy *et al.* 2000; Schwarzott and Schüßler 2001; Thines *et al.* 2004; Zhao *et al.* 2005); which unfortunately was not tested in these experiments.

In case study with fungi suspected of causing severe mortality in *Acacia mangium* seedlings in the nursery, FTA cards were used to test seeds, roots and soil for the presence of fungal pathogens. Several cultures isolated from foot rot (black stem lesions) in older seedlings were also included in the study. The aim of the study was to detect and identify potential pathogens and also to see if the FTA card could be used during subsequent disease events at the nursery located at least 12 hours by plane from any diagnostic facilities. As expected detection and identification of fungal pathogens was not straightforward from any of the samples except for cultures. Issues of PCR inhibition, low DNA and multiple sequencing templates are significant with seeds, roots and soil. Even the DNA captured on FTA cards from cultures required a change in fungal primers before there was successful amplification.

The cultures that were identified however were plausible causal agents (e.g. *Phoma* sp, *Fusarium oxysporum* and *Cylindrocladium/Calonectria* sp.) and *F. oxysporum* was also detected and identified from root samples. While it cannot be definitely concluded that the latter fungi are causing the disease, the information was useful to the nursery manager. In future FTA cards will be used by the manager by washing, grinding roots in extraction buffer and spotting onto the FTA card and by incubating material in a dish on a damp

tissue before scraping fungal mycelium onto the FTA card (the latter technique may assist in avoiding contamination).

Using the FTA cards as either a direct bait substrate for *Phytophthora* or for processing leaf baits without resorting to isolations gave some exciting preliminary results. It is obvious that this preliminary study needs more research to fully validate this possibility and will no doubt depend on DNA analysis involving the use of species specific primers, cloning and DNA sequencing. Given the international and Australian interest in species of *Phytophthora* and their potential involvement in forest decline under a changing climate, a methodology that in any way facilitates broad scale sampling of rivers and leaf baits is to be recommended. Currently there are many attempts at river sampling using a variety of natural and synthetic baits which are processed using molecular techniques. The FTA card has the advantage that after sample application it can be dried, then stored at room temperature and if there is no issue of biosecurity the cards can be sent to a laboratory by mail.

Leaf and soil are complex materials which contain many microorganisms and PCR inhibitory compounds and successful capture of DNA by cards is more problematic. It is well known that plant material contains PCR inhibitors and so requires specialised procedures to minimise these (Tsai and Olson 1992; Pandey *et al.* 1996).

A major failing was the omission of an internal standard in the reactions to test for inhibition of PCR. Chapter 5 included an internal control in analyses and it revealed that many of the negative reactions were in fact due to inhibition. Given the large number of false negatives it is hard to see how FTA cards could be used unless the procedure was modified substantially as one would never know whether a negative reaction was really negative or due to inhibition or lack of template DNA. There is also an issue involved in the non uniform distribution of the target fungal material in this complex type of material. PCR inhibition or low DNA should be tested for in future trials (e.g. by always including and IAC plasmid). Whether entire spores are trapped on the card and/or are too small to squash efficiently should be established and the viability of spore material applied to the card examined.

The improvements that need to be made in order to capture DNA from such material with FTA cards effectively does not negate their potential usefulness. These improvements are actually very similar to those that could also be used for any material that poses extraction and PCR amplification problems e.g. the use of an IAC plasmid to determine the cause of PCR failure; grinding a small amount of material in an eppendorf containing an extraction buffer prior to spotting the solution from the ground mixture onto the card; dilution of a solution before application to the card; dilution of the DNA captured on the card; investigate different DNA polymerases; different target region; and different primers which specifically target a fungal group or species; nested PCR or cloning and sequencing.

The use of FTA cards to capture fungal DNA from soil will remain difficult because of the impurities in DNA samples from soil (Smalla *et al.* 1993). Soil is particularly notorious for PCR inhibition (Porteous and Armstrong 1991; Claassen *et al.* 1996; Yeates *et al.* 1997; Schneegurt *et al.* 2003). The addition of the IAC plasmid in PCR indicated both inhibition and low DNA. These problems may be solved as attempted in this thesis modifying the DNA extraction method: mixing soil samples with extraction buffer or mixing with sterile water before spotting the solution onto FTA cards. Soil is probably the least suitable material from which fungal DNA can be captured by FTA cards.

In summary, this research has shown that FTA cards could have some role to play in analytical procedures for detection of pathogens. It is possible to capture fungal DNA from a variety of fungal structures, plant material and water although methodology requires improvement especially in respect to the application of soil extracts to FTA cards. The effort given to improving techniques will depend on the advantages of using the technique versus the cost of the work involved to carry out the improvements and in the cost of the FTA cards themselves. The use of the cards will never replace traditional investigative techniques for fungal pathogens including microscopic examination and culturing. However when travelling long distances in Indonesia and working in remote locations, FTA card technology does offer a certain advantage - the possibility of collecting a large number of DNA samples from fresh material. The FTA cards can be used easily by untrained field assistants which are more likely to be available on such a trip than skilled

staff. The capture of DNA by FTA cards does not require any particular laboratory facilties which are unlikely to be available in remote locations. The ability to sample immediately and capture DNA will mean that contamination may be avoided e.g. the mycelium in root rot infections can often be peeled from under the bark in a root and could be ground in buffer at base camp and applied to a card. If a diseased root is transported in the topics, by the time isolations are carried out the material is usually contaminated even if vacuum sealed. FTA cards can be stored for long period of time at room temperature and are easily transported.

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APPENDICES

Appendix 3.1 PCR amplification of primers ITS-1F/ITS4 of DNA from pure fungal cultures and captured on FTA cards.

Code	Species/tag name	Fungal class	Age (Week)	PCR Amplification[1]
FF-24[1]	White paint, *Aleurodiscus*?	*Basidiomycota*	18	P,P,P
FF-59[1]	*	? *Basidiomycota*	6	P,N,N
FF-76[1]	White polypore	*Basidiomycota*	16	P,P,P
FF-120[1]	White cords	? *Basidiomycota*	3	P,P,P
FF-144[1]	Tiny, cottony rods	? *Basidiomycota*	15	P,P,P
FF-188[1]	*Hypoxylon crocopeplum*	*Ascomycota*	2	P,N,N
FF-214[1]	Creamy flat fungi	? *Basidiomycota*	16	P,P,P
FF-264[1]	Greyish snow	? *Basidiomycota*	91	N,P,P
FF-265[1]	Pink snow	? *Basidiomycota*	18	N,P,P
FF-266[1]	*	? *Basidiomycota*	16	N,P,P
FF-268[1]	Cream polypore	*Basidiomycota*	18	P,P,P
FF-269[1]	*	? *Basidiomycota*	107	P,P,P
M-U02[1]	*	? *Basidiomycota*	7	N,P,P
T-1394[1]	*Postia subcaesia*	*Basidiomycota*	7	P,N,P
T-1399[1]	*Postia subcaesia*	*Basidiomycota*	7	P,P,P
T-1400 A[1]	*Ganoderma.* aff. *australe*	*Basidiomycota*	7	P,P,P
T-1400 B[1]	*Ganoderma.* aff. *australe*	*Basidiomycota*	7	P,P,P
W-25 (iii)[1]	*Gymnopilus tyallus*	*Basidiomycota*	16	P,P,P
W-163[1]	*Chondrostereum purpureum.*	*Basidiomycota*	8	N,N,N
W-190[1]	*Crepidotus* sp.	*Basidiomycota*	107	N,P,N
W-225[1]	*Postia dissecta*	*Basidiomycota*	13	P,P,P
W-234[1]	*Ryvardenia crustacea*	*Basidiomycota*	39	P,P,P
W-276[1]	*Trametes hirsuta*	*Basidiomycota*	2	P,P,P
W-344[1]	*Panellus ligulatus*	*Basidiomycota*	7	P,P,P
Armillaria Cas[2]	*Armillaria luteobubalina*	*Basidiomycota*	43	P,P,P
Cyclaneusma SN 815[3]	*Cyclaneusma* sp.	*Ascomycota*	43	N,N,N
Cyclaneusma[3]	*Cyclaneusma* sp.	*Ascomycota*	43	N,N,N
IPC 10[3]	*Penicillium* sp.	*Ascomycota*	39	N,P,P
IPC 20/1[3]	*	*?Ascomycota*	99	N,P,P
IPC (32)[3]	*Lophodermium pinastri*	*Ascomycota*	16	P,P,P

[1] P indicates amplification of a fragment of approximately 600 bp (primer ITS 1F/4), N indicates no amplification *=unidentified macrofungus

Appendix 3.1 (Continued) PCR amplification of primers ITS-1F/ITS4 of DNA from pure fungal cultures and captured on FTA cards.

Code	Species/tag name	Fungal class	Age (Week)	PCR Amplification[1]
M-U01[3]	*Cyclaneusma minus*	*Ascomycota*	17	P,P,P
Mycosphaerella St. Marys[4]	*Mycospherella* sp.	*Ascomycota*	18	P,P,P
Mycosphaerella 80/1[4]	*Mycospherella* sp.	*Ascomycota*	16	P,P,P
10 B T 205[5]	*Ganoderma* sp.	*Basidiomycota*	17	P,P,P
12 T 175 B1[5]	*Phlebia* sp.	*Basidiomycota*	17	P,P,P
5 T 160 A2-1[5]	*Ganoderma* sp.	*Basidiomycota*	14	P,P,P
5 T 168 A2[5]	*Ganoderma mastoporum*	*Basidiomycota*	17	P,P,P
6 T 172 A2[5]	*Phanerochaete* sp.	*Basidiomycota*	17	N,N,P
6 T 200 A2-1[5]	*Phlebia* sp.	*Basidiomycota*	17	P,P,P
7 T 170 A2[5]	*Ganoderma.* aff. *australe*	*Basidiomycota*	17	P,P,P
8 T 169 A2[5]	*Ganoderma mastoporum*	*Basidiomycota*	17	P,P,P
8 T 201 A1[5]	*Ganoderma philippii*	*Basidiomycota*	13	P,P,P
Co 227[5]	*Unknown basidiomycete*	*Basidiomycota*	10	N,N,N
E 8809 A1[5]	*Phellinus* sp.	*Basidiomycota*	19	N,P,P
E 8812 A1[5]	*Ganoderma* sp.	*Basidiomycota*	19	P,P,P
E 8822 C1[5]	*Unknown basidiomycete*	*Basidiomycota*	19	P,P,P
E 8823 A2[5]	*Ganoderma* sp.	*Basidiomycota*	19	P,P,P
E 8828 C1[5]	*Formitopsis feei*	*Basidiomycota*	13	P,P,P
E 8831 A1[5]	*Ganoderma philippii*	*Basidiomycota*	17	P,P,P
E 8831 B1[5]	*Gymnopilus* sp.	*Basidiomycota*	17	P,P,P
E 8832 A1[5]	*Ganoderma philippii*	*Basidiomycota*	17	N,P,P
E 8832 B1[5]	*Ganoderma phiippii*	*Basidiomycota*	18	N,N,P
E 8842 C1[5]	*Ganoderma philpipii*	*Basidiomycota*	17	P,P,P
E 8851 A1[5]	*Ganoderma* aff. *australe*	*Basidiomycota*	17	P,P,P
E 8852 B1[5]	*Amauroderma rugosum*	*Basidiomycota*	20	N,P,N
E 8861 C2[5]	*Ganoderma* sp.	*Basidiomycota*	19	P,P,P
FB 1 A2[5]	*Phlebia* sp.	*Basidiomycota*	13	P,P,P
FB 16 B1[5]	*Ganoderma philippii*	*Basidiomycota*	17	P,P,P
FB 17 A1[5]	*Ganoderma subresinosum*	*Basidiomycota*	17	P,P,P
FB 20 A2[5]	*Pycnoporus sp.*	*Basidiomycota*	16	P,P,P
FB 4 A1[5]	*Ganoderma philippii*	*Basidiomycota*	17	P,P,P

[1] P indicates amplification of a fragment of approximately 600 bp (primer ITS 1F/4), N indicates no amplification

*=unidentified macrofungus

Appendix 3.1 (Continued) PCR amplification of primers ITS-1F/ITS4 of DNA from pure fungal cultures and captured on FTA cards.

Code	Species/tag name	Fungal class	Age (Week)	PCR Amplification[1]
FB1 B2? [5]	*Antrodia* sp.	*Basidiomycota*	19	P,P,P
T 19 A1[5]	*Trametes* sp.	*Basidiomycota*	17	P,P,P
T 57 A1[5]	*Fomes* sp.	*Basidiomycota*	19	P,P,P
T 42 B1[5]	*Fomes* sp.	*Basidiomycota*	19	P,P,P
T 72 B1[5]	*Amauroderma rugosum*	*Basidiomycota*	13	N,N,N
T 75 A2[5]	*Fomes* sp.	*Basidiomycota*	17	N,N,N
2A-1[6]	**	? *Ascomycota*	7	N,N,N
2A-2[6]	**	? *Ascomycota*	7	N,N,N
2A-36	**	? Ascomycota	7	N,N,N
2B-16	**	? Ascomycota	7	N,N,N
2B-26	**	? Ascomycota	7	N,N,N
2B-36	**	? Ascomycota	7	N,N,N
2B-46	**	? Ascomycota	7	N,N,N
P 6	**	? Ascomycota	7	N,N,N
Q6	**	? Ascomycota	7	N,N,N
R6	**	? Ascomycota	7	N,N,N
S6	*possible Fusarium sp.*	Ascomycota	7	N,N,N
T6	*possible Fusarium sp.*	Ascomycota	7	N,N,N
U6	*possible Fusarium sp.*	Ascomycota	7	N,N,N
Isolate A7	*Phytophthora sp.*	Oomycota	2	P,N,N

[1] P indicates amplification of a fragment of approximately 600 bp (primer ITS 1F/4), N indicates no amplification
*=unidentified macrofungus

Appendix 3.2 PCR amplification using primers ITS1-F/ITS4 of DNA captured from basidiocarps collected in northern Tasmania

Code	Species	PCR [1]	Code	Species	PCR [1]
NE01	*	N,P,N	T 1278	*Lactarius eucalypti*	N,P,P
NE02	*	N,P,N	T 1279	*Thaxterogaster* sp. A	P,P,P
NE03	*	P,N,N	T 1280	*Thaxterogaster* sp.	P,P,P
NE04	*	N,P,N	T 1281	*Laccaria* sp. A	N,P,P
T 1245	*	P,N,N	T 1282	*Lactarius eucalypti*	P,P,P
T 1246	*Lactarius eucalypti*	P,P,P	T 1284	*Cortinarius* sp. A	P,P,P
T 1247	*Lactarius eucalypti*	N,P,P	T 1286	*	P,N,N
T 1248	*Laccaria* sp. B	N,P,P	T 1287	*Dermocybe* sp. A	P,P,P
T 1249	*	P,N,N	T 1289	*Cortinarius* sp. B	N,P,P
T 1250	*Lactarius eucalypti*	P,P,P	T 1290	*	P,N,N
T 1251	*Lactarius* sp. B	P,P,P	T 1291	*Cortinariaceae* sp. A	P,P,P
T 1252	*Descomyces* sp. A	P,P,P	T 1292	*	P,N,N
T 1253	*	**N,N,N**	T 1293	*Boletaceae* sp. A	N,P,P
T 1254	*	P,N,N	T 1295	*Lactarius* sp. A	P,P,P
T 1255	*Lycoperdon* sp. A	N,P,P	T 1297	*Boletaceae* sp. B	P,P,P
T 1256	*Laccaria* sp. C	N,P,P	T 1298	*Thaxterogaster* sp. B	P,P,P
T 1257	*	P,N,N	T 1299	*Lactarius eucalypti*	N,P,P
T 1258	*	**N,N,N**	T 1300	*Inocybe* sp. A	N,P,P
T 1259	*Hydnum umbilicatum*	P,P,P	T 1301	*Russula* sp. A	N,P,P
T 1260	*Basidiomycete* sp. A	N,P,P	T 1358	*Galerina sp.*	N,P,P
T 1261	*Cortinarius* sp. D	N,P,P	T 1359	*Mycena spp.*	P,P,P
T 1262	*Lactarius* sp. B	P,P,P	T 1360	*Hygrocybe astatogala*	N,P,P
T 1263	*Laccaria* sp. E	N,P,P	T 1361	*Mycena subgalericulata*	N,P,P
T 1264	*Russula* sp. B	P,P,P	T 1362	*Tremella fuciformis*	P,P,P
T 1265	*Cortinariaceae* sp. B	N,P,P	T 1363	*Gymnopilus feruginosus*	P,P,P
T 1266	*Lactarius eucalypti*	N,P,P	T 1364	*Panellus longinquus*	P,P,P
T 1267	*	P,N,N	T 1365	*Laccaria spp.*	P,P,P
T 1268	*	**N,N,N**	T 1366	*Ryvardenia campyla*	N,P,P
T 1269	*Boletaceae* sp. C	N,P,P	T 1367	*Cortinarius spp.*	P,P,P
T 1270	*Cortinarius* sp. E	P,P,P	T 1368	*Clavariaceae*	N,P,P

[1] P indicates PCR amplification of a fragment of approximately 600 bp (primer ITS 1F/4), N indicates no amplification

Appendix 3.2 (Continued) PCR amplification using primers ITS1-F/ITS4 of DNA captured from basidiocarps collected in northern Tasmania

Code	Species	PCR [1]	Code	Species	PCR [1]
T 1271	*	**N,N,N**	T 1369	*Collybia eucalyptorum*	N,P,P
T 1272	*Laccaria* sp. E	P,P,P	**T 1372**	*	**N,N,N**
T 1273	*Cortinarius* sp. F	P,P,P	T 1373	*	P,N,N
T 1275	*Cortinarius* aff. *schlerophyllarum*	N,P,P	**T 1374**	*	**N,N,N**
T 1276	*Laccaria* sp. D	P,P,P	T 1375	*	P,N,N
T 1277	*Cortinarius* sp. C	P,P,P	**T 1376**	*	**N,N,N**

[1] P indicates PCR amplification of a fragment of approximately 600 bp (primer ITS 1F/4), N indicates no amplification

Appendix 3.3 PCR amplification using primers ITS1-F/ITS4 of DNA capture on FTA cards from spore-prints

Sample Code	Sample	PCR amplification (ITS1F-ITS4)[1]		
		1	2	3
SP1	*Cortinarius* sp.	P	P	P
SP2	Spore-print of *Cortinarius*	P	P	P
SP3	Spore-print of *Cortinarius*	P	P	P
SP4	*Agaricus bisporus*	P	P	P
SP5	Unidentified	N	N	N
SP6	Unidentified	P	P	P
SP7	Spore-print of *Cortinarius*	P	P	P
SP8	Spore-print of *Cortinarius*	P	N	P
SP9	Spore-prints of *Psathyrella*	P	P	P
SP10	Spore-prints of *Psathyrella*	P	P	P

[1] P indicates PCR amplification of a fragment of approximately 600 bp (primer ITS 1F/4), N indicates no amplification

Appendix 3.4 PCR amplification using primers Bt 2a/Bt 2b and EF1/EF2 of DNA captured on FTA cards of fungal cultures isolated from *Acacia mangium* seedlings

Code	Name	PCR amplification[1]					
		Bt 2a/ Bt 2b			EF1/EF2		
		1	2	3	1	2	3
2A-1	Black lesion (brown myc)	**P**	**P**	**P**	N	N	N
2A-2	Black lesion (brown myc)	**P**	**P**	**P**	N	N	N
2A-3	Black lesion (brown myc)	**P**	**P**	**P**	N	**P**	N
2B-1	Black lesion (dark green myc)	**P**	**P**	N	N	N	N
2B-2	Black lesion (dark green myc)	N	N	**P**	N	N	N
2B-3	Black lesion (dark green myc)	**P**	**P**	**P**	N	N	**P**
2B-4	Black lesion (dark green myc)	**P**	**P**	**P**	N	N	N
P	Black lesion	**P**	**P**	**P**	N	N	N
Q	DJ sample 1A	**P**	**P**	**P**	N	N	N
R	DJ sample 1B	**P**	**P**	**P**	N	N	N
S	Possible Fusarium	N	**P**	N	**P**	**P**	**P**
T	Possible Fusarium	**P**	**P**	**P**	**P**	**P**	**P**
U	Possible Fusarium	**P**	**P**	**P**	**P**	**P**	**P**

[1] P indicates amplification of a fragment of approximately 550 bp (primer Bt2a/2b) and 400 bp (primer EF1/EF2), N indicates no amplification

Appendix 3.5 PCR amplification using species specific primers Gphil2F/Gphil6R and Gphil3F/Gphil4R of DNA captured on FTA cards from basidiomycete cultures

Code	Name	PCR Amplification[1]					
		Gphil 2F/6R			Gphil 3F/4R		
		1	2	3	1	2	3
10 B T 205	*Ganoderma* sp.	N	N	N	N	N	N
12 T 175 B1	*Phlebia* sp.	N	N	N	P	N	N
5 T 160 A2-1	*Ganoderma* sp.	N	N	N	N	N	N
5 T 168 A2	*Ganoderma mastoporum*	N	N	N	N	P	P
6 T 172 A2	*Phanerochaete* sp.	N	N	N	N	N	N
6 T 200 A2-1	*Phlebia* sp.	N	P	N	N	N	N
7 T 170 A2	*Ganoderma* aff. *australe*	N	P	N	N	N	N
8 T 169 A2	*Ganoderma mastoporum*	N	N	N	P	N	N
8 T 201 A1	*Ganoderma philippii*	P	P	P	P	P	P
Co 227	unknown basidiomycete	N	N	N	N	N	N
E 8809 A1	*Phellinus* sp.	N	N	N	N	N	N
E 8812 A1	*Ganoderma* sp.	N	N	N	N	N	N
E 8822 C1	unknown basidiomycete	N	N	N	N	N	N
E 8823 A2	*Ganoderma* sp.	N	N	N	N	N	N
E 8828 C1	*Formitopsis feei*	N	P	N	N	N	N
E 8831 A1	*Ganoderma philippii*	P	P	P	P	P	P
E 8831 B1	*Gymnopilus* sp.	N	N	N	N	N	N
E 8832 A1	*Ganoderma philippii*	P	P	P	P	N	P
E 8832 B1	*Ganoderma philippii*	P	N	P	P	P	N
E 8842 C1	*Ganoderma philippii*	P	P	P	P	N	P
E 8851 A1	*Ganoderma* aff. *australe*	N	N	N	N	N	N
E 8852 B1	*Amauroderma rugosum*	N	N	N	N	N	N
E 8861 C2	*Ganoderma* sp.	N	N	N	N	N	N
FB 1 A2	*Phlebia* sp.	N	N	N	N	N	N
FB 16 B1	*Ganoderma philippii*	P	P	P	P	N	P
FB 17 A1	*Ganoderma subresinosum*	N	N	N	N	N	N
FB 20 A2	*Pycnoporus* sp.	N	N	N	N	N	N
FB 4 A1	*Ganoderma philippii*	P	P	P	P	N	P
FB1 B2?	*Antrodia* sp.	N	N	N	N	N	N
T 19 A1	*Trametes* sp.	N	N	N	N	N	N
T 42 B1	*Fomes* sp.	N	N	N	N	N	N
T 57 A1	*Fomes* sp.	N	N	N	N	N	N
T 72 B1	*Amauroderma rugosum*	N	N	N	P	N	N
T 75 A2	*Fomes* sp.	N	N	N	N	N	N

[1] P indicates positive amplification, N indicates no amplification

Appendix 3.6 Sequencing of PCR products and identification based on BLAST search

Code	Sample details	BLAST RESULTS									Identification
		Best match			Second best match			Third best match			
		Accession No.	Species Name	Match *	Accession No.	Species Name	Match *	Accession No.	Species Name	Match *	
SP1	Spore-print of Cortinarius	AF389160	*Cortinarius amoenus*	371/405/404	DQ974717	*Cortinarius* cf. *glaucopus*	369/404/404	AF389159	*Cortinarius icterinus*	370/404/404	*Cortinarius* sp.
SP4	Spore-prints of *Agaricus bisporus*	EF460355 (+24)**	*Agaricus bisporus*	453/455/455	AF432882	*Agaricus bisporatus*	452/455/455	AJ884646 (+15)	*Agaricus bisporus*	450/452/455	*Agaricus bisporus*
SP9	Spore-prints of *Psathyrella*	AY228352	*Psathyrella* cf. *gracilis*	406/406/406	DQ389684 (+1)	*Psathyrella microrrhiza*	405/406/406	DQ389685	*Psathyrella lutensis*	390/406/406	*Psathyrella* sp.
Gr1	Grass rust	AY836372 (+6)	*Eudarluca caricis*	321/321/321	AJ550891 (+1)	*Leptosphaeria biglobosa*	263/277/321	LET58S RDNA	*Leptosphaeria maculans*	262/277/321	*Eudarluca* aff. *caricis*
FF59	unknown	EU486442 (+12)	*Hypholoma fasciculare*	395/405/405	FJ596780 (+1)	*Hypholoma capnoides*	382/405/405	DQ490634	*Nematoloma longisporum*	362/384/405	*Hypholoma* aff. *fasciculare*
W25 (iii)	*Gymnopilus tyallus?*	AJ608709	*Ganoderma applanatum*	366/367/367	AJ006685	*Ganoderma adspersum*	365/367/367	AF255158	*Ganoderma* sp.	362/363/367	*Ganoderma* sp.
Cu7	*Armillaria* Cas	AF394916 (+9)	*Armillaria luteobubalina*	430/434/434	FJ660940	*Armillaria mellea*	260/264/434	FJ660939 (+15)	*Armillaria* sp.	260/264/434	*Armillaria luteobubalina*
Cu8	*Mycosphaerella* sp.	DQ302951 (+39)	*Mycosphaerella cryptica*	307/307/307	DQ240187	*Colletogloeopsis* sp.	297/307/307	AY244420	*Coniothyrium zuluense*	297/307/307	*Teratosphaeria cryptica (= Mycosphaerella cryptica)*

[1] These accessions are considered to be misidentified as the sequence had low similarity to many other accessions from that species.
(*: indicates number of matching nucleotides/length of aligned region/ length of the sequence submitted **: indicates the number of accessions from the same species with the same sequence similarity)

Appendix 3.6 (Continued) Sequencing of PCR products and identification based on BLAST search

Code	Sample details	BLAST RESULTS									Identification
		Best match			Second best match			Third best match			
		Accession No.	Species Name	Match *	Accession No.	Species Name	Match *	Accession No.	Species Name	Match *	
Cu10	*Mycosphaerella* sp.	AY725574 (+1)	*Teratosphaeria nubilosa*	299/305/311	DQ665659 (+6)	*Mycosphaerella nubilosa*	299/305/311	AY534230 (+2)	*Mycosphaerella* sp.	299/305/311	*Teratosphaeria nubilosa (= Mycosphaerella nubilosa)*
FF214	Creamy flat fungi	FJ662408 (+48)	*Pichia guilliermondii*	300/332/332	AF209874[1]	*Debaryomyces hansenii*	300/332/332	FJ196619	*Pichia* sp.	300/332/332	*Pichia* sp.
FF 120	white cords fungi	AY805597	*Verticillium* sp.	333/338/347	AB378523	*Lecanicillium* sp.	335/347/347	AF324874(+8)	*Verticillium fungicola (= Lecanicillium fungicola)*	335/347/347	*Lecanicillium* sp.
W225	*Postia dissecta*	DQ132810 (+2)	*Pochonia bulbillosa*	336/336/336	DQ888743	*Verticillium* sp.	335/336/336	AJ292409	*Verticillium gonioides (= Pochonia gonioides)*	331/332/336	*Pochonia* sp.
W276	*Trametes hirsuta*	EF546240	*Trametes hirsuta*	348/353/352	FJ462762[1]	*Stereum hirsutum*	348/352/353		*Trametes hirsuta*	347/353/352	*Trametes hirsuta*
FF144	tiny, cottony rods	EU486442 (+12)	*Hypholoma fasciculare*	397/402/402	FJ596780 (+5)	*Hypholoma capnoides*	384/402/402	AY818349	*Hypholoma sublateritium*	379/402/402	*Hypholoma aff. fasciculare*
Cu21	Unknown	AY315402	*Xylariaceae sp.*	326/326/339	DQ658238	*Nemania diffusa*	328/329/339	AY909015	*Xylaria longipes*	323/336/339	*Xylariaceae* sp.
FF76	white polypore	AF515584	*Fomitiporia punctata*	68/77/358	AY313282	*Omphalotus subilludens*	68/77/358	FJ903378	*Basidiomycota sp.*	85/99/358	Basidiomycota sp.

[1] These accessions are considered to be misidentified as the sequence had low similarity to many other accessions from that species.

[2] Primer used to amplify the samples. EF indicates elongation factor primer, Bt indicates Beta-tubulin

(*: indicates number of matching nucleotides/length of aligned region/ length of the sequence submitted **: indicates the number of accessions from the same species with the same sequence similarity)

Appendix 3.6 (Continued) Sequencing of PCR products and identification based on BLAST search

Code	Sample details	BLAST RESULTS									Identification
		Best match			Second best match			Third best match			
		Accession No.	Species Name	Match *	Accession No.	Species Name	Match *	Accession No.	Species Name	Match *	
W344	*Panellus ligulatus*	EU486442 (+15)	*Hypholoma fasciculare*	397/453/531	EF530927 (+15)	*Hypholoma capnoides*	241/271/531	AF335450	*Hypholoma sp.*	222/252/531	*Hypholoma* sp
T1400	*Ganoderma australe*	AJ608709	*Ganoderma applanatum*	366/367/367	AJ006685	*Ganoderma adspersum*	365/367/367	AF255158.	*Ganoderma sp*	362/363/367	*Ganoderma* sp.
T1399	*Postia pelliculosa*	AJ006666 (+1)	*Postia balsamea*	190/196/354 (+ 101/112/354)	AY599566 (+1)	*Oligoporus balsameus*	190/196/354 (+ 101/112/354)	FJ403209	*Daedalea microsticta*	168/169/354	*Postia* sp.
Isolate A	Possible Phytophthora	EF601645 (+1)	*Mortierella* sp.	307/309/309	EF152528 (+2)	*Mortierella gamsii*	305/310/309	EU428773	*Zygomycete sp.*	304/310/309	*Zygomycete* sp.
U	Possible Fusarium (EF)[2]	EU246568	*Fusarium oxysporum*	641/653/669	EF452995 (+7)	*Fusarium* sp. (*F. oxysporum* species complex)	633/647/669	DQ452422	*Fusarium solani*	627/653/669	*Fusarium oxysporum*
2A-2	Possible Fusarium (Bt)[2]	EU541420 (+1)	*Phoma exigua*	295/307/313	EU541415 (+1)	*Phoma eupyrena*	294/306/313	EU541434	*Phoma sojicola*	287/299/313	*Phoma* sp.
2B-2	Possible Fusarium (Bt)	EU541420 (+1)	*Phoma exigua*	294/306/543	EU541415 (+1)	*Phoma eupyrena*	293/305/543	EU541434	*Phoma sojicola*	288/300/543	*Phoma sp.*
P	Possible Fusarium (Bt)	FJ466740 (+9)	*Fusarium oxysporum*	304/304/506	DQ092470	*Fusarium solani*	303/304/506	AY714095	*Fusarium oxysporum*	303/304/506	*Fusarium oxysporum*

[1] These accessions are considered to be misidentified as the sequence had low similarity to many other accessions from that species.
[2] Primer used to amplify the samples. EF indicates elongation factor primer, Bt indicates Beta-tubulin
(*: indicates number of matching nucleotides/length of aligned region/ length of the sequence submitted **: indicates the number of accessions from the same species with the same sequence similarity)

Appendix 3.6 (Continued) Sequencing of PCR products and identification based on BLAST search

Code	Sample details	BLAST RESULTS									Identification
		Best match			Second best match			Third best match			
		Accession No.	Species Name	Match *	Accession No.	Species Name	Match *	Accession No.	Species Name	Match *	
S	Possible Fusarium (Bt)	FJ466740 (+9)	*Fusarium oxysporum*	305/305/309	DQ092470	*Fusarium solani*	304/305/309	AY714095	*Fusarium oxysporum*	304/305/309	*Fusarium oxysporum*
U	Possible Fusarium (Bt)	FJ466740 (+9)	*Fusarium oxysporum*	303/304/459	DQ092470	*Fusarium solani*	302/304/459	AY714095	*Fusarium oxysporum*	302/304/459	*Fusarium oxysporum*
T	Possible Fusarium (Bt)	FJ466740 (+9)	*Fusarium oxysporum*	305/305/428	DQ092470	*Fusarium solani*	304/305/428	AY714095	*Fusarium oxysporum*	304/305/428	*Fusarium oxysporum*

[1] These accessions are considered to be misidentified as the sequence had low similarity to many other accessions from that species.
[2] Primer used to amplify the samples. EF indicates elongation factor primer, Bt indicates Beta-tubulin
(*: indicates number of matching nucleotides/length of aligned region/ length of the sequence submitted **: indicates the number of accessions from the same species with the same sequence similarity)

Appendix 3.7 Sequences data in FASTA format

>SQ1 SP1, Spore print of *Cortinarius*, ITS1F/ITS4

GATCATTATTGAAATAAATCTAGCTTGGTTGATGCTGGTCTTTTGGAAGACATGTG
CACACCTGGTCATCTTCTGGGTCATTTAGAGAAAGTAAAAGTCGTAACAAGTTTTC
GTTTGGTGAACCAGCGGAGGGATCATTACGGAGTTTACAATTCCCAAACCCCTGTA
AACATACCTATTTTTCTTTCGGCGGGATCGCCCCGGCGCCCTCGGCCCCGAACCCA
GGCCCCCCCCCGGAGGCCCCAAACTCTTTTCTTTAATGAGAATTTTTTGAGTAACAT
AAGCAAATAAATTAAAACTTTCAACAACGAATTTTTTGGTTCTGGCATCAATGAAG
AACGCAGCAAAATGCGATAAGTAATGTAAATTCCAAAATTCCGGGATTCATCGAA
TCTTTAAACGCCCATTGCCCCCGCCAGTATTCGGGCGGCCATCCCTGTTCAAGGGT
CATTT

>SQ2 SP2, Spore print of *Agaricus bisporus*, ITS1F/ITS4

GATCATTATTGAAATAAACCTGATGAGTTGCTGCTGGTTCTCTAGGGGACATGTGC
ACACTTGTCATCTTTATATCTCCACCTGTGCACCTTTTGTAGACCTGGAAGTCTTTT
CTGAATGGGCCCAATTCAGGTTTTGAGGATTGGCTCTCTGTCATTCCTTACATTTCC
AGGCCTATGTCTCTTCATATACTCTATTGTATGTCATAGAATGTAACTAATAGGAC
TTTGTGCCTATAATAAATCATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCAC
GATGAAGAACGCAGCGAAATGCGTTAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATTTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTT
GAGTGTCAT

>SQ3 SP4, Spore print of *Psathyrella*, ITS1F/ITS4

GATCATTAATGAATATCTATGGCGTTGGTTGTAGCTGGCTTCTAGGAGCATGTGCA
CACCCGCCATTTTTATCTTTCCACCTGTGCACTAAATGTAGATCTGGATAACTCTCG
CTCTCACGAGCGGATGCAAGGATTGCTGTGTCGCAAGACCGGCTCTCCTTGAACTT
CCAGGTCTATGTACCCTTTACACACCCCAATTGAATGATGAAGAATGTAGTCAATG
GGCTCTAAGCCTATAAAACAAAATACAACTTTCAGCAACGGATCTCTTGGCTCTCG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCC
TGTTTGAGTGTCAT

>SQ4 Gr1, Grass rust, ITS1F/ITS4

GATCATTACCCTTCTTAATCAGAGGTTTAGCCTTCATTGGCCGTATACCTTTCTGAT
TTTACCCATGATTTTGCGCACTATTTGTTTCCTCGGCGGGCTTGCCCGCCGATTGGA
CACCCTACAACCCTTGTAATTGCAATCAGCGTCAGTAACAAGTAATTATTACAACT
TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT
AAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
CCCCATGGTATTCCGTGGGGCATGCCTGTTCGAGCGTCAT

>SQ5 FF59, Unknown, ITS1F/ITS4

GATCATTATTGAATAAATCTGGCTTGGTTGATGCTGGTCTTTTCGAGGACATGTGC
ACATCTTGTCATCTCTTTATATCTCCACCTGTGCACCTTTTGTAGACCTGGATTCAA
CTTTCCGAGGAAACTCGGTTGTGGGGAATTGCTTGATAGGCTTTCCTTGTTAGTTTC
CAGGGCTATGTTTTTCATATACACCTTACGAATGTAACAGAATGTCATTAATTAGG
CTTAATTGCCTTATAAACTATATACAACTTTCAGCAACGGGATCTCTTGGCTCTCGC
ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCT
GTTTGAGTTTCAT

Appendix 3.7 (Continued) Sequences data in FASTA format

>SQ6 W25 (iii), *Gymnopilus tyallus* ?, ITS1F/ITS4

GATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCACGTGCACGCC
CTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTTTACGGGTCGCGAAACGG
GCTCGTTATTCGTGCTTGTGGAGCGCACTTGTTGCCTGCGTTTATCACAAACTCCAT
AAAGTATTAGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACG
GATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTA
TTCCGAGGAGC ATGCCTGTTTGAGTGTCAT

>SQ7 Cu7, *Armillaria*-Cas, ITS1F/ITS4

GATCATTATTGAAGCTTGAATCGTAGCGTTGAGAGCTGTTGCTGACCTGTTAAAGG
GTATGTGCACGTTCAAAGTGTTGCGTTTTATTCTTTTCCCCCTGTGCACCTTTGTAG
ACTTGGTTAAGGATGTCGCTGTTGAGTGTTGCTCTTGAGCTCCCTTTGATTTTTGAA
GGGTTGCTTTCGAGCTTCCCTTTCTTTGTCTACCAAGTCTATGTCTATAATCTCTTGT
ATGTGTAGAATGTCTTGTTTATTGGATGCTTGCGTCCTTTAAATCTTATACAACTTT
CAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAA
CTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACCTTGCGCC
CTTTGGTATTCCGAAGG GCATGCCTGTTTGAGTGTCAT

>SQ8 Cu8, *Mycosphaerella* sp., ITS1F/ITS4

GATCATTACCGAGTGAGGGCGCCCGCCCGACCTCCAACCCCATGTTTTCCAACCAT
GTTGCCTCGGGGGGCGACCCGGCCGCCGTGCCGGGGCCCCCGGCGGACCCCTCAAC
TCTGCATCTTTGCGTCTGAGTGATAACGAAAAATCAATCAAAACTTTCAACAACGG
ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTC
TGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCAT

>SQ9 Cu10, *Mycosphaerella* sp., ITS1F/ITS4

GATCATTTACTGAGTGCGGGCGCCAGCCCGACCTCCCACCCCCATTGTTTTCCCCA
CCACGTTGCCTCGGGGGGCGACCCGGCCACCGCGCCGGGGCCCTCGCAGGACGCC
TCAACGCTGCATCTGTGCGTCGGAGTAATACAACCAATCAATTAAAACTTTCAACA
ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTG
GTATTCCGGAGGGCATGCCTGTTCGAGCGTCAT

>SQ10 FF214, creamy flat fungi, ITS1F/ITS4

GATCATTACAGTATTCTTTATCCAGCGCTTAACTGCTCGGCGAAAACCGTTACACA
CAGCGTCTGTTTGATACAGAACTCTTGCCTGGGTCCCCCGTAGAGATAGCCGGGGC
CAGAGGTTTAACAAAACACAATTTAATTATTTTTACAGTTAGTCAAATTTGGAACT
CATCTTCAAAACTTTCAACAACGGATCTCTGGGTTCTCGCATCGAGGAAGAACGCA
GAAAAATGCGATAAGTAATATGAATTGCAAATTTTAGAGAATCATCGAATCTTTG
AACGCACATTGCGCCCTTTGGTCTTCCACAGGGCATGCCTCATTGAGCGTCAT

>SQ11 FF120, White cords fungi, ITS1F/ITS4

GATCATTACAGAGTTTACAACTCCCAAACCCAAATGTGAACATACCTATCGTTGCT
TCGGCGGACTCGCCCCGGCGTCCGGTCGACCTTGCGTCGGCCGCGGCCCGGAACC
AGGCGGCCGCCGGAGACCATCAAACTCTTTGTATTATCAGTATCTTCTGAATCCGC
CGCAAGGCAAAACAAATGAATCAAAACTTTCAACAACGGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCAATTGCGCCCGCCAGCATTCTGGCGGGCATGCCT
GTTCGAGCGTCAT

Appendix 3.7 (Continued) Sequences data in FASTA format

>SQ12 W225, *Postia dissecta*, ITS1F/ITS4

GATCATTACCGAGTTATTCTACTCCCAAACCCCTGTGAACTTATACCTTTACTGTTG
CTTCGGCGGGTTAACGCCCCGGAAGGCCCGCGAGGGCCGCCGGAACCAGGCGCCC
GCCGGGGGACCAAAACTCTTGTATCTTTTTTATAGCATGTCTGAGTGGAATCATAA
ACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGA
ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT
CTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTC
AT

>SQ13 W276, *Trametes hirsuta*, ITS1F/ITS4

GATCATTAACGAGTTTTGAAATGGGTGTTGCTGGCCTTCCGAGGCATGTGCAGGCC
CTGCTCATCCACTCTACACCTGTGCACTTACTGTAGGTTGGCGTGGGTTTGTAGCCT
CCGGGCTGGGAGCATTCTGCCGGCTTATGTACACTACAAACTCTAAAGTATCAGAA
TGTAAACGCGTCTAACGCATCTTAATACAACTTTCAGCAACGGATCTCTTGGCTCT
CGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATG
CCTTTTTGAGTGTCAT

>SQ14 FF144, Tiny, cottony rods, ITS1F/ITS4

GATCATTATTGAATAAATCTGGCTTGGTTGATGCTGGTCTTTTCGAAGACATGTGC
ACACCTGGTCATCTTTATATCTCCACCTGTGCACCTTTTGTAGACCTGGATTCAACT
TTCCGAGGAAACTCGGTTGTGAGGAGTTGCTTAATAGGCTTTCCTTGTTCGTTTCCA
GGGCTATGTTTTCATATACACCTTACGAATGTAACAGAATGTCATTATTAGGCTTA
ATTGCCTTATAAACTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTT
TGAGTGTCAT

>SQ15 Cu21, unknown, ITS1F/ITS4

GATCATTAAAGAGTGTAATAACTCCCAAACCCATGTGAACATACCTCATGTTGCCT
CGGCAGGTCGCGCCTACCCCGCAGACCCCTACCCTGTAGGGCCTACCCGGAAGGC
GCGGGTAACCCTGCCGGCGGCCCACGAAACTCTGTTTAGTATTGAATTCTGAACCT
ATAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCG
AATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGC
GTCAT

>SQ16 FF76, White polypore, ITS1F/ITS4

GAATCATTCCGAGTTTTGGATGGGTGGAGGTGCCTTTCGAAGCATGGGCGCGCAC
GGGTTCATCCACTCTGAACCTGTGAACTTCTTGGGGTGACGGGTGGAAAAAAGAC
GGGTTTATTCGGGCTAGTGGAGCGCCCGTGTTGCTGCGGTTTATCATAACCCCCAA
AAATATAAGAATGTGATTTTGGATAAAACGCATCAAAATAAAAGTTGCAGCAACG
GACCACTTGCTCCGGCATGATAAAGAATGCAGAGACTGGAATAAGTAATGTTAAT
TGCAGAGTTCATGAAATAATCAAATCTTGGAACGACCTTGCGGCCCTTGGTATTCC
GAGGGAGCAGCCGGTATG AGTGTCAT

Appendix 3.7 (Continued) Sequences data in FASTA format

>SQ17 W344, *Panellus ligulatus*, ITS1F/ITS4
GATCATTATGAATAAATCGGCTTGTTGATGCTGTCTTTCCGAGACATGTGCCCCCC
GGTCATCTTTAATTTTCACCTGGGCACCTTTTGGTGGTCTGGATTCCACTTTCCGAA
GAAACTTGGTTTGAGGAGGTTCCTTATTAGCCTTCCCTGGTTGTTTCCAAGGGTAA
GTTTTTATATACCCCTTACGAATGTAACCGAATGTCCTTATTAGGGTTAATTGCCTT
ATTAACCATATACCACTTTCAGCAACGGATTTTTTGGCTCTTGCATCGATGAAGAA
CGCAACGAAATGCGATAAGTAATGTGAATTGCCGAATTCAATGAATCATTGAATC
TTTGAACGCCCCCTGCGCTCCTTGGTATTTCGAGGGGCCAGCCTGTTTGAGGGTCC
TTAAATTTTCCACCTTTATTAACTTTTTTGGTTAGTAAATGGATTGGAAGGGGGGG
CAATGTTGGTTTCTTCACTGAAATAAACTCCCCTGAAATGCATTAGCTGGTTGCCTT
GTGCAAACATGTCTATTGAGTGTGAT

>SQ18 T1400, *Ganoderma australe*, ITS1F/ITS4
GATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCACGTGCACGCC
CTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTTTACGGGTCGCGAAACGG
GCTCGTTTATTCGTGCTTGTGGAGCGCACTTGTTGCCTGCGTTTATCACAAACTCCA
TAAAGTATTAGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAAC
GGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGT
ATTCCGAGG AGCATGCCTGTTTGAGTGTCAT

>SQ19 T1399, *Postia pelliculosa*, ITS1F/ITS4
GATCATTATTGAATTTTTGAAGGAGCTGTTTGCTGGCCCTTGACGGGGCATGTGCA
CGCTTCGTTTCAAATCTCCAACCTCTTCATACCCCTGTGCATCTTTTGTAGGGTCGC
ATCGGTCGAAAGGCCGGTGTGCTCTATGTCATATCACAAACTCTTGTATGTGTAGA
ATGTTCAATGCGCACGACGCATCTTTATACAACTTTCAGCAACGGATCTCTTGGCT
CTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATC
CAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCA
TGCCTGTTTGAGTGTCAT

>SQ20 Isolate A, possible *Phytophthora*, ITS1F/ITS4
GATCATTCATAATAAGTGTTTTATGGCACTTTTTAAATCCATATCCACCTTGTGTGC
AATGTCAGTTGATCTTCTTTATGGAGATCAACCAAACATCAACCTAATTTTTTAACT
CTTTGTCTGAAAAATATTATGAATAAATAATTCAAAATACAACTTTCAACAACGGA
TCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCATATTGCGCTCTTTGGTATTC
GAAGA GCATGCTTGTTTGAGTATCAG

>SQ21 U, Possible Fusarium, EF1/EF2
GTCTCAAGAAGTCGACTCTGGCAGTCGACCACTGTGAGTACTCTCCTCGACAATGA
GCTTATCTGCCATCGTCAATCCCGACGAAGACCTGGCGGGGTATGTCTCAAAGTCA
ACATACTGACATCGTTTCACAGACCGGTCACTTGATCTACCAGTGCGGTGGTATCG
ACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTTACTTTCCATTCAATCGCGC
GTCCTTTGCCCATCGATTTCCCCTACGACTCGAAACGTGCCCGCTACCCCTCTCGA
GACCAAAAATTTTGCAATATGACCGTAATTTTTTTGGTGGGGCATTTACCCCGCCC
CTCGGGTGCCGGGCGCGTTTGCCCTCTTACCATTCTCACAACCTCAATGAGCGCAT
CGTCACGTGTCAAGCAGTCACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGG
TAAGGGTTCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCTGAGCGTGAGC
GTGGTATCACCATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTC
ACCGTCATTGGTATGTTGTCGCTCATGCTTCATTCTACTTCTCTTCGTACTAACACA
TCACTCAGACGCTCCCGGTCACCGTGATTTCATCAAGAACATGATCATGGTACC

Appendix 3.7 (Continued) Sequences data in FASTA format

>SQ22 2A-2, Possible Fusarium, Bt2a/Bt2b

TCTCCCGCGAGCATGGCCTCGACGGCTCCGGTGTCTACAATGGCACCTCGGACCTC
CAGCTCGAGCGCATGAACGTCTACTTCAACGAGGTACTAGAAATGACACGCTGTC
CTTGGACGGACTGCGAGTGCTGACCCGCTTCTAGGCCTCCGGCAACAAGTTCGTTC
CCCGCGCCGTCCTCGTCGATTTGGAGCCCGGAACAATGGACGCCGTCCGCGCTGGC
CCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCGTCTTCGGCCAGTCTGGTGCTGGT
AACAATTGGGCCAAGGGTCACTACACTGAGGGTA

>SQ23 2B-2, Possible Fusarium, Bt2a/Bt2b

ATCTCGGCGAGCATGGCCTCGACGGCTCCGGTGTCTACAATGGCACCTCGGACCTC
CAGCTCGAGCGCATGAACGTCTACTTCAACGAGGTACTAGAAATGACACGCTGTC
CTTGGACGGACTGCGAGTGCTGACCGCTTCTAGGCCTCCGGCAACAAGTTCGTTCC
CCGCGCCGTCCTCGTCGATTTGGAGCCCGGAACAATGGACGCCGTCCGCGCTGGCC
CCTTCGGTCAGCTCTTCCGTCCCGACAACTTCGTCTTCGGCCAGTCTGGTGCTGGTA
ACAATTGGGCCAAGGGTCACTACACTGAGGGAAATAATATGTGTTAAGATCGATG
AAACACTGAATTCTGCAATTCACATTACTATCGCATTTCACTGCGTTCTCACGATG
CGAGAGCCAAACCCCCGGGGAGACAATGGATTTTGAAGTATTTCTCTCCCAAAAT
CTTCCTACGAACAGTCCAAAAATTAAGGTGATTTTGGCTGATCACCGCCCCAAGAT
TCGACCGACATAGCAAGAAATTGGATATCCATTTAATACTT

>SQ24 P, Possible Fusarium, Bt2a/Bt2b

CTCTGGCGAGCACGGCCTCGACAGCAATGGTGTCTACAACGGTACCTCCGAGCTCC
AGCTCGAGCGCATGAGTGTCTACTTCAACGAGGTATGCATTAACAGTCAATGTCAA
GAATTCCCAAGCTCACACAACTAGGCCTCTGGCAACAAGTACGTTCCCCGAGCCGT
CCTCGTCGATCTTGAGCCTGGTACCATGGACGCCGTCCGTGCTGGTCCCTTCGGTC
AGCTCTTCCGTCCCGACAACTTCGTTTTCGGTCAGTCCGGTGCTGGAAACAACTGG
GCCAAGGGTCACTACACTGAGGGTAACATCTAATACGCTGCGACACAGGGAGAGA
GAAGAAATAAATGCGTTTATGAAAGATAAGATATTCCTAGATTAAATTCAAGAGT
TGGGTAGGGTCCCATGGGGGGCCTCACCGTCGTGAGGAGGGCATCGCCCAGGAAA
CCACGCCAGACCATTCGCATTGATAAGCCAGTTTTTAATGGGTACCGACCCCTCGT
TGATC

>SQ25 S, Possible Fusarium, Bt2a/Bt2b

ACCCTCAGTGTAGTGACCCTTGGCCCAGTTGTTTCCAGCACCGGACTGACCGAAAA
CGAAGTTGTCGGGACGGAAGAGCTGACCGAAGGGACCAGCACGGACGGCGTCCA
TGGTACCAGGCTCAAGATCGACGAGGACGGCTCGGGGAACGTACTTGTTGCCAGA
GGCCTAGTTGTGTGAGCTTGGGAATTCTTGACATTGACTGTTAATGCATACCTCGT
TGAAGTAGACACTCATGCGCTCGAGCTGGAGCTCGGAGGTACCGTTGTAGACACC
ATGCTGTCGAGGCCGTGCTCGCCAGAGAGTCC

>SQ26 U, Possible Fusarium, Bt2a/Bt2b

CCTCTGGCGAGCACGGCCTCGACAGCAATGGTGTCTACAACGGTACCTCCGAGCTC
CAGCTCGAGCGCATGAGTGTCTACTTCAACGAGGTATGCATTAACAGTCAATGTCA
AGAATTCCCAAGCTCACACAACTAAGGCCTCTGGCAACAAGTACGTTCCCCGAGC
CGTCCTCGTCGATCTTGAGCCTGGTACCATGGACGCCGTCCGTGCTGGTCCCTTCG
GTCAGCTCTTCCGTCCCGACAACTTCGTTTTCGGTCAGTCCGGTGCTGGAAACAAC
TGGGCCAAGGGTCACTAACTGAGGGTCATCGCAATTCGCTGCGACACTCTTGGAA
GAACCATCAATTCCTTTAACAAAGCCCGAAAATATTATTCAAACTCCTTCCAGGGC
CGATCCCCAGAAGAGCCAACTCCTCAATTGCCCACCTCCGCCGAAGAAGCCCCCC
ACCACAGCCCAGCTA

Appendix 3.7 (Continued) Sequences data in FASTA format

>SQ27 T, Possible Fusarium, Bt2a/Bt2b

TCTCTGGCGAGCACGGCCTCGACAGCAATGGTGTCTACAACGGTACCTCCGAGCTC
CAGCTCGAGCGCATGAGTGTCTACTTCAACGAGGTATGCATTAACAGTCAATGTCA
AGAATTCCCAAGCTCACACAACTAGGCCTCTGGCAACAAGTACGTTCCCCGAGCC
GTCCTCGTCGATCTTGAGCCTGGTACCATGGACGCCGTCCGTGCTGGTCCCTTCGG
TCAGCTCTTCCGTCCCGACAACTTCGTTTTCGGTCAGTCCGGTGCTGGAAACAACT
GGGCCAAGGGTCACTACACTGAGGGTTAAGGCTTATATTGGGGTGTTTTTTCAAGA
AAAAAGAAGAAAGAAGGGTGAACTTTTTATTTTATTAGAAAAAGAAACATCTATG
GGGACAGGTTTCCCCACGAGGAAAATATTTCTTTTAGG

Appendix 4.1 PCR amplification of leaf, bark and root samples using primers ITS1F/ITS4 and ITS3/ITS4

Sample		DNA Extraction method	PCR Results[2]				
Code	Details		ITS1-F/ITS4			ITS3/ITS4	
			1	2	3	1	2
Sd1	*Pinus radiata* seed	Seed ground in DNA extraction buffer[1]	N	N	N	N	N
Sd2	*Pinus radiata* seed	Sterile water eluate applied to FTA card	N	N	N	N	N
Sd3	*Pinus radiata* seed	Glassmilk DNA extraction	N	N	N	N	N
Sd4	*Eucalyptus camaldulensis* seed	Seed ground in DNA extraction buffer	N	N	N	N	N
Sd5	*Eucalyptus camaldulensis* seed	Sterile water eluate applied to FTA card	N	N	N	N	N
Sd6	*Eucalyptus camaldulensis* seed	Glassmilk DNA extraction	N	N	N	N	N
Sd7	*Acacia mangium* seed	Seed ground in DNA extraction buffer	N	N	N	N	N
Sd8	*Acacia mangium* seed	Sterile water eluate applied to FTA card	N	N	N	N	N
Sd9	*Acacia mangium* seed	Glassmilk DNA extraction	N	N	N	N	N
Mb1	Mildewed *Phaseolus vulgaris* leaf	Direct squash onto FTA card	**P**	**P**	**P**	**P**	**P**
Mb2	Mildewed *Phaseolus vulgaris* leaf	Direct squash onto FTA card	N	N	**P**	**P**	**P**
Mb3	Mildewed *Phaseolus vulgaris* leaf	Direct squash onto FTA card	N	N	N	N	**P**
Mb4	Mildewed *Phaseolus vulgaris* leaf	Direct squash onto FTA card	**P**	**P**	**P**	**P**	**P**
Mp1	Mildewed *Pyrus communis* leaf	Direct squash onto FTA card	**P**	**P**	**P**	**P**	**P**
Mp2	Mildewed *Pyrus communis* leaf	Direct squash onto FTA card	N	**P**	**P**	**P**	**P**
Mp3	Mildewed *Pyrus communis* leaf	Direct squash onto FTA card	**P**	**P**	**P**	**P**	**P**
Mp4	Mildewed *Pyrus communis* leaf	Direct squash onto FTA card	**P**	**P**	**P**	**P**	**P**

[1]Samples ground in extraction buffer were applied to the FTA card

[2]P indicates amplification of a fragment of approx 500-600 bp with primers ITS1-F/ITS4 or approx. 300 bp with primers ITS3/ITS4; N indicates no amplification; NT indicates not tested.

Appendix 4.1 (Continued) PCR amplification of leaf, bark and root samples using primers ITS1F/ITS4 and ITS3/ITS4

Sample		DNA Extraction method	PCR Results[2]				
Code	Details		ITS1-F/ITS4			ITS3/ITS4	
			1	2	3	1	2
Rm3	Mildewed *Rosa* sp. leaf	Leaf ground in DNA extraction buffer	N	**P**	N	N	N
C12	Mildewed *Rosa* sp. leaf	Glassmilk DNA extraction	N	N	NT	NT	NT
Rm4	Mildewed *Rosa* sp. leaf	Direct squash onto FTA card	**P**	**P**	**P**	**P**	**P**
Fm1	Mildewed *Myosotis arvensis* leaf	Direct squash onto FTA card	**P**	**P**	N	N	**P**
Fm2	Mildewed *Myosotis arvensis* leaf	Leaf ground in DNA extraction	**P**	**P**	N	N	**P**
C11	Mildewed *Myosotis arvensis* leaf	Glassmilk DNA extraction	N	N	NT	NT	NT
Bs2	Mildewed *Brassica oleracea* leaf	Direct squash onto FTA card	N	N	**P**	**P**	N
Bs3	Mildewed *Brassica oleracea* leaf	Leaf ground in DNA extraction buffer	**P**	**P**	N	N	**P**
C14	Mildewed *Brassica oleracea* leaf	Glassmilk DNA extraction	N	**P**	NT	NT	NT
Euc1	*Aulographina* eucalypti in *Eucalyptus* leaf A	Direct squash onto FTA card	**P**	**P**	N	N	N
Euc2	*Aulographina* eucalypti in *Eucalyptus* leaf A	Leaf ground in DNA extraction buffer	N	N	N	**P**	N
C5	*Aulographina* eucalypti in *Eucalyptus* leaf A	Glassmilk DNA extraction	**P**	**P**	NT	NT	NT
Euc3	*Mycosphaerella* spp. in *Eucalyptus* leaf B	Direct squash onto FTA card	N	N	N	**P**	N
Euc4	*Mycosphaerella* spp. in *Eucalyptus* leaf B	Leaf ground in DNA extraction buffer	N	N	N	**P**	**P**
C6	*Mycosphaerella* spp. in *Eucalyptus* leaf B	Glassmilk DNA extraction	**P**	**P**	NT	NT	NT
Euc5	*Mycosphaerella* spp. in *Eucalyptus* leaf C	Direct squash onto FTA card	**P**	N	N	N	**P**
Euc6	*Mycosphaerella* spp. in *Eucalyptus* leaf C	Leaf ground in DNA extraction buffer	N	N	N	N	N

[1]Samples ground in extraction buffer were applied to the FTA card

[2]P indicates amplification of a fragment of approx 500-600 bp with primers ITS1-F/ITS4 or approx. 300 bp with primers ITS3/ITS4; N indicates no amplification; NT indicates not tested.

Appendix 4.1 (Continued) PCR amplification of leaf, bark and root samples using primers ITS1F/ITS4 and ITS3/ITS4

Sample		DNA Extraction method	PCR Results[2]				
Code	Details		ITS1-F/ITS4			ITS3/ITS4	
			1	2	3	1	2
C7	*Mycosphaerella* spp. in *Eucalyptus* leaf C	Glassmilk DNA extraction	P	N	NT	NT	NT
Euc7	*Mycosphaerella* spp. in *Eucalyptus* leaf D	Direct squash onto FTA card	N	**P**	N	N	N
Euc8	*Mycosphaerella* spp. in *Eucalyptus* leaf D	Leaf ground in DNA extraction buffer	N	N	**P**	**P**	**P**
C8	*Mycosphaerella* spp. in *Eucalyptus* leaf	Glassmilk DNA extraction	N	N	NT	NT	NT
Euc9	*Mycosphaerella* spp. in *Eucalyptus* leaf E	Direct squash onto FTA card	**P**	**P**	**P**	N	N
Euc10	*Mycosphaerella* spp. in *Eucalyptus* leaf E	Leaf ground in DNA extraction buffer	N	N	**P**	**P**	**P**
C9	*Mycosphaerella* spp. in *Eucalyptus* leaf	Glassmilk DNA extraction	N	P	NT	NT	NT
Euc11	*Mycosphaerella* spp. in *Eucalyptus* leaf F	Direct squash onto FTA card	**P**	**P**	N	N	N
Euc12	*Mycosphaerella* spp. in *Eucalyptus* leaf F	Leaf ground in DNA extraction buffer	N	**P**	**P**	N	N
C10	*Mycosphaerella* spp. in *Eucalyptus* leaf	Glassmilk DNA extraction	N	N	NT	NT	NT
Bt1	*Armillaria* sp. mycelium under bark of *Prunus persica*	Mycelium picked off and squashed onto FTA card	**P**	**P**	N	N	N
Bt2	*Armillaria* sp. mycelium under bark of *Prunus persica*	Mycelium picked off and squashed onto FTA card	**P**	**P**	**P**	N	N
Bt3	*Armillaria* sp. mycelium under bark of *Prunus persica*	Mycelium picked off and squashed onto FTA card	N	N	N	N	**P**
Bt4	*Armillaria* sp. mycelium under bark of *Prunus persica*	Mycelium picked off and squashed onto FTA card	**P**	**P**	**P**	**P**	**P**
Bt5	*Armillaria* sp. mycelium under bark of *Prunus persica*	Mycelium picked off and ground in DNA extraction buffer	N	N	N	**P**	N
Lr1	*Armillaria luteobubalina* in *Eucalyptus nitens* root 13	Root ground in DNA extraction buffer	**P**	N	**P**	**P**	**P**
Lr2	*Armillaria luteobubalina* in *Eucalyptus nitens* root 23	Root ground in DNA extraction buffer	N	N	N	**P**	N
Lr3	*Armillaria luteobubalina in Eucalyptus nitens root 25*	Root ground in DNA extraction buffer	P	N	N	**P**	P

[1]Samples ground in extraction buffer were applied to the FTA card; [2]P indicates amplification of a fragment of approx 500-600 bp with primers ITS1-F/ITS4 or approx. 300 bp with primers ITS3/ITS4; N indicates no amplification; NT indicates not tested.

Appendix 4.1 (Continued) PCR amplification of leaf, bark and root samples using primers ITS1F/ITS4 and ITS3/ITS4

Sample		DNA Extraction method	PCR Results[2]				
Code	Details		ITS1-F/ITS4			ITS3/ITS4	
			1	2	3	1	2
Lr4	*Armillaria luteobubalina* in *Eucalyptus nitens* root 27	Root ground in DNA extraction buffer	N	N	N	**P**	N
Pn1	*Cyclaneusma* sp. in *Pinus radiata* G2617	Leaf ground in DNA extraction buffer	N	N	N	N	N
C15	*Cyclaneusma* sp. in *Pinus radiata* G2617	Glassmilk DNA extraction	P	N	NT	NT	NT
Pn2	*Cyclaneusma* sp. in *Pinus radiata* G2682	Leaf ground in DNA extraction buffer	**P**	N	**P**	**P**	**P**
C16	*Cyclaneusma* sp. in *Pinus radiata* G2682	Glassmilk DNA extraction	N	N	NT	NT	NT
Pn3	*Cyclaneusma* sp. in *Pinus radiata* G2685	Leaf ground in DNA extraction buffer	N	N	**P**	N	N
C13	*Cyclaneusma* sp. in *Pinus radiata* G2685	Glassmilk DNA extraction	N	N	NT	NT	NT
Pn4	*Cyclaneusma* sp. in *Pinus radiata* G2683	Leaf ground in DNA extraction buffer	N	**P**	N	N	N
C4	*Cyclaneusma* sp. in *Pinus radiata* G2683	Glassmilk DNA extraction	**P**	**P**	NT	NT	NT
Pn5	*Cyclaneusma* sp. in *Pinus radiata* G2686	Leaf ground in DNA extraction buffer	**P**	**P**	**P**	**P**	**P**
C2	*Cyclaneusma* sp. in *Pinus radiata* G2686	Glassmilk DNA extraction	N	**P**	NT	NT	NT
C1	*Armillaria* sp. mycelium under bark of *Prunus persica*	Glassmilk DNA extraction	N	N	NT	NT	NT
Gr1	Grass (*Arrhenatherum elatius* var. bulbosum) infected with rust (undet.)	Leaf squash on FTA card	N	N	N	NT	NT
Gr2	Grass (*Arrhenatherum elatius* var. bulbosum) infected with rust (undet.)	Leaf squash on FTA card	N	N	N	NT	NT
Gr4	Grass (*Arrhenatherum elatius* var. bulbosum) infected with rust (undet.)	Leaf squash on FTA card	N	N	N	NT	NT
Gr5	Grass (*Arrhenatherum elatius* var. bulbosum) infected with rust (undet.)	Leaf squash on FTA card	N	N	N	NT	NT
Sb1	*Beta vulgaris* infected with *Uromyces betae*	Leaf squash on FTA card	N	N	N	NT	NT

[1]Samples ground in extraction buffer were applied to the FTA card

[2]P indicates amplification of a fragment of approx 500-600 bp with primers ITS1-F/ITS4 or approx. 300 bp with primers ITS3/ITS4; N indicates no amplification; NT indicates not tested

Appendix 5.1 List of samples obtained from nursery

Code	Sample Description
GP SPA 2071	*Acacia mangium* seed
CM 0580	*Acacia mangium* seed
CM 0773	*Acacia mangium* seed
CM 0774	*Acacia mangium* seed
S4-H	Soil sample from 4 week-old, healthy *Acacia mangium* seedlings
S4-S	Soil sample from 4 week-old, symptomatic *Acacia mangium* seedlings
S4-D	Soil sample from 4 week-old, dying *Acacia mangium* seedlings
S6-H	Soil sample from 6 week-old, healthy *Acacia mangium* seedlings
S6-S	Soil sample from 6 week-old, symptomatic *Acacia mangium* seedlings
S6-D	Soil sample from 6 week-old, dying *Acacia mangium* seedlings
S8-H	Soil sample from 8 week-old, healthy *Acacia mangium* seedlings
S8-S	Soil sample from 8 week-old, symptomatic *Acacia mangium* seedlings
S8-D	Soil sample from 8 week-old, dying *Acacia mangium* seedlings
S10-H	Soil sample from 10 week-old, healthy *Acacia mangium* seedlings
S10-S	Soil sample from 10 week-old, symptomatic *Acacia mangium* seedlings
S10-D	Soil sample from 10 week-old, dying *Acacia mangium* seedlings
R1, R2	Roots from 10 week-old *Acacia mangium* seedlings with black stem lesions
R3 – R9	Roots from 10 week-old *Acacia mangium,* symptomatic but not dying, seedlings
R10	Roots from 9 week-old *Acacia mangium* seedlings with black stem lesions
R11-R14	Roots from 9 week-old, healthy *Acacia mangium* seedlings
R15 – R20	Roots from 8 week-old, diseased and dying *Acacia mangium* seedlings
R21 – R23	Roots from 8 week-old, healthy *Acacia mangium* seedlings
R24 – R35	Roots from 6 week-old, diseased and dying *Acacia mangium* seedlings

Appendix 5.2 PCR amplification results for the cultures isolated from *Acacia mangium* root black lesions samples using ITS, Beta-tubulin and EF primers on DNA extracted by different methods.

Code	Details	PCR Amplification[1]																	
		FTA Card									Glassmilk DNA Extraction								
		ITS1F/4			Bt 2a/ Bt 2b			EF1/EF2			ITS1F/4			Bt 2a/ Bt 2b			EF1/EF2		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2A-1	Brown mycelium isolated from black lesion	N	N	N	P	P	P	N	N	N	NT	NT	NT	P	P	P	P	P	N
2A-2	Brown mycelium isolated from black lesion	N	N	N	P	P	P	N	N	N	NT	NT	NT	P	P	P	P	P	N
2A-3	Brown mycelium isolated from black lesion	N	N	N	P	P	P	N	P	N	NT	NT	NT	P	P	P	P	P	N
2B-1	Dark green mycelium isolated from black lesion	N	N	N	P	P	N	N	N	N	NT	NT	NT	P	P	P	N	P	N
2B-2	Dark green mycelium isolated from black lesion	N	N	N	N	N	P	N	N	N	NT	NT	NT	N	N	N	N	N	N
2B-3	Dark green mycelium isolated from black lesion	N	N	N	P	P	P	N	N	P	NT	NT	NT	P	P	P	N	N	P
2B-4	Dark green mycelium isolated from black lesion	N	N	N	P	P	P	N	N	N	NT	NT	NT	P	P	P	N	N	N
P	Isolated from black lesion	N	N	N	P	P	P	N	N	N	NT	NT	NT	P	P	P	N	N	N
Q	Possible Fusarium 1A	N	N	N	P	P	P	N	N	N	NT	NT	NT	P	P	P	N	N	N
R	Possible Fusarium 1B	N	N	N	P	P	P	N	N	N	NT	NT	NT	P	P	P	N	N	N
S	Possible Fusarium	N	N	N	N	P	N	P	P	P	NT	NT	NT	P	P	P	P	P	P
T	Possible Fusarium	N	N	N	P	P	P	P	P	P	NT	NT	NT	N	N	N	P	P	P
U	Possible Fusarium	N	N	N	P	P	P	P	P	P	NT	NT	NT	P	P	P	P	P	P

[1]P indicates PCR amplification of a fragment approx. 500-600 bp (primers ITS1F/4), 550 bp (primers Bt2a/2b) and 440 bp (primers EF1/EF2), N indicates no amplification and NT indicates samples were not tested

Appendix 5.3 PCR amplification of the *Acacia mangium* root seedlings samples using ITS primers in different DNA extraction methods

Samples	DNA Extraction Method	PCR Results[1]					
		ITS 1F-ITS 4			ITS 3-ITS 4		
		1	2	3	1	2	3
R1-10 week-old *A. mangium* seedling with black stem lesion	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R2-10 week-old *A. mangium* seedling with black stem lesion	Glassmilk DNA extraction	**P**	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
	FTA and extraction buffer and IAC	N	N	N	NT	NT	NT
	FTA and sterile water and IAC	N	N	N	NT	NT	NT
R3-10 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	P	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R4-10 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	**P**	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
	FTA and extraction buffer and IAC	**PC**	**PC**	**PC**	NT	NT	NT
	FTA and sterile water and IAC	C	C	C	NT	NT	NT
R5-10 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	N	**P**	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
	FTA and extraction buffer and IAC	N	N	N	NT	NT	NT
	FTA and sterile water and IAC	C	C	C	NT	NT	NT
R6-10 week-old *A .mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	N	N	**P**	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
	FTA and extraction buffer and IAC	**PC**	**PC**	**PC**	NT	NT	NT
	FTA and sterile water and IAC	C	C	C	NT	NT	NT
R7-10 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	**P**	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N

[1]P indicates PCR amplification of a fragment ca. 500-600 nt, N indicates no amplification, C indicates amplification of IAC (ca. 1100 nt), NT indicates samples were not tested.

Appendix 5.3 (Continued) PCR amplification of the *Acacia mangium* root seedlings samples using ITS primers in different DNA extraction methods

Samples	DNA Extraction Method	PCR Results[1]					
		ITS 1F-ITS 4			ITS 3-ITS 4		
		1	2	3	1	2	3
R8-10 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	**P**	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
	FTA and extraction buffer and IAC	C	C	C	NT	NT	NT
	FTA and sterile water and IAC	N	N	N	NT	NT	NT
R9-10 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	**P**	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
	FTA and extraction buffer and IAC	N	N	N	N	N	N
	FTA and sterile water and IAC	N	N	N	N	N	N
R10-10 week-old *A. mangium* seedling with black stem lesion	Glassmilk DNA extraction	**P**	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
	FTA and extraction buffer and IAC	C	C	C	NT	NT	NT
	FTA and sterile water and IAC	**PC**	**PC**	**PC**	NT	NT	NT
R11-9 week-old , healthy *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R12-9 week-old , healthy *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R13-9 week-old , healthy *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R14-9 week-old , healthy *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N

P indicates PCR amplification of a fragment ca. 500-600 nt, N indicates no amplification, C indicates amplification of IAC (ca. 1100 nt), NT indicates samples were not tested.

Appendix 5.3 (Continued) PCR amplification of the *Acacia mangium* root seedlings samples using ITS primers in different DNA extraction methods

Samples	DNA Extraction Method	PCR Results[1]					
		ITS 1F-ITS 4			ITS 3-ITS 4		
		1	2	3	1	2	3
R15-8 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	N	N	**P**	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
	FTA and extraction buffer and IAC	C	C	C	NT	NT	NT
	FTA and sterile water and IAC	N	N	N	NT	NT	NT
R16-8 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R17-8 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R18-8 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R19-8 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R20-8 week-old *A. mangium* symptomatic but not dying, seedling	Glassmilk DNA extraction	N	**P**	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
	FTA and extraction buffer and IAC	N	N	N	NT	NT	NT
	FTA and sterile water and IAC	C	C	C	NT	NT	NT
R21-8 week-old, healthy, *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R22-8 week-old, healthy *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N

[1]P indicates PCR amplification of a fragment ca. 500-600 nt, N indicates no amplification, C indicates amplification of IAC (ca. 1100 nt), NT indicates samples were not tested.

Appendix 5.3 (Continued) PCR amplification of the *Acacia mangium* root seedlings samples using ITS primers in different DNA extraction methods

Samples	DNA Extraction Method	PCR Results[1]					
		ITS 1F-ITS 4			ITS 3-ITS 4		
		1	2	3	1	2	3
R23-8 week-old, healthy *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R24-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R25-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R26-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R27-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R28-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R29-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R30-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R31-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R32-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R33-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N

[1]P indicates PCR amplification of a fragment ca. 500-600 nt, N indicates no amplification, C indicates amplification of IAC (ca. 1100 nt), NT indicates samples were not tested.

Appendix 5.3 (Continued) PCR amplification of the *Acacia mangium* root seedlings samples using ITS primers in different DNA extraction methods

Samples	DNA Extraction Method	PCR Results[1]					
		ITS 1F-ITS 4			ITS 3-ITS 4		
		1	2	3	1	2	3
R34-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N
	FTA and sterile water	N	N	N	N	N	N
R35-6 week-old, diseased and dying *A. mangium* seedling	Glassmilk DNA extraction	N	N	N	N	N	N
	FTA and extraction buffer	N	N	N	N	N	N

[1]P indicates PCR amplification of a fragment ca. 500-600 nt, N indicates no amplification, C indicates amplification of IAC (ca. 1100 nt), NT indicates samples were not tested.

Appendix 5.4 PCR amplification of soil samples from around the *Acacia mangium* seedlings using ITS primers in different DNA extraction methods.

Code	Details	DNA Extraction Method[1]					
		1	2	3	4	52	62
S4-H	Soil sample from 4 week-old, healthy *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	NT,NT[3]	NT,NT[3]
S4-S	Soil sample from 4 week-old, symptomatic *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	N,N[3]	N,N[3]
S4-D	Soil sample from 4 week-old, dying *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	N,N[3]	N,N[3]
S6-H	Soil sample from 6 week-old, healthy *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	NT,NT[3]	NT,NT[3]
S6-S	Soil sample from 6 week-old, symptomatic *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	N,N[3]	N,N[3]
S6-D	Soil sample from 6 week-old, dying *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	N,N[3]	N,N[3]
S8-H	Soil sample from 8 week-old, healthy *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	NT,NT[3]	NT,NT[3]
S8-S	Soil sample from 8 week-old, symptomatic *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	N,N[3]	N,N[3]
S8-D	Soil sample from 8 week-old, dying *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	N,N[3]	N,N[3]
S10-H	Soil sample from 10 week-old, healthy *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	NT,NT[3]	NT,NT[3]
S10-S	Soil sample from 10 week-old, symptomatic *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	N,N[3]	N,N[3]
S10-D	Soil sample from 10 week-old, dying *Acacia mangium* seedlings	N,N[3]	**P,P[3]**	N,N[3]	N,N[3]	N,N[3]	N,N[3]

[1]Results are given for ITS1-F/ITS4 PCR, ITS3/ITS4 PCR. DNA extraction methods are: 1, standard glassmilk DNA extraction; 2. Powersoil DNA extraction kit; 3 and 5, ground in extraction buffer, incubated at RT before application to FTA card; 4 and 6, ground in sterile water, incubated at RT before application to FTA card.
[2]IAC plasmid was added to the reaction mix.
[3]PCR results from primer ITS3/4. P indicates PCR amplification, N indicates no amplification and NT indicates samples were not tested

Appendix 6.1 Phytophthora Selective Media

1. CORN MEAL AGAR

17 gr Difco corn meal agar
1 l distilled water
Boil to completely dissolve
Autoclave 15 minutes at 121 °C

2. V8 JUICE AGAR

200 mL V8 Juice
2.5 g $CaCO_3$
Combine the two ingredients and centrifuge to clarify about 5 minutes at 2000 rpm ((Eppendorf Centrifuge 5804 Germany), decant, discard pellet
Add 20 gr agar and distilled water to make 1 litre.
Autoclave 15 minutes at 121 °C

3. PARPH

17 gr Difco corn meal agar
1 l distilled water
Boil to completely dissolve
Autoclave for 15 minutes at 121 °C. Cool to 45 °C
Add:
10 mg Pimaricin
125 mg Ampicilin
10 mg Rifampicin
100 mg/l pentacholoronitrobenzene
50 mg/l hymexazol

4. PARPH-V8

20 g agar
200 mL filtered V8 broth
800 mL deionized water
50 gr hymexazol
5 mg pimaricin
10 mg rifampicin
250 mg ampicilin
125 mg pentachloronitrobenzene

Appendix 6.2 PCR results using ITS primers from cultures, baits and cultures isolated from baits

Code	Species	PCR Results[1]			
		Glassmilk DNA Extraction		FTA Direct Squash	
		ITS1F ITS4	ITS1 ITS2	ITS1F ITS4	ITS1 ITS2
PC	*Phytophthora cinnamomi*	P,P,P	P,P,P	P,P,P	P,P,P
NY 001	*Phytophthora cryptogea*	P,P,P	P,P,P	N,N,N	P,P,P
Hansen 133	*Phytophthora drechsleri*	P,N,N	P,P,P	N,N,N	P,N,N
Hansen 139	*Phytophthora drechsleri*	P,P,P	P,P,P	P,P,P	N,P,P
Hansen 266	*Phytophthora drechsleri*	P,N,N	P,P,P	P,N,N	P,N,N
P 501	*Phytophthora gonapodyides*	N,N,N	N,P,N	P,N,N	P,N,N
S 5	*Phytophthora gonapodyides*	P,N,N	P,P,P	N,N,N	N,N,N
S 42	*Phytophthora gonapodyides*	N,N,N	N,N,N	N,N,P	N,P,P
IAF	Isolated from *Saintpaulia* sp. leaf bait	N,N,N	N,N,N	N,N,N	N,P,P
ICO	Isolated from *Pseudotsuga menziesii* needle bait	P,P,N	P,P,P	N,N,N	N,P,P
IAL	Isolated from *Medicago sativa* sprout bait	P,P,P	P,P,P	P,N,N	P,P,P
ILE	Isolated from *Citrus limon* leaf bait	P,P,P	P,P,P	P,N,N	N,P,P
IPE	Isolated from *Cupressus sempervinens* needle bait	P,P,P	P,P,P	N,N,N	N,N,P
IRH	Isolated from *Rhododendron* sp. leaf bait	P,P,P	P,P,P	N,N,N	P,N,P
IRO	Isolated from *Rosa* sp. Petal Bait	P,P,P	P,P,P	P,N,N	N,P,N
IST	Isolated from *Fragaria* sp. leaf bait	P,P,P	P,P,P	N,N,N	N,N,P
IMB	Isolated from *Phaseolus mungo* sprout bait	N,N,N	N,N,N	P,P,P	N,P,P
ICA	Isolated from *Camellia* sp. leaf bait	P,N,N	P,P,N	P,N,N	N,P,N
CF	*Pseudotsuga menziesii* needle bait	P,P,P	P,P,P	N,N,N	N,N,N
SL	*Fragaria* sp. leaf bait	P,P,P	P,N,P	N,N,N	P,N,N
PL	*Cupressus sempervinens* needle bait	P,N,N	P,P,P	N,N,N	N,N,P
AL	*Medicago sativa* sprout bait	P,N,N	P,P,P	P,P,P	N,P,N
RL	*Rhododendron* sp. leaf	N,N,N	N,N,N	N,N,N	N,N,P
MB	*Phaseoulus mungo* sprout bait	P,P,P	P,P,P	P,P,P	N,P,P
LL	*Citrus limon* leaf bait	P,P,P	P,P,P	N,N,N	N,P,N
CL	*Camellia* sp. leaf bait	N,N,N	N,N,N	N,N,N	N,N,P
RP	*Rosa* sp. Petal Bait	P,P,P	P,P,P	N,N,N	N,N,P
AV	*Saintpaulia* sp. leaf bait	P,P,P	P,P,P	P,P,P	P,P,P
EL	*Eucalyptus leaf bait*	NT	NT	N,N,N,	N,N,N,

[1] P indicates amplification of a fragment of approximately 600 bp (primers ITS1-F/ITS4) or 300 bp (primers ITS1/ITS2) for at least one of the three replicate samples, N indicates no amplification in any of the three replicates, NT indicates that samples were not tested .

Appendix 6.3 PCR results using species-specific primers of cultures, cultures isolated from baits and baits samples from glassmilk DNA extraction method

Code	Species	Primer pairs[1]		
		1	2	3
PC	*Phytophthora cinnamomi*	N,N,N	N,N,N	N,N,N
NY 001	*Phytophthora cryptogea*	**P,P,P**	**P,P,P**	N,N,N
Hansen 133	*Phytophthora drechsleri*	**P,P,P**	**P,P,P**	N,N,N
Hansen 139	*Phytophthora drechsleri*	**P,P,P**	**P,P,P**	N,N,N
Hansen 266	*Phytophthora drechsleri*	**P,P,P**	**P,P,P**	N,N,N
P 501	*Phytophthora gonapodyides*	**P,P,P**	N,N,N	N,N,N
S 5	*Phytophthora gonapodyides*	**P,P,P**	**P,P,P**	N,N,N
S 42	*Phytophthora gonapodyides*	**P,P,P**	**P,P,P**	N,N,N
IAF	Isolated from *Saintpaulia* sp. leaf bait	N,N,N	N,N,N	N,N,N
ICO	Isolated from *Pseudotsuga menziesii* needle bait	N,N,N	**P,N,P**	N,N,N
IAL	Isolated from *medicago sativa* sprout bait	N,N,N	N,N,N	N,N,N
ILE	Isolated from *Citrus limon* leaf bait	N,N,N	N,N,N	N,N,N
IPE	Isolated from *Cupressus sempervirens needle* bait	N,N,N	N,N,N	N,N,N
IRH	Isolated from *Rhododendron* sp. leaf bait	N,N,N	N,N,N	N,N,N
IRO	Isolated from *Rosa* sp.petal Bait	N,N,N	N,N,N	N,N,N
IST	Isolated from *Fragaria* sp. leaf bait	N,N,N	N,N,N	N,N,N
IMB	Isolated from *Phaseoulus mungo* sprout bait	N,N,N	N,N,N	N,N,N
ICA	Isolated from *Camellia* sp.leaf bait	N,N,N	N,N,N	N,N,N
CF	*Pseudotsuga menziesii* needle	**P,P,P**	N,N,N	N,N,N
SL	*Fragaria* sp. leaf	**P,P,P**	N,N,N	N,N,N
PL	*Cupressus sepervirens needle*	**P,P,P**	N,N,N	N,N,N
AL	*Medicago sativa sprout*	N,N,N	N,N,N	N,N,N
RL	*Rhododendron* sp. leaf	N,N,N	N,N,N	N,N,N
MB	*Phaseolus mungo* sprout	N,N,N	N,N,N	N,N,N
LL	*Citrus limon* leaf	N,N,N	N,N,N	N,N,N
CL	*Camellia* sp. leaf	N,N,N	N,N,N	N,N,N
RP	*Rosa* sp. petal	N,N,N	**P,P,P**	**P,P,P**
AV	*Saintpaulia* sp. leaf	**P,P,P**	**P,P,P**	N,N,N

[1] Primers used were 1. YPh1F/2R; 2. YMeg1F/2R; 3. YPtc1F/2R [2] P indicates amplification of a fragment of approximately 470 bp (primers YPh1F/YPh2R), 196 bp (primers YMeg1F/YMeg2R), and 247 bp (primers 247 bp (primers YPtc1F/2R. N indicates no amplification

Appendix 6.4 PCR results using species specific primer of cultures, cultures isolated from baits and baits samples from FTA direct squash method

Code	Species	PCR amplifications[1]	
		1	2
PC	*Phytophthora cinnamomi*	N,N,N	N,N,N
NY 001	*Phytophthora cryptogea*	**P,P,P**	**P,P,P**
Hansen 133	*Phytophthora drechsleri*	**P,P,P**	**P,P,P**
Hansen 139	*Phytophthora drechsleri*	**P,P,P**	**P,P,P**
Hansen 266	*Phytophthora drechsleri*	**P,P,P**	**P,P,P**
P 501	*Phytophthora gonapodyides*	**P,P,P**	N,N,N
S 5	*Phytophthora gonapodyides*	**P,P,P**	**P,P,P**
S 42	*Phytophthora gonapodyides*	**P,P,P**	**P,P,P**
IAF	Isolated from *Saintpaulia* sp. leaf bait	N,N,N	N,N,N
ICO	Isolated from *Pseudotsuga menziesii* needle bait	**P,N,P**	N,N,N
IAL	Isolated from *medicago sativa* sprout bait	N,N,N	N,N,N
ILE	Isolated from *Citrus limon* leaf bait	N,N,N	N,N,N
IPE	Isolated from *Cupressus sempervirens needle* bait	N,N,N	N,N,N
IRH	Isolated from *Rhododendron* sp. leaf bait	N,N,N	N,N,N
IRO	Isolated from *Rosa* sp.petal Bait	N,N,N	N,N,N
IST	Isolated from *Fragaria* sp. leaf bait	N,N,N	N,N,N
IMB	Isolated from *Phaseoulus mungo* sprout bait	N,N,N	N,N,N
ICA	Isolated from *Camellia* sp.leaf bait	N,N,N	N,N,N
CF	*Pseudotsuga menziesii* needle	N,N,N	N,N,N
SL	*Fragaria* sp. leaf	**P,P,P**	N,N,N
PL	*Cupressus sempervirens needle*	N,N,N	N,N,N
AL	*Medicago sativa sprout*	N,N,N	N,N,N
RL	*Rhododendron* sp. leaf	N,N,N	N,N,N
MB	*Phaseolus mungo* sprout	N,N,N	N,N,N
LL	*Citrus limon* leaf	N,N,N	N,N,N
CL	*Camellia* sp. leaf	N,N,N	N,N,N
RP	*Rosa* sp. petal	N,N,N	N,N,N
AV	*Saintpaulia* sp. leaf	N,N,N	N,N,N
EL	*Eucalyptus* leaf	N,N,N	N,N,N

[1] Primers used were 1.YPh1F/2R; 2.YMeg1F/2R

[2] P indicates amplification of a fragment of approximately 470 bp (primers YPh1F/YPh2R) or 196 bp (primers YMeg1F/YMeg2R), N indicates no amplification

Appendix 6.5 PCR results using ITS primers of water samples from different sample preparation method

Sample Preparation Method	PCR Results[1]		
	ITS 1F/4	**1TS1/2**	**YMeg1F/2R**
FTA card left in stream with baits	**P,P,P**	N,N,N	**N,N,N**
Water dripped onto FTA card	N,N,N	**P,P,N**	**P,P,P**
FTA card stirred in water	N,N,N	**P,P,N**	**P,P,P**
Pellets from centrifuged water onto FTA card	N,N,N	**P,P,P**	**P,P,P**
Whatman filter paper eluate, pelleted and squashed onto FTA card	N,N,N	N,N,N	N,N,N
Whatman filter paper eluate, pelleted and DNA extracted by glassmilk method	N,N,N	N,N,N	**P,P,P**
Millipore filter eluate, pelleted and squashed onto FTA card	N,N,N	N,N,N	N,N,N
Millipore filter eluate, pelleted and DNA extracted by glassmilk method	N,N,N	N,N,N	**P,P,P**

[1] P indicates amplification of a fragment of approximately 600 bp (primers ITS1F/ITS4), 300 bp (primers ITS1/ITS2) and 196 bp (primers YMeg1F/YMeg2R) for at least one of the three replicate samples; N indicates no amplification in any of the three replicates