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ANTIMICROBIALS FROM THE FUNGAL ENDOPHYTES OF SANTALUM LANCEOLATUM

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Abstract

The emergence of drug resistance in organisms is a serious human health concern due to the subsequent reduction in treatment options. One way to combat resistance is the discovery and development of novel antimicrobial compounds with novel modes of action. Fungal endophytes live within the tissues of plants and cause no visible symptoms of infection. Research already conducted in the field indicates that fungal endophytes are diverse, largely undiscovered and are potential key sources of novel bioactive compounds. High probability sources of bioactive endophytes include plants used for medicinal purposes. *Santalum lanceolatum* has been used by the Indigenous Australian population to treat skin and upper respiratory infections, making it a prime candidate for investigation.

This study isolated 67 fungal and 4 bacterial isolates from the leaves, bark and fruit from five *S. lanceolatum* plants at five sites located in the Darling Downs. Histology of leaf samples showed fungal hyphae residing in the epidermal layer of the leaf, with projections into the mesophyll layer. *Nigrospora* spp. accounted for 45 isolates across the five sites, indicating possible host preference. Eight fungal genera in total were identified; *Aspergillus, Diaporthe*, *Fusarium*, *Nigrospora*, *Pestalotiopsis*, *Preussia*, *Pyronema* and *Xylaria*. One bacteria was identified as a *Bacillus* sp.

Initial antimicrobial activity was observed in 5 isolates – 2 unidentified bacteria, 2 *Pyronema* sp. and *Xylaria grammica*. *X. grammica* and one of the *Pyronema* sp. bulked sufficiently to fractionate for further antimicrobial testing. All fractions of both fungi, as well as the *Pyronema* crude extract, showed bacteriostatic activity to *S. marcescens* at varying minimum inhibitory concentrations (MIC). The *X. grammica* crude extract showed bactericidal activity against *S. marcescens* at a MIC of 1.25mg/ml. Bacteriostatic activity towards *S. aureus* was also seen in *Pyronema* sp. fractions S4R 4 and S4R 5-7 at MIC of 1mg/ml and 0.125mg/ml

respectively. These results indicate that *S. lanceolatum* as found on the Darling Downs is a source of potential novel fungal endophytes which produce secondary metabolites with antimicrobial properties against clinically significant pathogens.

Declaration

I certify that the experimental work, results, analyses and conclusions are entirely my own effort, except where otherwise acknowledged. I also certify that this work is original and has not been previously submitted for assessment or award in any other subject or University.

Candidate Date

ENDORSEMENT

Supervisor, Dr Mark Lynch Date

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List of Abbreviations

AGRF	-	Australian Genome Research Facility
EtOAc	-	Ethyl acetate
EtOH	-	Ethanol
HPLC	-	High Performance Liquid Chromatography
ITS	-	Internal Transcribed Spacer
MEB	-	Malt Extract Broth
MEGA	-	Molecular Evolutionary Genetics Analysis
MHB	-	Muller-Hinton Broth
MIC	-	Minimum inhibitory concentration
MeOH	-	Methanol
MRSA	-	Methicillin resistant *Staphylococcus aureus*
NMR	-	Nuclear magnetic resonance
PCR	-	Polymerase Chain Reaction
PDA	-	Potato Dextrose Agar
STA	-	Sensi-test Agar
TSB	-	Tryptone Soy Broth

1.0 Introduction

Bacteria capable of causing serious infections are becoming resistant to current chemotherapeutic approaches. Currently it is still possible to treat these infections with alternative medications to which the bacteria involved are not resistant. However, it is imperative to search for alternatives as the current treatments may no longer be useful options due to the ever continual development of drug resistance. One bacteria which poses a high risk to health care providers is methicillin-resistant *Staphylococcus aureus* (MRSA) (Schito 2006). Even more concerning is that there are some strains of *S. aureus* that are multidrug resistant due to their developed resistance to vancomycin as well as methicillin (Mongkolrattanothai et al. 2003; Sieradzki & Tomasz 1997; Welte & Pletz 2010). Imipenem (Carbapenem) or linezolid (Oxazolidone) are currently alternative treatments, administered orally and intravenously respectively, though these are generally only used when other treatments have not been effective (Welte & Pletz 2010). In the hospital setting, *Klebsiella pneumoniae* and *Enterobacter* spp. have both shown reduced susceptibility to treatment with ceftazidime from 1990-93 until 1994-2000, with decreases ranging from 93%-87% and 67%-63% respectively (Neuhauser et al. 2003). *Pseudomonas aeruginosa* also had reduced susceptibility to ciprofloxacin, with a reduction from 89% to 68% over 1990/1993 to 2000 (Neuhauser et al. 2003). The development of novel antimicrobials is an endeavour that needs to be continual to ensure that treatment options will always be available. It must be assumed that the more a treatment has been used, the higher the likelihood that resistance will follow, rendering that drug, and possibly the class of drugs ineffective. As fungi was the original source of modern antibiotics, there is a logical progression to the continued search for novel compounds from fungi. The model of penicillin discovery and development should serve as an example for

future developments from fungi. This review will therefore discuss modern treatments of infection, fungal endophytes and the possible medical uses of fungal endophytes.

1.1 History of Antibiotic Use and Resistance

The advent of treatment with modern antimicrobials began with penicillin. Discovered in 1929 by Alexander Fleming at St Mary's Hospital, Paddington, United Kingdom, the extent of the uses of penicillin were not truly captured until the 1940's with the work of Howard Florey and associates. In a paper by Chain et al. (1940) penicillin was trialled as a chemotherapeutic agent, with success in treating 3 pathogens *in vitro* – *streptococci*, *staphylococci* and *Clostridium septicum*. Abraham et al. (1941) and Florey and Florey (1943) expanded on the uses of penicillin as an antibacterial agent. A number of observations were made by the group as to how the mould grows and penicillin is produced. Traces of penicillin can be found within culture media when the colour of the surface mycelium changes from white to blue-green, around 5 to 6 days after culturing (Abraham et al. 1941). The pH of the medium decreases to 3 as the mycelium is produced, rising in conjunction with the colour change. Optimal production of penicillin occurs when the pH of the media has risen to 7 (Abraham et al. 1941). As the concentration of penicillin increases, it causes a yellowing of the media. It was noted that penicillin was not produced under anaerobic conditions and that attempting to maintain a constant pH did not increase the yield (Abraham et al. 1941). The yellow media can be harvested and replaced with fresh media and penicillin extracted (Abraham et al. 1941).

Testing of penicillin against *S. aureus* resulted in zones of inhibition that increased in diameter as the units/cm^3 increased; though the rate of increase tapered off significantly after 4 units/cm^3. Further testing showed that there was resistance to penicillin by certain strains of *S. aureus*. A

test strain was grown in culture with very low levels of crude penicillin, with the concentration of the penicillin being increased over the duration of the test period of nine weeks. Over this time the bacteria were able to continue growing in penicillin concentrations 30-fold more than the original concentration. After a further seven weeks, the culture of *S. aureus* was able to grow in a concentration one-thousand times greater than that which inhibited the parent strain (Abraham et al. 1941). At the conclusion of this experiment, the adapted strain was grown in a large volume, ground up, centrifuged and the extract incubated with penicillin. It was concluded that there was no loss of activity by the penicillin and that staphylococcus was not producing an enzyme which was inactivating penicillin. It can be concluded, that the resistance of *S. aureus* to penicillin was, in this experiment at least, not due to the activity of a ß-lactamase. This conclusion is also not likely to have been due to ineffective technique, as in 1940, Abraham & Chain showed, using the same method, that *Escherichia coli* produced an enzyme – later named as penicillinase – that prevented the efficacy of penicillin to restrict the growth of susceptible *S. aureus* (Abraham & Chain 1940). Stewart (1947) in conducting experiments on Gram-negative bacteria noted that while some strains produced penicillinase (ß-lactamase) others did not yet show resistance.

It was noted by Gardner (1940) that by using concentrations of penicillin which were low enough to be insufficient to kill the bacterium (if gram positive), definite morphological changes could be seen in the bacterial cells. These changes involved the lengthening of rods and enlargement of cocci, and could be seen in both gram negative and gram positive species. The ability of amoxicillin to act on gram negative cells, by being able to fully permeate the outer lipopolysaccharide layer, may just be an enhanced action than that which was seen by Gardner (1940) with penicillin. Gardner speculated these morphological changes were due to the continued growth and attempted reproduction of the cells, with incomplete fission causing

the appearance of engorgement. This lack of fission is consistent with interference in the construction of the peptidoglycan layers of the cell wall.

The use of penicillin to treat human infections was effective for *S. aureus* and streptococcal infections in a range of clinical settings; over a wide patient demographic; and at various stages of disease (Florey & Florey 1943). Many of these cases were treated with penicillin as a final option, having previously being treated with sulphapyridine to no great effect. The treatment with penicillin in general did not result in adverse reactions for the patients and there was a noticeable reduction in the white blood cell count of the patients as treatment progressed, indicating the rate at which the penicillin reduced the presence of the pathogen (Florey & Florey 1943).

The success of penicillin as a treatment for human infection was the beginning of a medical revolution and the beginning of modern antibiotic treatment. The chemical structure of penicillin was later confirmed in 1945 as a ß-lactam by the work of Dorothy Hodgkin using x-ray crystallography (Douglas & Kellar 1947). The ß-lactam class of drugs includes the penicillins, carbenapems, cephalosporins and the monobactams. The developing of resistance to one of the drugs within the ß-lactam class of antibiotics indicates that resistance was likely to develop against all antibiotics within the ß-lactam group; as ß-lactamases act by breaking the ß-lactam ring. Following on from the success of penicillin, derivatives, both synthetic and natural were also developed.

Currently there is an extensive range of antibacterial agents available: penicillins, cephalosporins, carbenapems, monobactams, tetracyclines, quniolones, isoniazids,

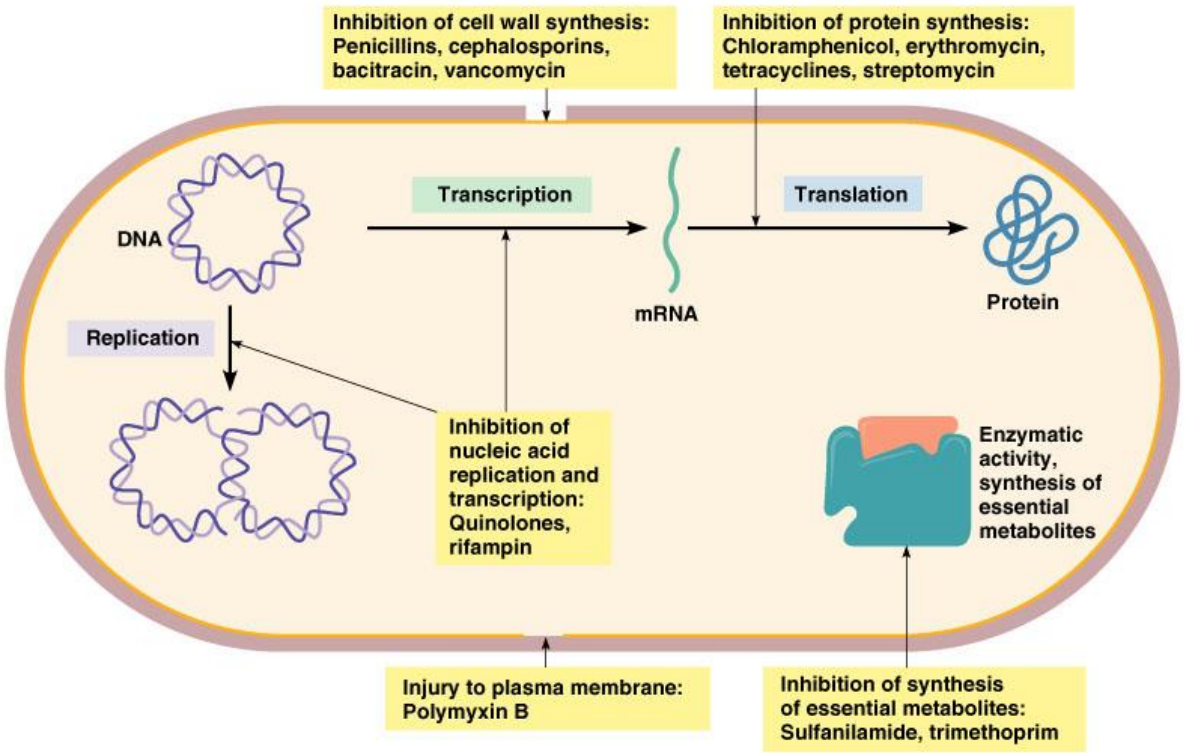

Figure 1. The locations at which antibiotics act upon the cell. (Tortora, Funke & Case 2004)

aminoglycosides, glycopeptides. Such an extensive range infers extensive and diverse mechanisms of action, however current treatments act by inhibiting one of five normal functions of the bacterium (Figure 1). ß-lactams act by inhibiting the synthesis of the cell wall, while other drugs inhibit DNA synthesis (quinolones), protein synthesis (tetracyclines), folate synthesis (sulphonamides) or cause injury to the plasma membrane (Polymyxin B) (Kaufman 2011; Yoon et al. 2004). New antibiotics may fall into one of these classes of inhibition or they may involve a new mode of bacterial interference. Butler and Buss (2006) state that drugs that function by novel mechanisms of action should be at an advantage to novel drugs which use currently described mechanisms of action. The continued development of a novel mode of activity would be desirable as the eventual rate of resistance development would most probably be slower than the current rate of resistance propagation observed.

From 1995 to 2005 there were only 22 new antibiotics released onto the market, 12 were natural products or were derived from a natural product, and the other 10 were synthetic. The 12

naturally derived drugs fall into 5 structural classes while the 10 synthetic drugs fall only into two – 9 of which were quinolones (Butler & Buss 2006). While these drugs may be effective methods of treatment, the fact that they are structurally related to, and share modes of action with current treatments means that their longevity is tethered to their counterparts.

To find out how antibiotics may be rendered ineffective, the modes of antibiotic resistance need to be understood. As stated previously, Abraham and Chain discovered that *E. coli* had resistance to treatment with penicillin by the action of an enzyme named penicillinase. Penicillinase is now called a ß-lactamase, and prevents the ß-lactam antibiotic from functioning by hydrolysing the ß-lactam ring (Thatcher 1975). It is the transmission between bacteria of plasmids carrying the gene that codes for the ß-lactamase enzyme that spreads this type of resistance (Thatcher 1975). Resistance to ß-lactams can also be due to changes in or absence of the penicillin-binding protein (PBP) on the target bacterium (Kaufman 2011).

Since being made available in the 1980s, resistance has developed to quinolones (Robicsek, Jacoby & Hooper 2006). Quinolones work by inhibiting the ability of gyrase or topoisomerase IV from resealing the break created in dsDNA when removing supercoiling. The resistance to quinolones comes in three forms. The first involves mutations to gyrase and topoisomerase IV, whereby the sites to which the quinolones bind have one or more altered amino acids, reducing efficacy or preventing binding altogether. The second involves the up-regulation of natural efflux pumps to reduce the amount of intracellular accumulation of the drug (Robicsek, Jacoby & Hooper 2006). The third mechanism is one that is easily transferrable as it is located on a plasmid. The QnrA protein binds to the gyrase, preventing it from binding to DNA and stopping the quinolone-gyrase-DNA complex forming (Robicsek, Jacoby & Hooper 2006).

Resistance to vancomycin is due to a change in the carboxy terminal of the disaccharide pentapeptide cell wall precursor. A transposon located enzyme changes the structure by altering the end of the carboxy terminal from D-alanyl-D-alanine to the depsipeptide D-alanyl-D-lactate (Weigel et al. 2003). This causes a 1000 fold reduction in the affinity of vancomycin for the protein, leaving the cell relatively uninhibited.

While the increase in antibiotic resistance is of great concern, the rate of community acquired infections caused by resistant strains is lower than the number of infections caused by resistant strains in the hospital setting. However it is important not to disregard any concern towards resistant strains should they become a major player in the community. Antimicrobial therapy must always assume resistance will occur eventually and that new drugs will always be required. A possible source of novel antimicrobial agents are endophytic fungi. Endophytes have been shown to be useful against a number of human pathogens (Aly et al. 2010; Vaz et al. 2009) due to the production of secondary metabolites with antimicrobial properties.

1.2 Fungal Endophytes

Fungal endophytes live in all types of plants in a mostly symbiotic relationship. These fungi can aid in increasing the tolerance of the plant to environmental stressors (Rodriguez et al. 2009). They differ from mycorrhizal fungi in that they inhabit the tissues of the plant itself, leaves, stems and roots, rather than just colonising the root and extending into the rhizosphere. There is also a distinct differentiation between endophytes and epiphytic fungi in that the epiphytes inhabit the surface and not the internal structures. Endophytic fungi generally do not cause the host plant to become diseased as they rely on the plant to provide environmental protection (Strobel 2003). However, if the health of the plant becomes compromised then the

fungi may become pathogenic. There are many agricultural applications that can be derived from studying endophytic fungi, particularly in the prevention of infectious colonisation by other fungal or bacterial species. An example being endophytic prevention of Coffee Tracheomycosis (vascular wilt) in *Coffea arabica* (Mulaw et al. 2013). However this review focuses on the medical aspects of endophytes.

Fungal endophytes can be broken down into two major classes, clavicipitaceous and nonclavicipitaceous (Rodriguez et al. 2009; Sieber 2007). The clavicipitaceous endophytes (Hypocreales; Ascomycota) were first identified in *Lolium* spp. seeds in the 19th century. This class has a narrow host range, inhabiting the shoot and rhizome of grasses, rushes and sedges. They are known to produce secondary metabolites – which stimulate plant growth – and bioactive alkaloids. The production of alkaloids is the cause of toxicosis in cattle, as the fungi act as defensive mutualists (Rodriguez et al. 2009). Clavicipitaceous endophytes are primarily transmitted vertically, from host plant to seed, usually with only one species of endophyte per host plant (Ganley, Brunsfeld & Newcombe 2004; Rodriguez et al. 2009).

Nonclavicipitaceous endophytes are less well studied when compared to clavicipitaceous endophytes as they do not have as great an agricultural impact, due to their inability to infect grasses, even though they make up a majority of endophytes. They can be broken down further into 3 functional groups, with this differentiation being based on colonisation patterns of the host; the mode of transmission between generations of the host; ecological function; and the in-host species diversity levels (Rodriguez et al. 2009). They are non-systemic and are mostly Ascomycota, as well as some Basidomycota (Rodriguez et al. 2009). Like the clavicipitaceous endophytes, they produce bioactive compounds that express beneficial phenotypic traits, such as tolerance to both biotic and abiotic stress (Aly et al. 2010; Sieber 2007) which aids in

acquisition of nutrients to increase both plant growth and yield (Ganley, Brunsfeld & Newcombe 2004; Rodriguez et al. 2009).

In cases where there is disease caused by endophytic fungi, it is either due to the introduction of a foreign endophyte to which the plant has no mutualistic association or influence by external vectors, possibly causing damage to the host plant. Endophytes that cause no harm to their host plant can be extremely pathogenic if introduced to another host, such as those that cause Dutch elm disease (Sieber 2007). High frequency colonisation of healthy plants is an indicator of low pathogenicity of the fungus. When a plant is put under either abiotic or biotic stress, the levels of reactive oxygen species (ROS) increase. Ravindran et al. (2012) tested the effectiveness of endophytic fungi in reducing levels of ROS in mangrove plants. Their results showed that the fungi were able to convey a reduction in ROS, possibly increasing the tolerance of the plant to abiotic stressors such as drought or high salinity.

In cases where host and endophytic fungi produce the same bioactive compound, it is thought that this is due to horizontal gene transfer from either the fungi to the host, or host to fungi. However the complexity of some genes or gene clusters decreases the likelihood of horizontal transfer. Taxol requires around 20 genes to be produced; therefore it is probably more likely that the fungi and plant developed similar gene sets separately to defend from a common pathogen (Zhi-Qiang et al. 2013). Taxol is also able to be produced by fungi which do not reside in the yew tree; therefore the origins of the taxol genes in those fungi is not likely to be due to horizontal gene transfer (Yanfang et al. 2014).

1.3 Medical Uses of Endophytes

There is a great scope of development for medically important compounds from the bioactive products of endophytic fungi. (Strobel 2003) states that just over half (51%) of the compounds isolated from endophytic fungi are compounds that were previously unknown. There is estimated to be approximately 1 million fungal species worldwide, approximately 100,000 of which have been described, leaving 900,000 to be classified (Strobel 2003). This provides the research sector with a sound rationale for investigating this ecological niche as a possible source of novel compounds for the treatment of microbial pathogenesis, viral infection or cancer. As further incentive for investigating endophytes, many of the drugs currently in use today are natural products or the derivatives of natural products (Butler & Buss 2006). A recent discovery from the endophyte streptomycete associated with *Grevillea pteridifolia* in the Northern Territory are broad spectrum antibiotics called kakadumycins (Basha et al. 2012; Strobel 2003). Other medically significant compounds are the immunosuppressive drugs subglutinol A and subglutinol B from the endophyte *Fusarium subglutinans* (Basha et al. 2012). It is important to test fungal endophytes against bacteria, cancer cell lines and other fungi as it would be advantageous for the endophytic fungi to produce one compound which provides protection from multiple pathogens, rather than multiple compounds which are selective for a particular organism.

Worldwide there are a number of research groups studying bioactive compounds from endophytic fungi. Vaz et al. (2009) studied the *Orchidaceae* in Brazilian tropical areas. They were able to isolate 395 endophytes from 1224 plant samples of 54 orchid species. 382 of these isolates were filamentous while the other 13 were yeasts. Of the pathogens against which they were tested (*E. coli*, *S. aureus*, *Candida albicans*, *Candida krusei*), 124 displayed antimicrobial activity against one or more target species. From these 124, 22 stable extracts were derived

with 6 showing activity against *E. coli*, 5 against *S. aureus*, 9 against *C. albicans* and 8 against *C. krusei*. In particular, an isolate identified as an *Alternaria sp*. showed strong activity against *S. aureus* and *B. cereus*. This study illustrates that plants host a wide spectrum of endophytes which may contain medically important compounds. Testing of the isolated compounds against cancer cell lines or viruses may also show activity. However Vaz et al. (2009) only focused on bacteria and fungi.

A study in Thailand by Chaeprasert et al. (2010) of the fungal endophytes present in mangrove trees which had been previously used as traditional medicine to treat infection, yielded 1921 isolates from 3900 leaf segments. Eighty-seven extracts were tested for anticancer properties. There was cytotoxic activity shown by 26 of these extracts and the cancer cell lines against which the extracts were tested were: human malignant melanoma, human colorectal carcinoma, human gastric carcinoma, human liver hepatoblastoma and human acute T cell leukaemia. Most of the isolates were effective against the gastric and leukaemia lines. *Pestalotiopsis* sp. and *Cladosporium* sp. both showed broad spectrum activity. Seventy-one extracts were also tested against *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli*. Twenty-two showed activity towards at least 1 pathogen, while 3 were active against the gram negative bacteria. *Pestalotiopsis microspora* produces an antifungal called ambuic acid, and may be the species isolated by Chaeprasert (Basha et al. 2012). A study of the endophytes from *Terminalia brownii*, a plant used in Eritrea to treat bacterial and viral infections by Basha et al. (2012) yielded 2 antimicrobial compounds which were particularly active against *S. aureus*. In India, the plant *Nycanthes arbor-tristis* was studied by Gond et al. (2012). Twleve isolates showed activity to multiple bacterial pathogens while 9 isolates were active against at least one fungal pathogen. *Nigrospora oryzae* was particularly effective against *Salmonella paratyphi*, the cause of paratyphoid fever as well as *Shigella flexnii*.

The endophytes of *Phyllostachys edulis* were studied by Xiaoye et al. (2012). 30 isolates were identified using GenBank as belonging to 12 genera. Of the 30 isolates, 5 were shown to have antibiotic and/or antifungal activity against a combination of the following pathogens; S. *aureus*, *B. subtilis*, *L. monocytogenes*, *C. albicans*, and *R. rubra*. The isolate with the greatest zones of inhibition was identified as belonging to the *Penicillium* genus, it was also active against all 5 pathogens.

Of the studies listed above, many of the bioactive endophytes could only be identified to their genus, indicating that they had not been previously described. It is also possible that other bioactive compounds produced by the endophyte are not expressed under *in vitro* conditions due to there being no chemical signalling by the plant (Aly et al. 2010). There is also the possibility that some species of endophytic fungi are not able to be grown *in vitro* due to the requirement of niche host conditions.

A few of the species listed above produced secondary metabolites which expressed bioactivity towards multiple pathogens. Whether these are single compounds, or endophytic fungi capable of producing more than one bioactive compound is not defined by the research conducted.

It is evident that there are fungal endophytes capable of producing antimicrobial compounds. The use of these compounds for the treatment of infection *in vivo* is yet to be tested so the mode of administration, dose and response to metabolism has not been evaluated. Isolates that showed activity towards a wide range of pathogens and cancer cell lines may not be able to be used for the treatment of humans as they have wide ranging toxicity.

1.4 Project Outline

This project aims to investigate the antimicrobial properties of endophytes obtained from the native Australian plant, *Santalum lanceolatum*. This plant has been used by the Indigenous population as treatment for skin and respiratory infections (Palombo & Semple 2001).

Tree samples will be taken from sites within the Darling Downs region of southern Queensland and endophytes grown from these samples. Endophytes will be isolated and tested against a range of type strains of clinically significant pathogens, *Escherichia coli* (ATCC 25922), *Enterobacter aerogenes* (ATCC 13048), *Enterococcus faecalis* (ATCC 19433), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (ATCC 25923) and *Serratia marcescens* (ATCC 14756). Endophytes which display initial activity will be grown in bulk and extracted with EtOAc. Extractions will be fractioned by HPLC and tested again for antimicrobial activity. Analytical HPLC will be run of bioactive fractions to identify active compounds. If possible and time permitting, compounds will then be identified by nuclear magnetic resonance (NMR). Endophytes will be identified by either morphological or molecular means.

1.5 Project Objectives

It is hypothesised that *S. lanceolatum* should potentially yield a diverse range of fungal endophytes, of which some may be new species and have antimicrobial secondary metabolites. Projects similar to this one have been conducted worldwide on a range of plant species growing in a wide variety of ecological settings, with most investigations identifying at least one endophyte displaying bioactivity against at least one pathogen with significant clinical impact.

The intended objectives of this project are to; isolate endophytes from *S. lanceolatum* tissues; identify endophytes by either morphological or molecular means; produce and identifiy at least one bioactive compound. This project will potentially add to current knowledge of fungal derived secondary metabolites and Indigenous medicinal plant usage. The identification and characterisation of new fungal endophyte species will also add to what is known about the biodiversity of endophytes.

2.0 Materials and Methods

2.1 Acquisition of Endophytes

2.1.1 Sample Sites

Leaf samples of *Santalum lanceolatum* were taken from 5 sites within the Darling Downs from March – July, 2014. Approximate locations of sample sites can be seen in Figure 2. Site 1 was located near Felton, south-west of Toowoomba. The plant sampled was in open scrub on the north face of a small hill within 200m of a residence. Site 2 was located on a property at Gowrie Junction, north of Toowoomba. The tree sampled was in the house yard of the property, but had grown naturally. Site 3 was Duggan Park, on the eastern edge of Toowoomba. The plant sampled was within 100m of residences and on the north-eastern side of the slope. It appeared that plants within 10m may have been subject to spraying by pesticides, but the tree itself did not appear to be affected. Site 4 was located at Preston, south of Toowoomba. The plant sampled was located on the verge within 10m of a road and backing onto a paddock, the general topography of the area was flat. Site 5 was located at Boodua, 30km north-west of Toowoomba. The plant sampled was situated on the lower northern slopes of a hill in open scrub. Bark samples were also taken from sites 1 and 4, and fruit samples from site 3.

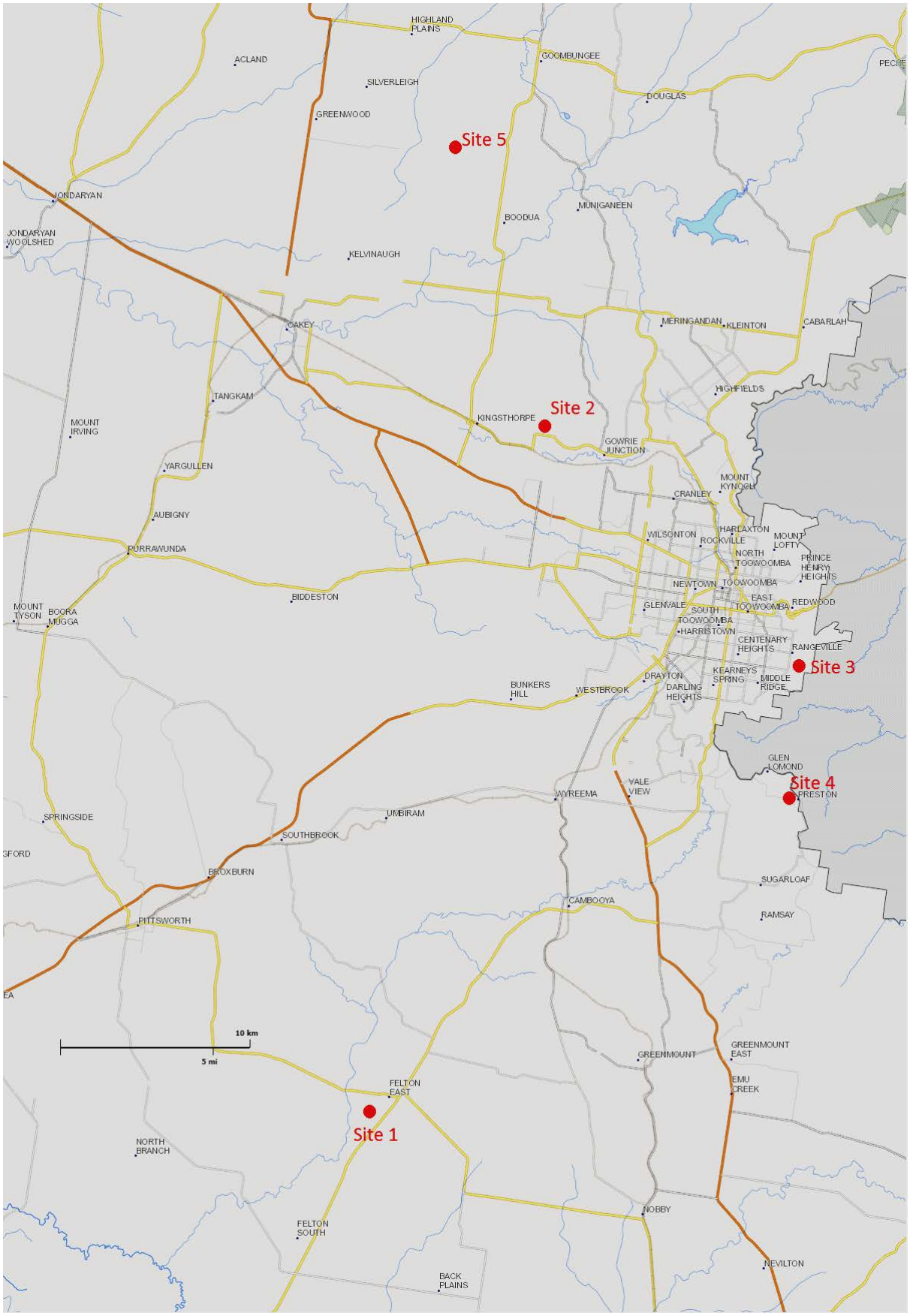

Figure 2 Approximate locations of sample sites within the Toowoomba region (map from Toowoomba Regional Council).

2.1.2 Sample Collection

S. lanceolatum is a tree which grows approximately 3-5m in height. At these heights, trunk diameter can vary from ~6-9cm. Leaves are a dull green in colour and have a silver sheen, as seen in Figure 3. The fruit of the tree is approximately 2-3cm in diameter, has a large "scar" on the lower facet (Figure 3) and is hard to the touch. Foliage generally starts at 1-1.5m from the ground. Whole leaf samples were collected from three foliage heights; lower canopy, middle canopy and upper canopy, this was done so as to gain a more complete overview of endophytes present. Fruit samples were typically taken from the upper canopy of the tree. Bark samples of approximately 1-2cm^2 were taken approximately 1m from the ground and were only collected from trees with a trunk diameter greater than 7cm to avoid the possibility of ringbarking. All samples collected were placed in separate plastic bags and stored on ice until they were able to be processed. Processing of samples occurred within 3 hours of collection.

Figure 3 Leaves, buds and fruit (circled) of S. lanceolatum.

2.1.3 Endophyte Isolation

Collected samples were washed by half filling collection bags with tap water and shaking vigorously for 1min. This process was conducted 3 times and the water replaced after each wash. The samples were then relocated to a biohazard cabinet where they were soaked in sterile water for 5min before being surface sterilised. Surface sterilisation was conducted by soaking the leaf in 95% ethanol (EtOH) for 1min and then passing the leaf through a blue flame to remove excess EtOH and any biological contaminants. Leaves were then pressed against a potato dextrose agar (PDA; Sigma-Aldrich, Castle Hill, NSW) plate to ensure successful sterilisation had taken place. The leaf was then sectioned by the use of a sterile hole punch. Eight leaf sections were placed on each PDA plate, with 2 plates per canopy height. Plates were then sealed with parafilm and incubated in the dark at 23°C. Plates were checked twice daily for growth of fungal or bacterial endophytes from the leaf sections. As fungi or bacteria appeared, they were subcultured onto new PDA plates by sectioning the growing edge with a sterile scalpel blade. These pure culture plates were also incubated at 23°C in the dark.

2.2 Identification of Fungi

2.2.1 Morphological Identification of Fungal Isolates

A small section, approximately 1-2cm^3 of the fungi grown on PDA was removed with a sterile scalpel, placed on a slide with a drop of water and covered with a coverslip. The slide was then examined using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan). Fungal genus identification was conducted as described by Malloch (1997). Photos of isolates were taken with CellSens imaging software.

2.2.2 Molecular Identification of Fungal Isolates

Endophytic isolates which were unable to be identified to the genus level by microscopy or which showed activity in the streak plate test were identified by rDNA taxonomic regions. Bacterial isolates were identified via small subunit (SSU) sequencing and fungi by internal transcribed spacer (ITS) sequencing. DNA of the isolates was extracted using Sigma-Aldrich XNAP-2 kit. The fungal primers used were ITS1F and ITS4 (Gardes & Bruns 1993; Manter & Vivanco 2007), and the bacterial primers used were 27F and 1492R (Galkiewicz & Kellogg 2008). Polymerase Chain Reaction (PCR) was run in duplicate 20µl samples containing 1µl of each respective forward and reverse primer, 10µl extract-N-Amp™ PCR ReadyMix™ (Sigma-Aldrich), 4µl sterile H_2O and 4µl of extracted DNA. A negative control was also included with 4µl sterile H_2O instead of DNA. PCR was run on a Thermo Hybaid PCR Express Thermal Cycler. The DNA was amplified using 35 cycles of 95°C for 1min, 50°C for 1min and 72°C for 1min, followed by a final incubation at 72°C for 10min and a holding temperature of 4°C once the cycle was complete. PCR products were then purified using Diffinity Rapid Tips 2 (Fisher Biotech, Wembley, WA), whereby the sample was drawn up and expressed from the pipette approximately 15 times over the course of 1min. 1µl of the purified product along

with 0.5µl loading dye was then run on a 2% (w/v) agarose gel with iNtRON RedSafe (Gyeonggi-do, Korea) for 20min at 280 volts and 350mA. Visualization of the gel was conducted under UV light. Molecular ladders were also run on the gel alongside the PCR products at 1µl for Fermentas MassRuler™ Express DNA Ladder, LR Forward and 1.5µl for Fermentas MassRuler™ Express DNA Ladder, LR Reverse. Sequencing of extracted purified DNA was conducted by the Brisbane laboratory of the Australian Genome Research Facility (AGRF). The 12µl samples sent for sequencing contained 10µl of 18-30ng/µl of purified DNA and 2µl of the appropriate forward primer (ITS1F for fungi and 27F for bacteria), sterile H_2O was added to make samples up to volume if required. Returned sequences were analysed using the BLASTn search on GenBank (http://www.ncbi.nlm.nih.gov/) to identify the closest genus/species match for each isolate.

2.2.3 Phylogenetic Analysis

The closest matches to sequenced isolates (≥97% similarity) from the GenBank BLASTn search were obtained and were used in sequence alignment and phylogenetic analysis using Molecular Evolutionary Genetics Analysis (MEGA) version 6. For most of the trees produced, outgroups belonging to the same family but separate genus were used, in some cases an outgroup belonging to the same order but different family was used if a closer related match was not available. Sequences were aligned using MUSCLE with a gap open penalty of -400 and an extension penalty of 0. The alignment was manually checked and modified so that all sequences were the same size. Phylogenetic analysis was conducted using the neighbour-joining method. All trees were created using the Kimura 2-parameter model with bootstrap analysis of 1,000 replications.

2.3 Leaf Histology

2.3.1 Formalin Histology

To further characterise the endophytic microbes of *S. lanceolatum*, leaf samples collected from Duggan Park (Site 4) were surface sterilised, sectioned with a scalpel to approximately 1.5cm^2 and placed in formalin. The leaf segments were left in formalin for at least 24hr. Each segment was then placed into a Simport tissue processing/embedding cassette and soaked in water for 20min. Segments were then soaked in 70% EtOH for 45min, 90% EtOH for 45min, and twice in 100% EtOH for 45min each wash. The samples were then soaked in two washes of xylene for a total of 90min. Samples were placed into molten paraffin wax (60°C) and left to solidify for 18hr. The wax was re-melted and the sample embedded in a mould. Once set, the wax was sliced by hand to obtain thin layers for mounting on slides. The layer was placed on a slide on a 50°C hot plate. A cover slip was placed over the sample to draw away the wax, which was then removed with tissue and replaced with water. The stain, either Toluidine Blue or Lacto-Fuchsin was then drawn across the sample for 1min and then replaced with glycerol. Slides were viewed under a Nikon Eclipse E600 microscope at a number of magnifications.

2.4 Antimicrobial Activity

2.4.1 Pathogens

Type strains of *Escherichia coli* (ATCC 25922), *Enterobacter aerogenes* (ATCC 13048), *Enterococcus faecalis* (ATCC 19433), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (ATCC 25923) and *Serratia marcescens* (ATCC 14756) were grown on Sensitest agar (STA) plates for use in the *in vitro* streak plate test. Once cultured, the plates were incubated at 37°C overnight and later stored at 23°C until required for streak plate testing. Pathogens were re-cultured every 3-4 weeks onto new STA.

2.4.2 Streak Plate Test

A 0.5cm^3 section of each endophyte isolated from the leaf samples were transferred from the isolate plate onto the centre of a STA plate. Fungi were transferred by sectioning with a sterile scalpel blade and bacterial isolates were transferred by the use of a sterile inoculation loop and streaked in the centre of the plate. Each isolate was cultured onto STA in duplicate. Plates were incubated in the dark at 23°C. Once the cultures were approximately 2cm in diameter, the test pathogens were streaked via sterile bamboo skewers from the edge of the endophytic colony towards the edge of the agar plate, as seen in Figure 4. Plates were then sealed with parafilm and incubated in the dark at 37°C. They were checked at 24, 36 and 48 hr for signs of inhibition. Isolates that showed inhibition of pathogens on both duplicate plates were investigated further.

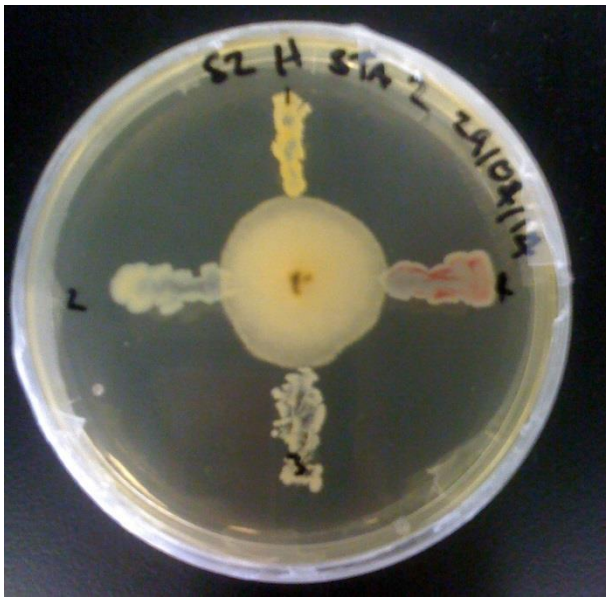

Figure 4 Streak plate growth after approximately 24 hours.

2.5 Bioassay

2.5.1 Liquid Fermentation

Fungi that returned a repeatable positive result for the streak plate test were next cultured in duplicate McCartney bottles containing 10ml of malt extract broth (MEB; Sigma-Aldrich). Bacteria which returned a repeatable positive result were cultured similarly except tryptone soy broth (TSB) was used. The bottles were inoculated with approximately 1cm^3 of fungal mycelia or bacteria and were incubated at 23°C for 1 week with daily gentle agitation. One bottle of MEB was also prepared without fungi as a negative control. After one week, the 10ml inoculum was added to an autoclaved 1L Erlenmeyer flask containing 490ml of MEB or TSB to a total volume of 500ml. Samples were prepared in duplicate with the aim of increasing yield, providing both cultures grew and there was no contamination. Erlenmeyer flasks were blocked with autoclaved cotton stoppers sprayed in 70% EtOH and capped with alfoil to prevent contamination. The flasks were incubated in the dark at 23°C and were swirled daily. After 1 week growth, 2ml of autoclaved pathogens in 0.9% saline were added to the Erlenmeyer flasks. The pathogens added to the flasks were the same as that which had been inhibited by the isolate in the streak plate test. In cases where inhibition was seen against all pathogens, flasks were only treated with autoclaved *E. coli*, *S. aureus*, and *S. marcescens*. The flasks were incubated at 23°C in the dark for a further 2 weeks, with daily mixing.

2.5.2 Extraction

After the growth period, the broth was filtered through 6 layers of gauze into two 500ml separatory funnels. The mycelial mat remaining in the Erlenmeyer flask was then subject to 1hr of sonication while submerged in 200ml of ethyl acetate (EtOAc), with the EtOAc being replaced halfway through the sonication procedure. The separatory funnels, each containing

250ml of broth then had an equal amount of EtOAc added. The funnels were shaken up so as to mix the two layers. Once settled, the broth layer was released into a beaker and the EtOAc layer into a separate flask. This process was conducted on the broth 4 times. The EtOAc used in sonication was added to the flask containing the EtOAc collections from the separatory funnels. The EtOAc extract was then placed on the Heildolph LABOROTA rotary evaporator (Schwabach, Germany) to remove the solvent under vacuum until only the dried crude extract remained. The fungal mycelia and broth layer were also evaporated under vacuum.

2.5.3 Fractionation of EtOAc Extract

For HPLC fractionation of the crude extract, Alltech Davisil 40–60 µm 60 Å C18 bonded silica was used for pre-adsorption work (Alltech, Deerfield, IL, USA). A Shimadzu LC-20AD pump equipped with a Shimadzu SPD-M20A PDA detector and a Shimadzu SIL-20A autosampler were fitted to the HPLC machine. A Phenomenex C18 Onyx Monolithic semi-preparative column (10 mm x 100 mm; Phenomenex, Torrance, CA, USA) and a Phenomenex C18 Onyx Monolithic analytical column (4.6 mm x 100 mm) were used for compound separation. All solvents used for chromatography, were Lab-Scan HPLC grade (RCI Lab-Scan, Bangkok, Thailand), and the H_2O was Millipore Milli-Q PF filtered (Millipore, Billerica, MA, USA). All synthetic reagents were purchased from Sigma Aldrich and used without further purification.

A portion of the crude extract (67mg) was pre-adsorbed to C18-bonded silica (1g) then packed into a stainless steel guard cartridge (10 × 30mm) that was subsequently attached to a C18 semi-preparative HPLC column. Isocratic HPLC conditions of H_2O-ACN-CF_3COOH (90:10:0.1) were initially employed for the first 5min, then a linear gradient to H_2O-ACN (0.1% CF_3COOH; 5:95:0.1) was run over 15min, followed by isocratic conditions of H_2O-ACN (0.1% CF_3COOH; 5:95:0.1) for a further 5min, all at a flow rate of 4ml/min. Seven fractions were

collected manually at 5min intervals from the start of the run, then prepared for bioassay testing.

2.5.4 Analytical HPLC

A portion of the crude extract obtained from the ethyl acetate extraction was resuspended in methanol. Isocratic HPLC conditions of H_2O-ACN-CF_3COOH (90:10:0.1) were initially employed for the first 5min, then a linear gradient to H_2O-ACN (0.1% CF_3COOH; 5:95:0.1) was run over 15min, followed by isocratic conditions of H_2O-ACN (0.1% CF_3COOH; 5:95:0.1) for a further 5min, all at a flow rate of 1ml/min and at 40°C.

2.5.5 Testing of Crude Extracts

Fungal extract fractions were dried using a REACTI-THERM and REACTI-VAP (Thermo Scientific, United States of America) to obtain dry weight. Fractions with insufficient mass were combined with neighbouring fractions if required. The fractions were then resuspended in EtOH to a concentration of 12mg/ml. Three further dilutions of fractions were also made at concentrations of 6mg/ml, 3mg/ml and 1.5mg/ml. The fungal crude extracts were made up at concentrations of 120mg/ml, 60mg/ml, 30mg/ml and 15mg/ml. Test pathogens *E. coli*, *S. aureus* and *S. marcescens* were cultured onto STA and incubated at 37°C overnight. Each pathogen was then suspended in Muller-Hinton Broth (MHB) to a 0.5 McFarland standard. Assays were conducted in separate 96-well plates for each extract with all dilutions of all extracts being conducted in duplicate against each of the three bacteria. 50µl of MHB and 37.5µl of 0.7% saline were added to each test well, along with 12.5µl of extract in EtOH and 50µl of pathogen, to a total volume of 150µl. As a positive control, 50µl of 12µg/ml vancomycin was added to 50µl of MHB and 50µl of each prepared pathogen. For a negative

control, 50µl of sterile H_2O was added to 50µl of MHB and 50µl of each prepared pathogen. As a solvent control, 12.5µl of EtOH and 37.5µl of 0.7% saline were added to 50µl of MHB and 50µl of each prepared pathogen. As a sterility control of the MHB, 100µl of 0.7% saline was added to 50µl of MHB. The 96-well plates were then incubated at 37°C for 18-24hr. Assay wells which showed no growth of pathogen after the 18-24hr incubation period were then streaked onto STA plates and incubated at 37°C overnight to determine if observed extract activity was bactericidal or bacteriostatic.

2.6 Plant Analysis

2.6.1 Leaf Extraction

To test the antimicrobial activity of whole *S. lanceolatum* leaves, leaves from site 3 were dried in a food dehydrator for 36hr to remove all liquid. A mass of dried leaves was then crushed and 1g was placed into each of three 25ml vials with 10ml of either H_2O, EtOAc or MeOH. These vials were then subject to sonication for 30min and left to stand for 24hr. 1ml of the liquid from each of the 25ml leaf matter vials was pipetted into separate vials for HPLC analysis. The remaining 9ml was then pipetted into a pre-weighed vial and the leaf matter flushed with solvent to ensure all extracted material was collected. The liquid extract was then dried using a REACTI-THERM and REACTI-VAP. The weights of the vials was then taken again to determine the amount of extracts collected.

2.6.2 Leaf Analysis HPLC

For HPLC analysis of the leaf extracts, a Shimadzu LC-20AD pump equipped with a Shimadzu SPD-M20A PDA detector and a Shimadzu SIL-20A autosampler were fitted to the HPLC machine. A Phenomenex C18 Onyx Monolithic analytical column (4.6mm x 100mm) was used for compound separation. All solvents used for chromatography, were Lab-Scan HPLC grade, and the H_2O was Millipore Milli-Q PF filtered (Millipore, Billerica, MA, USA). All synthetic reagents were purchased from Sigma Aldrich and used without further purification. Isocratic HPLC conditions of H_2O-ACN-CF_3COOH (90:10:0.1) were initially employed for the first 5min, then a linear gradient to H_2O-ACN (0.1% CF_3COOH; 5:95:0.1) was run over 15min, followed by isocratic conditions of H_2O-ACN (0.1% CF_3COOH; 5:95:0.1) for a further 5min, all at a flow rate of 1 ml/min and at 40°C.

2.6.3 Disc Diffusion

Whatman filter paper was sectioned using a hole punch and autoclaved. The MeOH and H_2O leaf extracts were resuspended with MeOH to 50mg/ml and discs were prepared with 40µl to equate to an extract amount of 20µg per disc. 0.5 McFarland standard preparation of *E. coli*, *S. aureus* and *S. marcescens* in 0.9% saline were spread over 2 STA plates each and let dry under a Bunsen burner. 3 discs of the MeOH and H_2O extracts were placed on the pathogen plates along with a 10ng gentamycin positive control and a methanol disc negative control. The plates were then incubated at 37°C in the dark for 24hr.

3.0 Results

3.1 Acquisition of Endophytes

In total, 71 endophytic isolates were cultured from the five *S. lanceolatum* plants, as seen in Figure 1. Eleven isolates were obtained from bark samples, 6 from fruit, 16 from lower canopy leaves, 18 from middle canopy leaves and 20 from upper canopy leaves (Table 1). Of the isolates obtained, 4 were bacterial and 67 were fungal. Both sites 3 and 4 yielded two bacterial isolates each, with most of the bacterial isolates originating from leaves. Not all of the leaf sections plated onto PDA produced culturable isolates, with some hyphae visibly presenting from the edge of the leaf section but not growing more than 1mm in length. Some plates of leaf sections did not grow any endophytes at all, while on other plates, some leaf segments yielded multiple endophytes, the most obtained from one leaf segment was 3 isolates. On average, the leaves yielded 10.6 endophytes per site. Site 4 produced the highest number of isolates with 21 (7 from bark samples, 14 from leaf sections), while site 5 only yielded 8 isolates, all from leaf sections.

Table 1 Number of endophytes isolated from each part of the plant for all sites.

	Site 1	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Total**
Upper Canopy	2	4	3	6	5	20
Middle Canopy	4	3	5	4	2	18
Lower Canopy	3	2	6	4	1	16
Fruit	-	-	6	-	-	6
Bark	4	-	-	7	-	11
Total	13	9	20	21	8	71

Fungal growth of a single colony was seen on the control plate for site 3, indicating incomplete surface sterilisation of the leaf; injury to the epidermal layer of the leaf, or post sterilisation contamination of the control plate. The fungus on the control plate was identified as a *Nigrospora* sp. (labelled as S3M) and was not subject to further testing.

Each isolate obtained was given a code relating to the site from which it was sampled and in what order they were each isolated from the plant sections. For example, S1A was the first isolate obtained from site 1.

Site 3 was sampled a second time to obtain extra plant material for histology, and extra leaf sections were also taken. From these sections a number of endophytes grew, including a bacterial isolate, DP1, which showed an approximate 1cm zone of fungal inhibition. The colouration of the bacteria in Figure 5 is likely due to growth on PDA with *S. lanceolatum* leaf tea being substituted for water in the preparation of PDA. When on PDA made with water, the bacteria grew with a white colour rather than the pink colour observed previously.

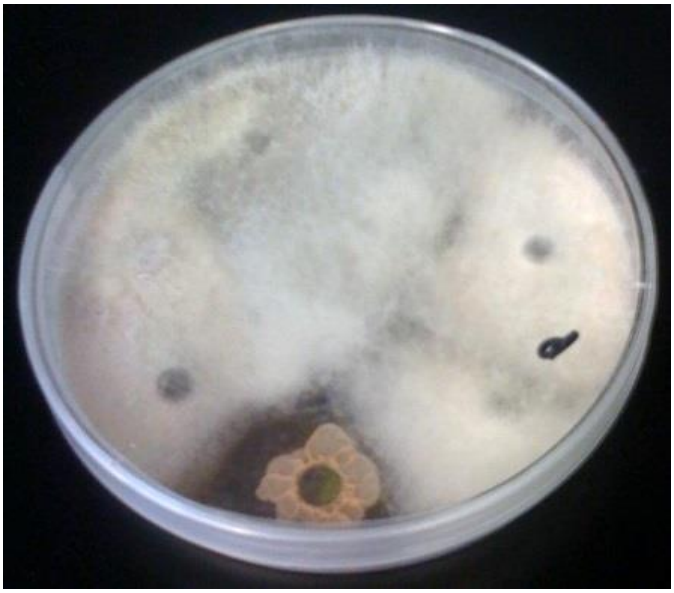

Figure 5 the bacterial isolate obtained from a second visit to Site 3. Note the distinct zone of inhibition between the bacteria and fungi.

3.2 Identification of Fungi

3.2.1 Morphological Identification of Fungal Isolates

Most of the fungi grown were varying shades of grey or cream, but isolates S2H, S4S, S5F and S5H, were green/cream, cream/pink, white/green and cream/brown respectively. All fungi from sites 1-4 were viewed under the microscope, however only the fungi which had sporulated were able to be identified by this method, these are listed in Table 3. Major phenotypic differences in fungi began to become apparent after 3 days growth eg; colour, shape of the leading hyphal edge, hyphal density, vertical projections and growth down into the agar. Notable structurally distinct isolates include S1D, S1H, S2H, S4U and S5F.

Table 2 Isolates able to be identified morphologically.

Site	Plate	Identification	Site	Plate	Identification
S1	A	*Pestalotiopsis* sp.	S3	E	*Nigrospora* sp.
S1	B	*Pestalotiopsis* sp.	S3	F	*Nigrospora* sp.
S1	C	*Pestalotiopsis* sp.	S3	G	*Nigrospora* sp.
S1	D	*Pestalotiopsis* sp.	S3	H	*Nigrospora* sp.
S1	G	*Nigrospora* sp.	S3	I	*Nigrospora* sp.
S1	H	*Aspergillus* sp.	S3	J	*Nigrospora* sp.
S1	I	*Nigrospora* sp.	S3	M	*Nigrospora* sp.
S1	J	*Nigrospora* sp.	S3	R	*Nigrospora* sp.
S1	K	*Nigrospora* sp.	S3	S	*Nigrospora* sp.
S1	L	*Nigrospora* sp.	S3	T	*Nigrospora* sp.
S1	M	*Nigrospora* sp.	S4	B	*Nigrospora* sp.
S2	A	*Nigrospora* sp.	S4	D	*Nigrospora* sp.
S2	C	*Nigrospora* sp.	S4	G	*Nigrospora* sp.
S2	D	*Nigrospora* sp.	S4	J	*Nigrospora* sp.
S2	E	*Nigrospora* sp.	S4	L	*Nigrospora* sp.
S2	F	*Nigrospora* sp.	S4	M	*Nigrospora* sp.
S2	G	*Nigrospora* sp.	S4	N	*Nigrospora* sp.

S2	I	*Nigrospora* sp.	S4	O	*Nigrospora* sp.
S3	A	*Nigrospora* sp.	S4	Q	*Nigrospora* sp.
S3	B	*Pestalotiopsis* sp.	S4	S	*Fusarium* sp.
S3	C	*Nigrospora* sp.			

S1D was identified as *Pestalotiopsis* sp. by spore shape and the presence of hairs at the ends of the spores (Figure 6). The fungus itself was white and developed in concentric circles, seen from below as dark rings on the agar (Figure 7, Image A). S1H had long brown conidiophores at the end of which were black spores visible to the naked eye and was identified as an *Aspergillus s*p. S2H was dark green with cream areas, it grew in a way which deformed the agar in a wave like fashion and turned the agar yellow. S2H was unable to be identified by morphological means. S4U was cream with relatively robust vertical hyphal growth. After 5 days the central area of the colony around the sectioned agar began to darken and hyphae appeared to die back. This change to the appearance of the isolate only affected an area ~2cm in diameter. Initially S5F was white

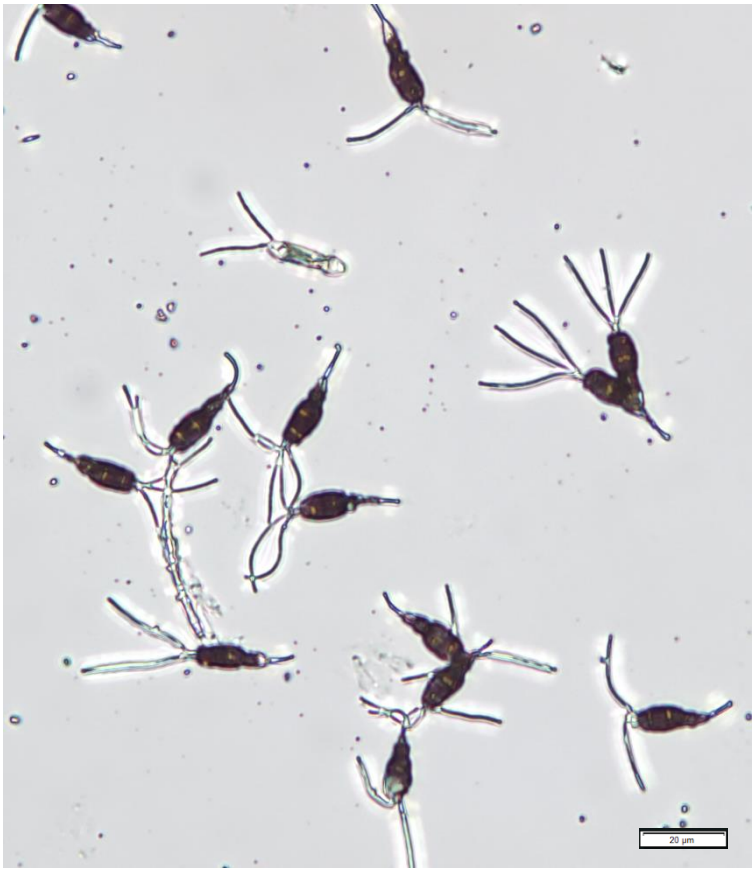

Figure 6 Spores of Pestalotiopsis sp. (S3B).

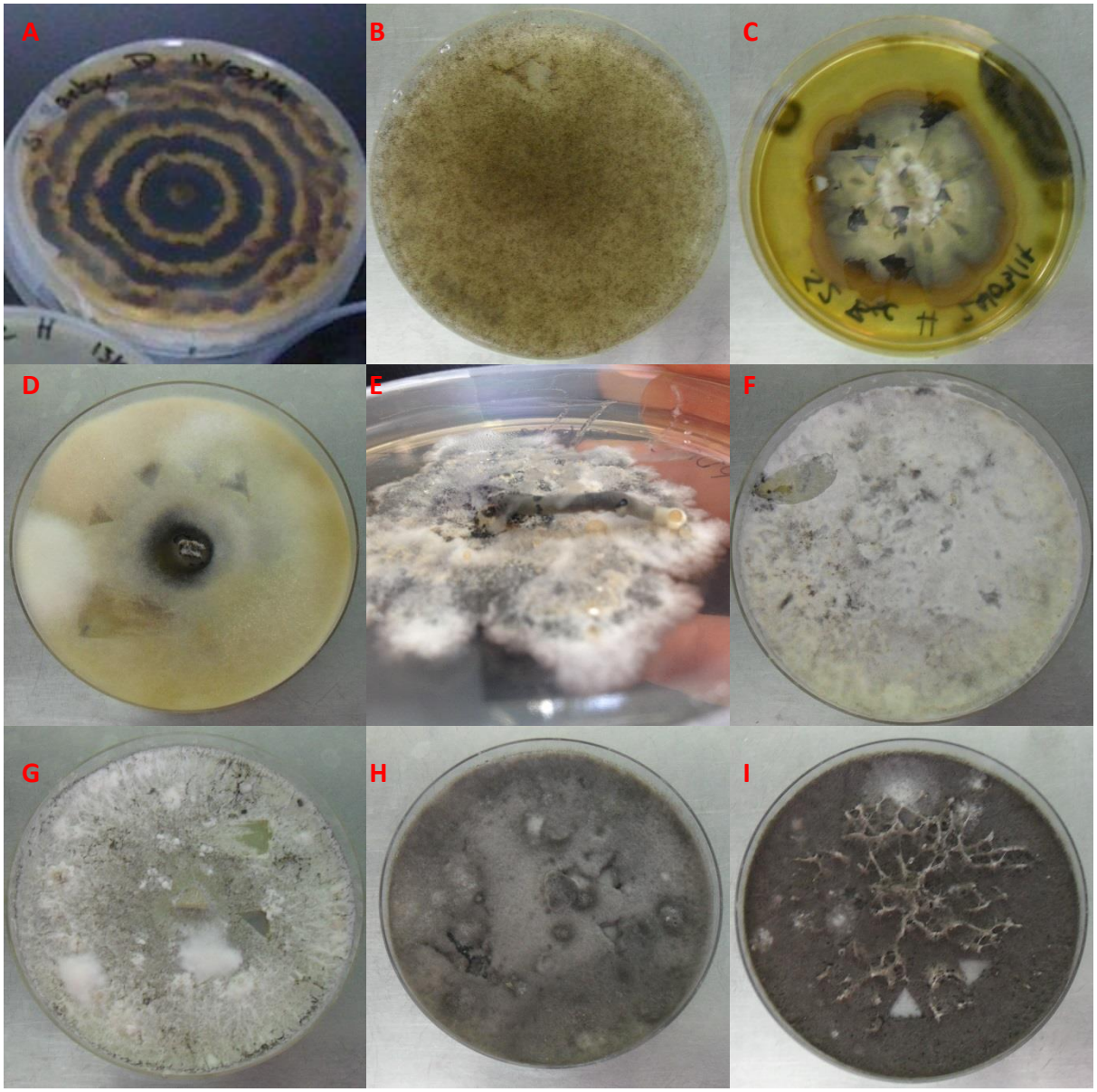

Figure 7 Images A-E, S1D, S1H, S2H, S4U, S5F. Images F-I, S1F, S3J, S3I and S4O.

in colour, with green and grey tones developing after 5-7 days. The leading hyphal edge was irregular in shape, with the fungus having little vertical hyphal growth. After approximately 2 weeks, a large stromata, characteristic of a fungi of the *Xylaria* genus, began to develop in the centre of the plate (Figure 7, image E).

The *Nigrospora* isolates obtained covered the full greyscale spectrum from off white, to black (Figure 7, images F-I). The darker isolates were fluffier in appearance – having greater mass of aerial hyphae. Lighter coloured colonies grew with a lower profile and a lesser hyphal mass

than the darker *Nigrospora* isolates, as can be seen in Figure 7. Most *Nigrospora* isolates also grew hard, dark projections down into the agar, rooting the surrounding surface mycelia to the agar.

Microscopic study of samples revealed many different structural aspects of each fungus. While differences in spore shape, colour and size were used to identify genera, there were also differences observed in the arrangement of hyphae. A majority of the *Nigrospora* spp. observed had short, multi-branched hyphae with the dark spores being located at the end of squat hyphal branches (Figure 9). S1H, identified as an *Aspergillus* sp., had long hyphae with less frequent branching than was seen in the *Nigrospora* isolates. Spores from S1H were held in sacs at the end of conidiophores. Figure 8 shows the hyphal arrangement, conidiophores and spores of S1H, in the course of slide preparation, the spore chains have dislodged and the

Figure 8 Hyphae, conidiophores and spores of S1H (Aspergillus sp.).

spores are free floating in the glycerol solution. All of the *Diaporthe* spp. (S3N, S3O, S3P and S4A) did not show evidence of sporulation and required identification by molecular means. The same was true for the *Pyronema* spp. (S4R and S4U) and *Xylaria* sp (S5F).

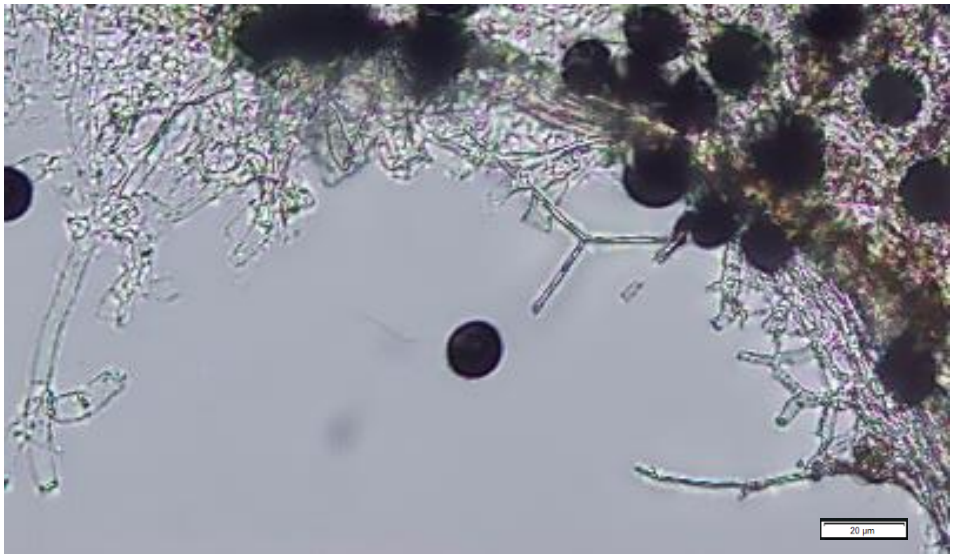

Figure 9 Hyphae and spores of Nigrospora sp. (S1G).

3.2.2 Molecular Identification of Fungal Isolates

31 isolates were unable to be identified via morphology and were subject to DNA extraction and PCR. Twenty-two isolates were able to be sequenced by the AGRF and identified by using the nBLAST search tool on NCBI. Four of the fungi identified as *Nigrospora* spp. by microscopic methods were also sequenced for use in phylogenetic analysis. Figure 10 shows an example gel, an electrophoretic run of both bacterial and fungal amplified DNA.

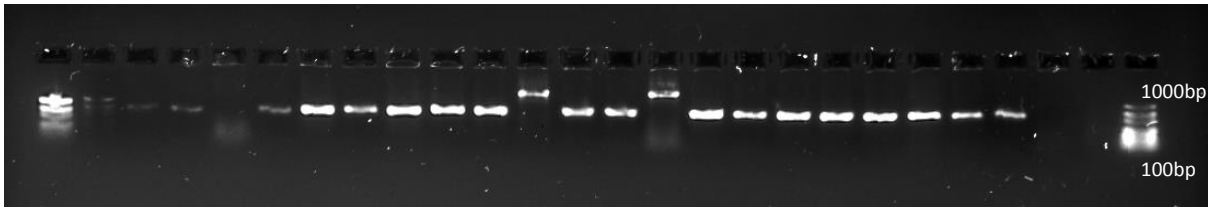

Figure 10 Electrophoresis gel 2, containing both fungal and bacterial amplified DNA, MW ladders are at both ends. The bacterial bands are ~1000bp and the fungal bands are 500-600bp.

Of the 67 fungal endophytes isolated across the five sites, 7 were unable to be identified by either morphological methods or by ITS sequence analysis. 45 of the 60 fungi identified (75%) were identified as being of the genus *Nigrospora*. 6 fungi were identified as *Pestalotiopsis* spp, 4 as *Diaporthe* spp, 2 as *Pyronema* spp, and 1 each as *Aspergillus* sp, *Fusarium* sp, *Preussia* sp. and *Xylaria* sp. Only one bacterium was able to be identified, S4H, as a *Bacillus* sp.

Table 3 Closest two matches from BLAST search of sequenced microbial isolates.

Isolate	GenBank Nº	Name	% Similarity
S1D	EF055203.1	*Pestalotiopsis lespedezae* strain PN3DH	99%
	EF055200.1	*Pestalotiopsis lespedezae* strain EC12A	99%
S1E	KC505176.1	*Nigrospora sphaerica* strain CL-OP30	98%
	JN211105.1	*Nigrospora oryzae* strain HBM1	98%
S1F	EU579801.1	*Nigrospora oryzae* strain SMCD.049	100%
	EU579800.1	*Nigrospora oryzae* strain SMCD.043	100%
S2H	KF557658.1	*Preussia minimoides* isolate MEXU26355	99%
	AY943053.1	*Sporomiellia isomera* strain CBS166.73	99%
S3K	HQ608152.1	*Nigrospora oryzae* isolate TR171	99%
	HQ608090.1	*Nigrospora oryzae* strain TR058	99%
S3N	KJ869137.1	*Diaporthe vanqueriae* strain CPC22703	99%
	FJ441631.1	*Phomopsis theicola* strain xz06	97%
S3O	KJ869137.1	*Diaporthe vanqueriae* strain CPC22703	100%
	KC343125.1	*Diaporthe inconspicua* strain LGMF931	98%
S3P	KJ869137.1	*Diaporthe vanqueriae* strain CPC22703	100%
	KC343125.1	*Diaporthe inconspicua* strain LGMF931	98%
S3Q	JX854541.1	*Pestalotiopsis neglecta* isolate CCTU12	99%
	JN314418.1	*Pestalotiopsis microspora* strain RA1-2	99%
S4A	KJ869137.1	*Diaporthe vanqueriae* strain CPC22703	99%
	KC343125.1	*Diaporthe inconspicua* strain LGMF931	98%
S4E	KC505176.1	*Nigrospora sphaerica* strain CL-OP30	98%
	JN211105.1	*Nigrospora oryzae* strain HBM1	98%

S4F	KC505176.1	*Nigrospora sphaerica* strain CL-OP30	98%
	JN211105.1	*Nigrospora oryzae* strain HBM1	98%
S4H	JN631035.1	*Bacillus licheniformis* strain CHW102	98%
	HM163535.1	*Bacillus licheniformis* strain 4TL12	98%
S4I	JN211105.1	*Nigrospora oryzae* strain HBM1	99%
	HQ608152.1	*Nigrospora oryzae* isolate TR171	99%
S4J	HQ608030.1	*Nigrospora sphaerica* isolate CY202	100%
	FJ478134.1	*Nigrospora sphaerica* strain xsd08093	100%
S4K	KC505176.1	*Nigrospora sphaerica* strain CL-OP30	98%
	JN211105.1	*Nigrospora oryzae* strain HBM1	98%
S4P	KC505176.1	*Nigrospora sphaerica* strain CL-OP30	98%
	JN211105.1	*Nigrospora oryzae* strain HBM1	98%
S4R	HM016895.1	*Pyronema domesticum* strain A10	98%
	HQ115722.1	*Pyronema domesticum* isolate ofi712	98%
S4U	HM016895.1	*Pyronema domesticum* strain A10	99%
	HQ115722.1	*Pyronema domesticum* isolate ofi712	99%
S5C	HQ608030.1	*Nigrospora sphaerica* isolate CY202	100%
	FJ478134.1	*Nigrospora sphaerica* strain xsd08093	100%
S5D	HQ608030.1	*Nigrospora sphaerica* isolate CY202	100%
	FJ478134.1	*Nigrospora sphaerica* strain xsd08093	100%
S5E	HQ608063.1	*Nigrospora sphaerica* isolate CY256	100%
	KF924041.1	*Nigrospora oryzae* isolate BY-D	99%
S5F	AB625411.1	*Xylaria grammica* BCC1002	98%
	GU300097.1	*Xylaria grammica* isolate 479	98%
S5G	KC505176.1	*Nigrospora sphaerica* strain CL-OP30	98%
	JN211105.1	*Nigrospora oryzae* strain HBM1	98%

3.2.3 Phylogenetic Analysis

Sequences which were identified as belonging to the same genus were phylogenetically analysed together. S1E, S1F, S3K, S4E, S4F, S4I, S4J, S4K, S4P, S5C, S5D, S5E and S5G were all identified as belonging to the *Nigrospora* genus and were analysed along with 13 identified *Nigrospora* species and *Arthrinium xenocordella* as an outgroup. Figure 11 shows how these endophytes fit within the tree. Of note are S1E, S4E, S4F, S4K, S4P and S5G which, although they all matched to 98% with *Nigrospora sphaerica* strain CL-OP30 and *Nigrospora oryzae* strain HBM1 they did not cluster with them when in the tree and have formed a separate

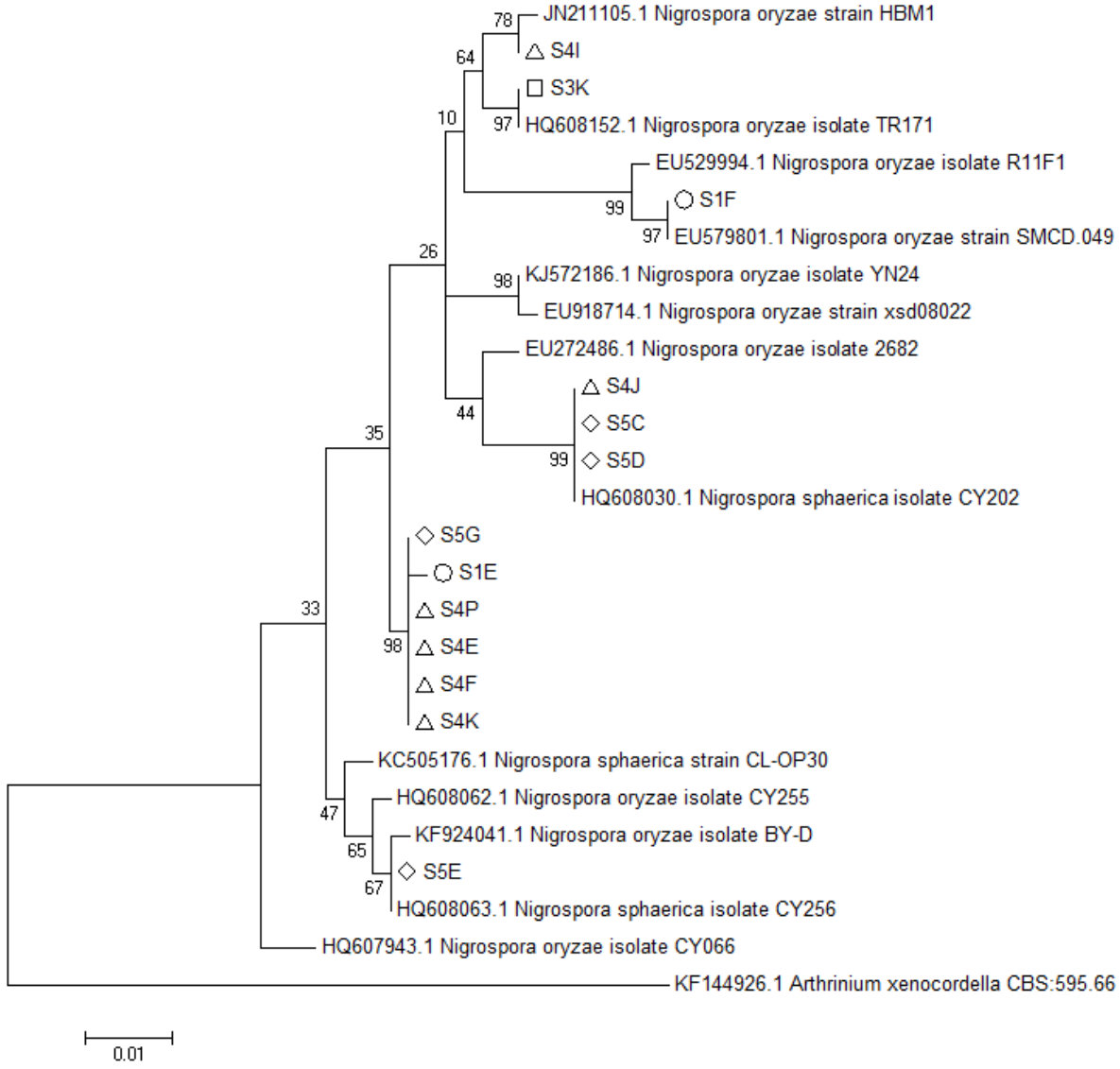

Figure 11 Nigrospora phylogenetic tree.

branch. S4E, S4F, S4P and S4K were all isolated from the same tree but were taken from all three sampled heights, upper canopy – S4E, S4F, middle canopy – S4K, and lower canopy – S4P. This may indicate that the isolates obtained are all the same fungal species which is systemic in the plant.

Figure 12 shows the phylogenetic tree for *Preussia*, including isolate S2H. Interestingly both *Preussia minimoides* isolate MEXU26355 and *Sporormiellia isomera* strain CBS166.73 to which S2H showed a 99% similarity are on a separate branch and S2H appears to have greater similarity to *Preussia africana* strains s12. The intermingling of *Preussia* and *Sporormiella* throughout the phylogenetic tree is most probably due to them both being of the family Sporormiaceae and having similar ITS sequences. However the outlier, *Westerdykella multispora* strain CBS 391.51 is also of the same family, yet is distant to the main branching of the tree. This could indicate improper identification of sequences uploaded to GenBank.

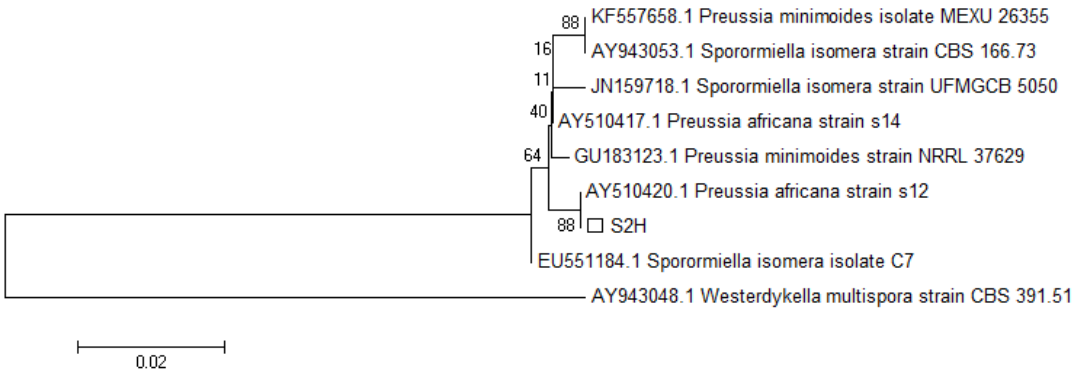

Figure 12 Preussia phylogenetic tree.

The *Xylaria* phylogenetic tree in Figure 13 shows that while *Xylaria grammica* BCC1002 and *Xylaria grammica* isolate 479 were the closest matches, S5F is on its own branch and may be a different taxon to those identified. As the closest matches were all *Xylaria grammica*, *Xylaria arbuscula* was also included, along with *Clypeosphaeria mamillana* as outgroups.

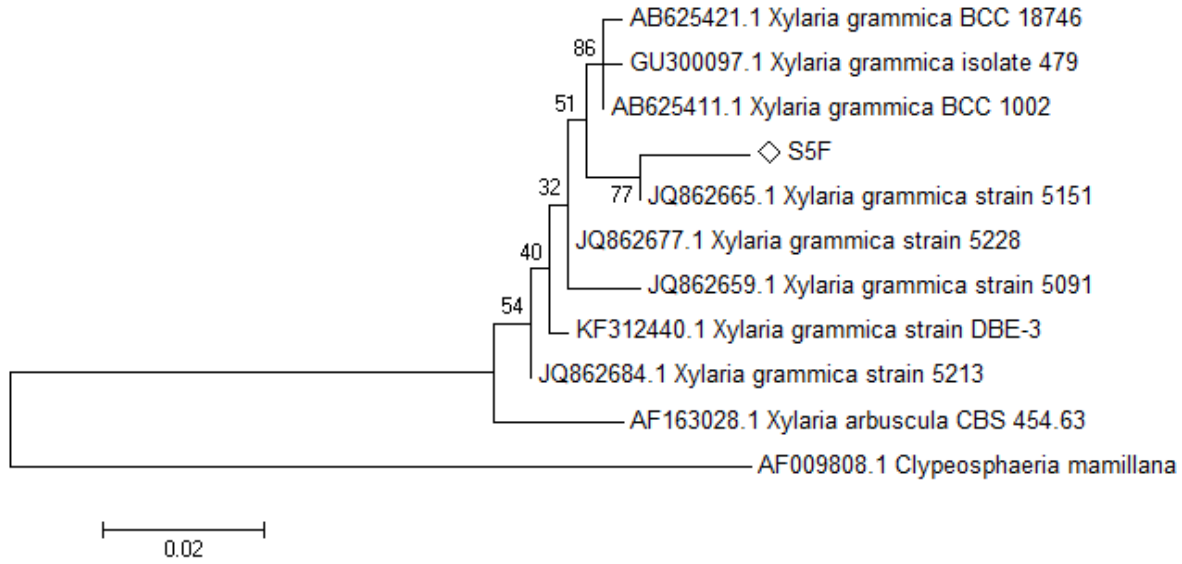

Figure 13 Xylaria phylogenetic tree.

The two sequenced *Pestalotiopsis* isolates returned different best matches. On the tree, seen in Figure 14, S3Q is on the same branch as *Pestalotiopsis neglecta* isolate CCTU12 and *Pestalotiopsis microspora* strain RA1-2 to which it returned a similarity of 99%. The two closest matches to S1D were *Pestalotiopsis lespedezae* strain PN3DH and *Pestalotiopsis lespedezae* strain EC12A, these can be seen at the top of the tree, while S1D has formed a separate branch near the bottom.

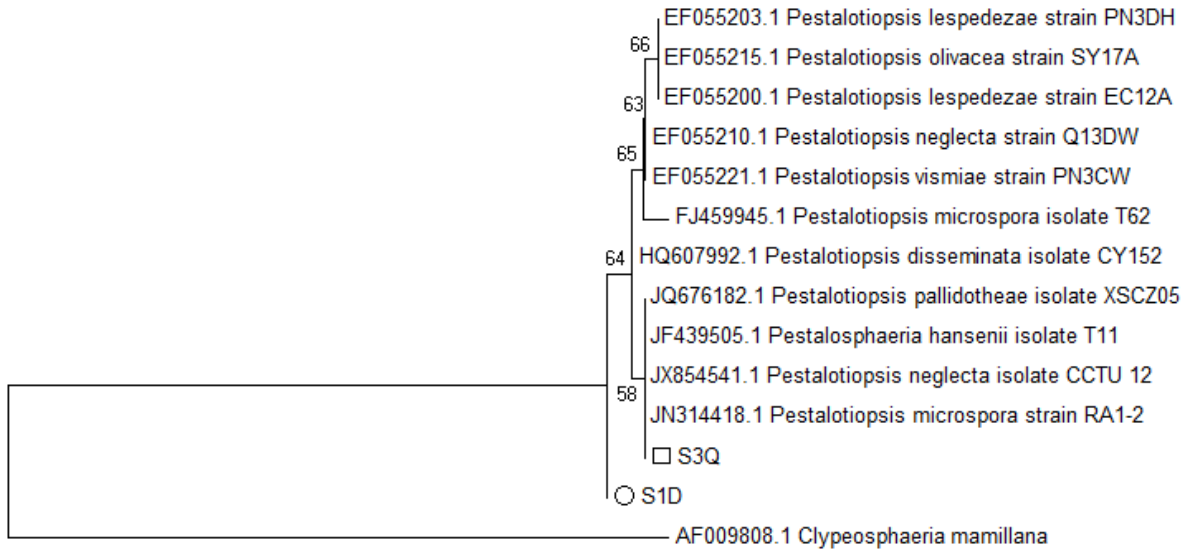

Figure 14 Pestalotiopsis phylogenetic tree.

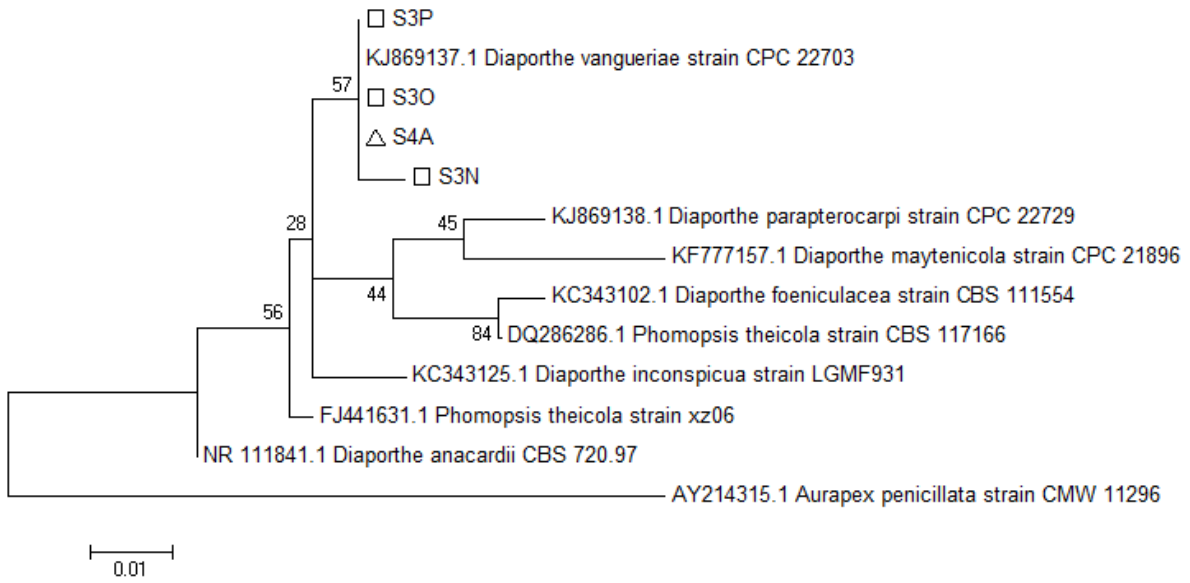

Figure 15 Diaporthe phylogenetic tree.

Figure 15 shows the phylogenetic tree for *Diaporthe*. S3N, S3O, S3P and S4A all grouped with *Diaporthe vanqueriae* strain CPC22703, to which they all had 99-100% similarity. The presence of *Phomopsis* within the tree may indicate that improper identification of sequences uploaded to GenBank has occurred or that there is high familial similarity of ITS regions. *Aurapex penicillata* (family *Cryphonectriaceae*) was included as an outgroup due to all tested sequences of the *Diaporthaceae* family intended as outgroups intermingling with the main clade of the tree.

The *Pyronema* phylogenetic tree in Figure 16 was limited in construction due to few sequences of useful identified species being present in GenBank. Both S4R and S4U showed 98%

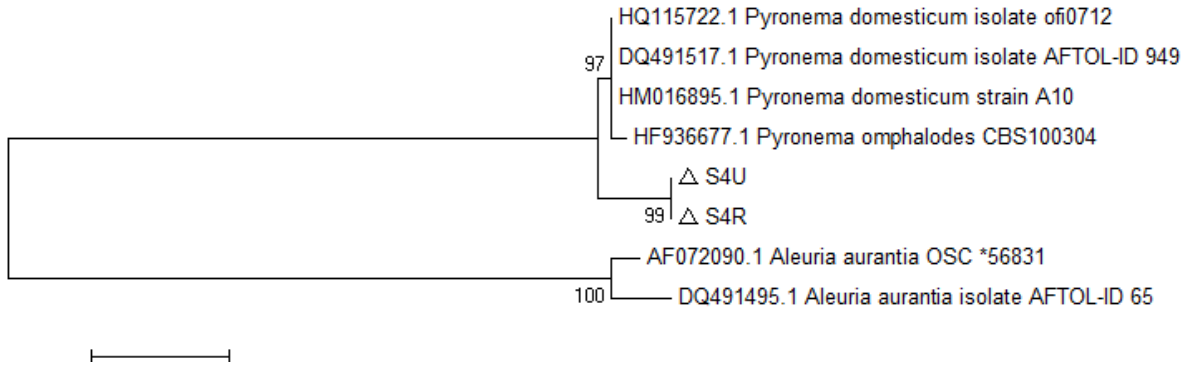

Figure 16 Pyronema phylogenetic tree.

similarity to *Pyronema domesticum* strain A10 and *Pyronema domesticum* isolate ofi712. On the tree it can be seen that S4R and S4U form their own branch, separate to their closest matches. Two *Aleuria aurantia* sequences were included as outgroups.

3.3 Leaf Histology

3.3.1 Formalin Histology

Fungal hyphae were easily identifiable at 200x magnification. They appeared to be more prevalent at the epidermal layer of the leaf, with projections into the mesophyll. At 1000x magnification, hyphal growth was able to be distinguished. Figures 17, 18 and 19 show fungal hyphae within leaves using both 400x and 1000x magnification and utilising both toluidine blue and lacto-fuchsin stains. Fungi were unable to be identified from these preparations as there were no obvious reproductive structures present and there was no distinguishing shape to the hyphae.

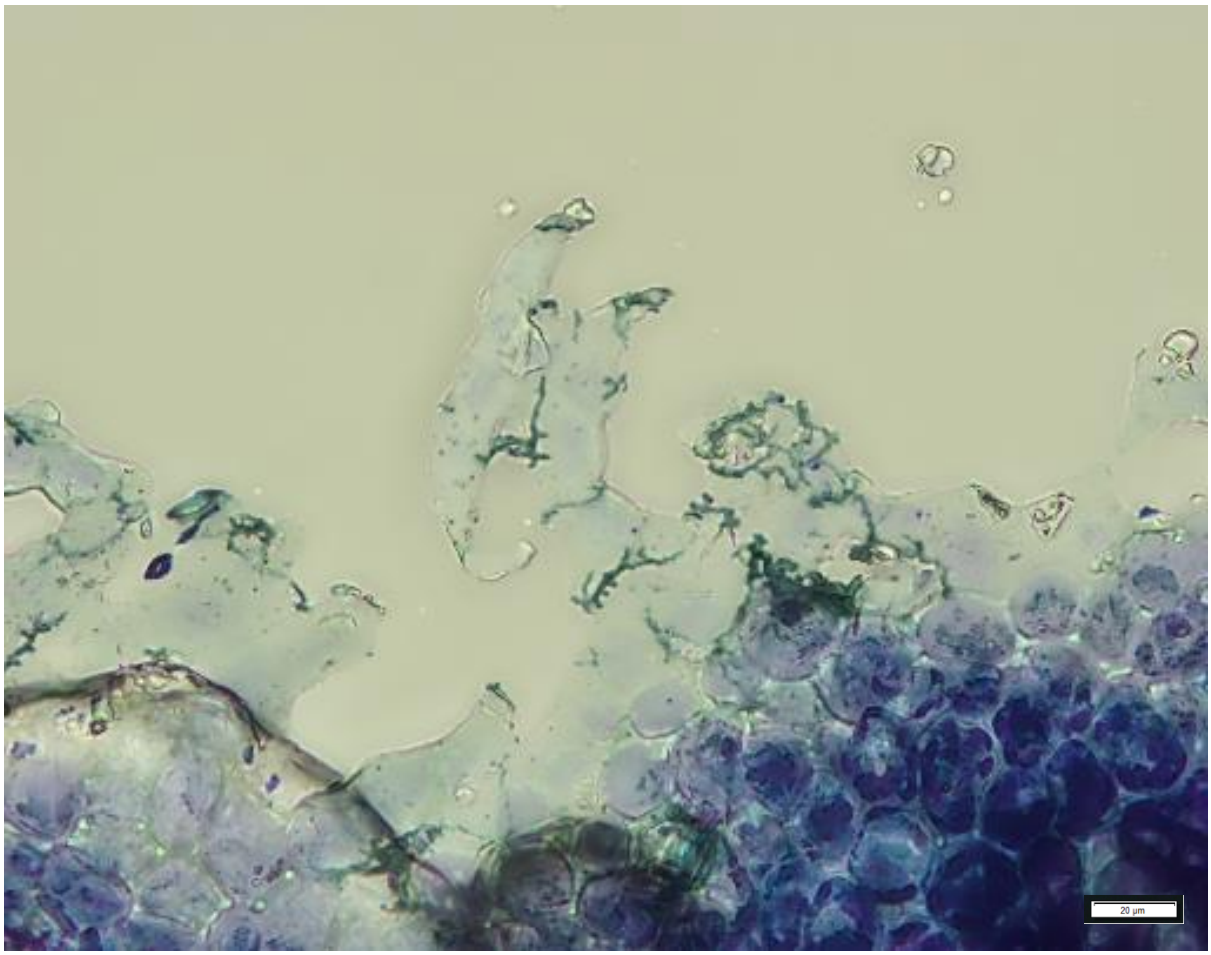

Figure 17 Leaf segment stained with toluidine blue at 400x magnification. Fungal hyphae can be seen through the epidermal layer and cuticle of the leaf.

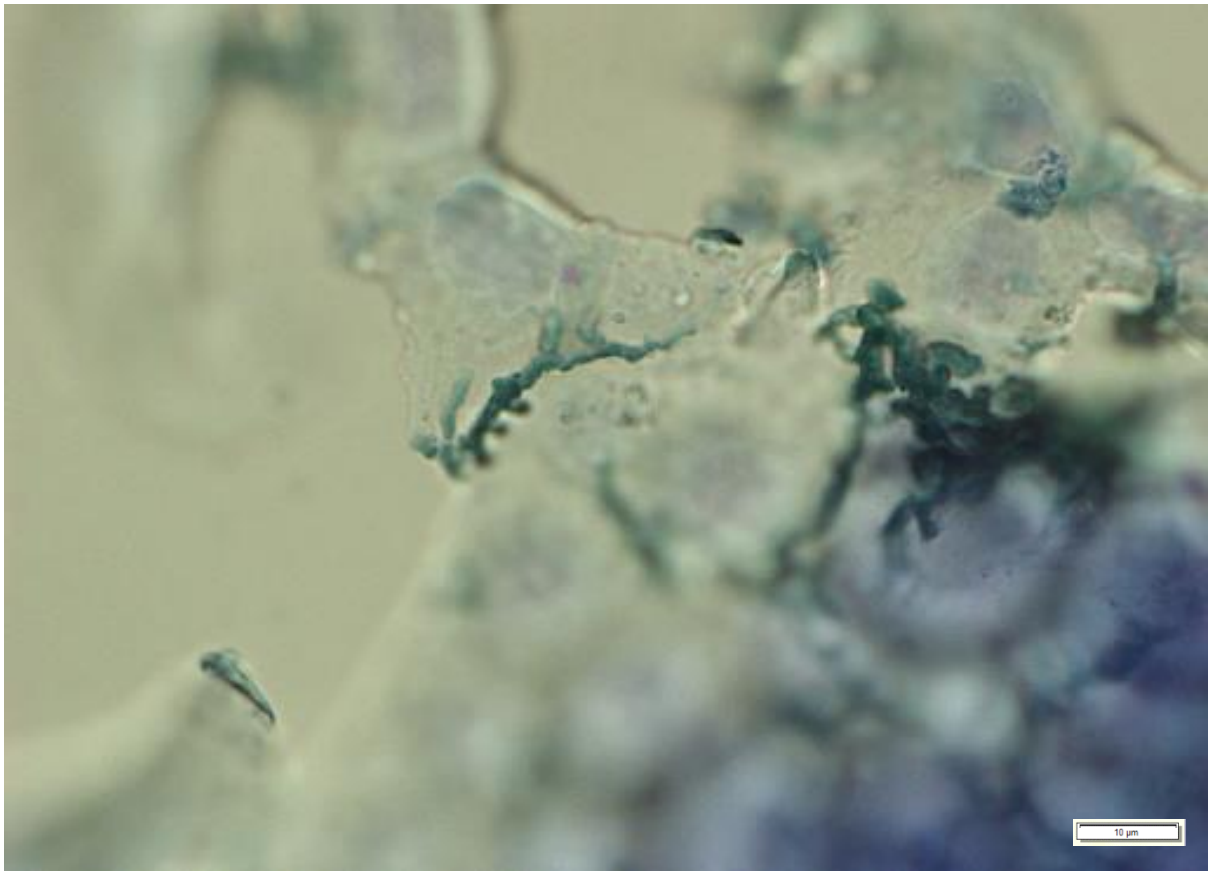

Figure 18 Leaf segment stained with toluidine blue at 1000x magnification. The growing tip of the fungal hyphae can be seen in the centre of the photo.

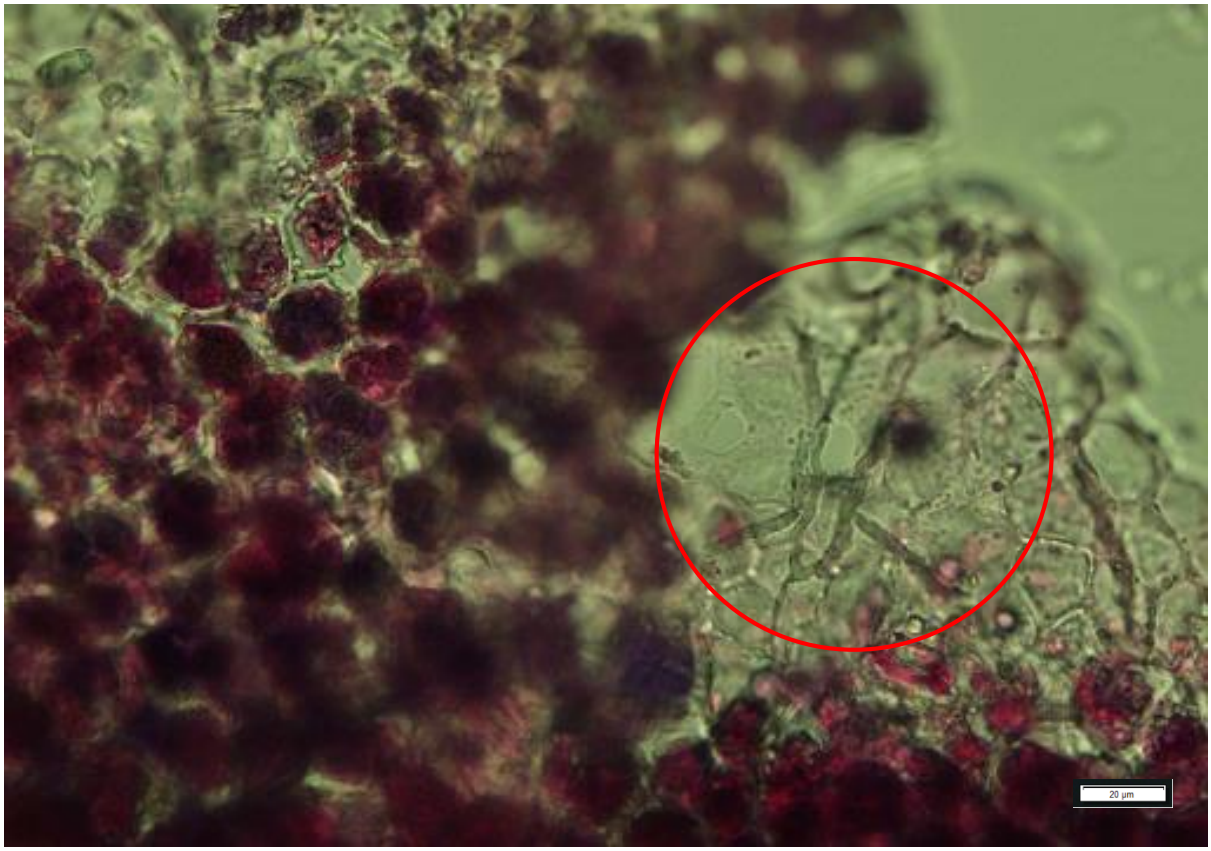

Figure 19 Leaf segment stained with lacto-fuchsin at 400x magnification. Hyphae can be seen growing between leaf cells (circled).

3.4 Antimicrobial Activity

3.4.1 Streak Plate Test

There was a noticeable difference in how some of the isolates grew on PDA and when cultured on STA. Many fungi spread at a reduced rate on STA with the hyphae occurring in a denser, centralised formation than on PDA. However, some of the fungi (S3F, S4K) grew quickly when on STA and did not develop a central mass, yet had hyphae reaching 4-5cm from the centre of the plate within 24-36 hours of being cultured. Most fungi took 36-48 hours to reach the 2cm diameter requirement for bacterial streaking. However, there were some fungi, notably a *Pestalotiopsis* sp. and a *Diaporthe* sp. which took 96 hours to reach the required diameter. Only one fungus, S5H, was unable to be tested as it did not grow at all on the STA.

All *Nigrospora* sp. tested showed no inhibition of the tested pathogens. None of the isolates, bacterial or fungal, showed distinct zones of inhibition between the isolate and the pathogen. Reduced growth of some pathogens was seen with S3D, S4R, S4U, S5F and DP1. Table 2 shows isolate activity against tested pathogens. A + indicates normal growth (growth consistent with that observed on a pure culture pathogen plate), - indicates reduced growth.

Table 4 Isolate activity against pathogens in the streak plate test.

	Test Microbe					
Isolate	***E.coli***	***E. faecalis***	***E. aerogenes***	***P. aeruginosa***	***S. aureus***	***S. marcescens***
S1A	+	+	+	+	+	+
S1B	+	+	+	+	+	+
S1C	+	+	+	+	+	+
S1D	+	+	+	+	+	+
S1E	+	+	+	+	+	+
S1F	+	+	+	+	+	+
S1G	+	+	+	+	+	+
S1H	+	+	+	+	+	+

	E.coli	*E. faecalis*	*E. aerogenes*	*P. aeruginosa*	*S. aureus*	*S. marcescens*
S1I	+	+	+	+	+	+
S1J	+	+	+	+	+	+
S1K	+	+	+	+	+	+
S1L	+	+	+	+	+	+
S1M	+	+	+	+	+	+
S2A	+	+	+	+	+	+
S2B	+	+	+	+	+	+
S2C	+	+	+	+	+	+
S2D	+	+	+	+	+	+
S2E	+	+	+	+	+	+
S2F	+	+	+	+	+	+
S2G	+	+	+	+	+	+
S2H	+	+	+	+	+	+
S2I	+	+	+	+	+	+
S3A	+	+	+	+	+	+
S3B	+	+	+	+	+	+
S3C	+	+	+	+	+	+
S3D	-	-	-	-	-	-
S3E	+	+	+	+	+	+
S3E	+	+	+	+	+	+
S3F	+	+	+	+	+	+
S3G	+	+	+	+	+	+
S3H	+	+	+	+	+	+
S3I	+	+	+	+	+	+
S3J	+	+	+	+	+	+
S3K	+	+	+	+	+	+
S3L	+	+	+	+	+	+
S3N	+	+	+	+	+	+
S3O	+	+	+	+	+	+
S3P	+	+	+	+	+	+
S3Q	+	+	+	+	+	+
S3R	+	+	+	+	+	+
S3S	+	+	+	+	+	+

	E.coli	*E. faecalis*	*E. aerogenes*	*P. aeruginosa*	*S. aureus*	*S. marcescens*
S3T	+	+	+	+	+	+
S4A	+	+	+	+	+	+
S4B	+	+	+	+	+	+
S4C	+	+	+	+	+	+
S4D	+	+	+	+	+	+
S4E	+	+	+	+	+	+
S4F	+	+	+	+	+	+
S4G	+	+	+	+	+	+
S4H	+	+	+	+	+	+
S4I	+	+	+	+	+	+
S4J	+	+	+	+	+	+
S4K	+	+	+	+	+	+
S4L	+	+	+	+	+	+
S4M	+	+	+	+	+	+
S4N	+	+	+	+	+	+
S4O	+	+	+	+	+	+
S4P	+	+	+	+	+	+
S4Q	+	+	+	+	+	+
S4R	-	-	-	+	-	-
S4S	+	+	+	+	+	+
S4T	+	+	+	+	+	+
S4U	-	-	-	+	-	-
S5A	+	+	+	+	+	+
S5B	+	+	+	+	+	+
S5C	+	+	+	+	+	+
S5D	+	+	+	+	+	+
S5E	+	+	+	+	+	+
S5F	-	-	-	-	-	-
S5G	+	+	+	+	+	+
S5H	Fungi didn’t grow on STA					
DP1	-	-	-	-	-	-

3.5 Bioassay

3.5.1 Liquid Fermentation

Isolates DP1, S4R, S4U and S5F were all grown in triplicate in McCartney bottles in MEB or TSB, with the 2 bottles showing greatest growth then being transferred into 1L Erlenmeyer flasks after 1 week. Once transferred into Erlenmeyer flasks, DP1 and S4U did not continue to grow and were excluded from further testing. Of the two S4R flasks, one showed signs of contamination after 3 days and was not continued. S5F grew the slowest of all the isolates when in the McCartney bottles. Of the 2 S5F Erlenmeyer flasks, one colony did not increase in size from when in the McCartney bottle and was therefore also excluded from further testing. After being exposed to the autoclaved pathogens (*E. coli*, *S. aureus* and *S. marcescens*) the remaining S4R and S5F isolates continued to grow. At the end of 3 weeks growth in the Erlenmeyer flasks, S4R had formed a mycelial mat 1cm thick over the surface of the MEB with the same texture as that when grown on PDA. There was little fungal growth observed in the broth itself. S5F grew around the edge of the flask in colonies approximately 3cm in diameter, covering half the surface area of the broth. The texture and colour of these was the same as when grown on PDA. Unlike when grown on PDA, the fungus did not develop any stromata over this period of time. As with S4R, S5F had very little observable growth in the broth itself; most mycelial growth was on the surface.

3.5.2 Extraction and Fractionation of EtOAc Extract

For both S4R and S5F EtOAc extracts, fractions were collected in 5min intervals of a 32.5min run (Figure 20). Dry weights of fractions obtained can be seen in Table 5. S5F had a total extract dry weight over 4 times greater than that of S4R. Both fungi had the highest extract dry weights between 15 and 25mins. Figure 19 shows that between 12.5min and 20min, S5F had high absorbance readings when compared to S4R. This indicates that the compounds present in the extract are soluble in the mid solvent range between H_2O-ACN-CF_3COOH and H_2O-CAN. This is in comparison to the S4R extract which shows less activity in the mid solvent range and greater activity across the spectrum. The readings around 2500 mAU correspond with high fraction yield around these times. For both S4R and S5F the colouration of the fractions between 10 and 25min was yellow while other fractions appeared clear.

Table 5 Dry weights of fractions from S4R and S5F.

Fraction	S4R	S5F
1 (0-5min)	0.0014g	0.0006g
2 (5-10min)	0.0076g	0.0412g
3 (10-15min)	0.0035g	0.0161g
4 (15-20min)	0.0113g	0.0254g
5 (20-25min)	0.0060g	0.0332g
6 (25-30min)	0.0017g	0.0206g
7 (30+ min)	0.0011g	0.0039g
Total	0.0326g	0.1410g

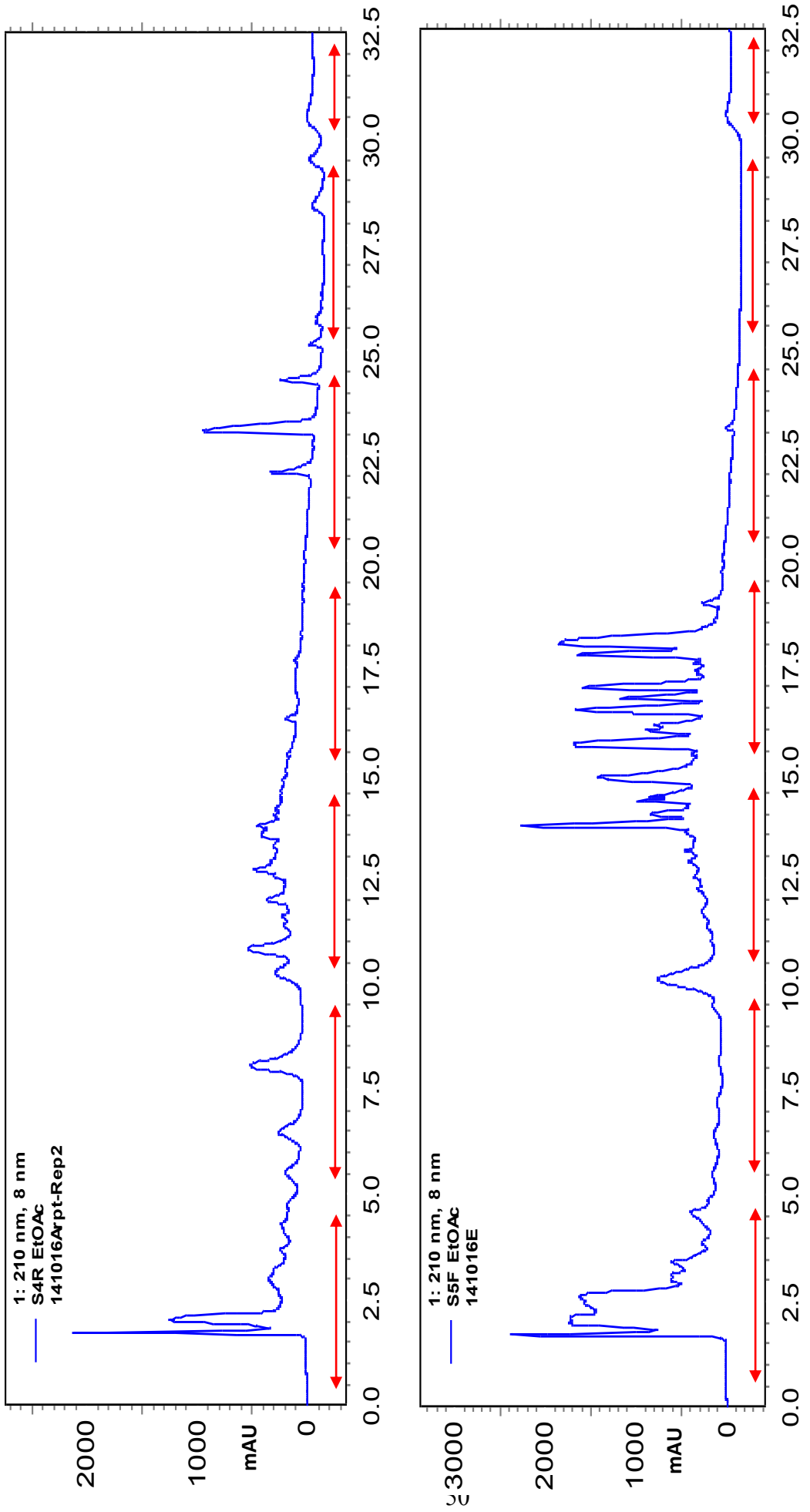

Figure 20 Analytical HPLC of S4R (top) and S5F (bottom). Fractions were collected over 5min intervals as shown in red.

3.5.3 Analytical HPLC

Analytical HPLC of extracted fractions show that each of the extracts expressed different profiles. All chromatograms show an injection peak at 2min. Profiles of all S4R fractions can be seen in Figure 21, profiles of all S5F fractions can be seen in Figure 22. S4R 1-3 analytical HPLC had no distinct peaks, S4R 4 had small peaks around the 250mAU range from 2.5 to 7.5min. S4R 5-7 showed a highest peak of 250 mAU around 7.5min and did not show any absorbance after 20min. S5F fraction 1-2 had no distinguishable peaks, save for a small 100 mAU peak at 2.5min. S5F fraction 3-4 had a highest peak of 400 mAU at 2.5min and several

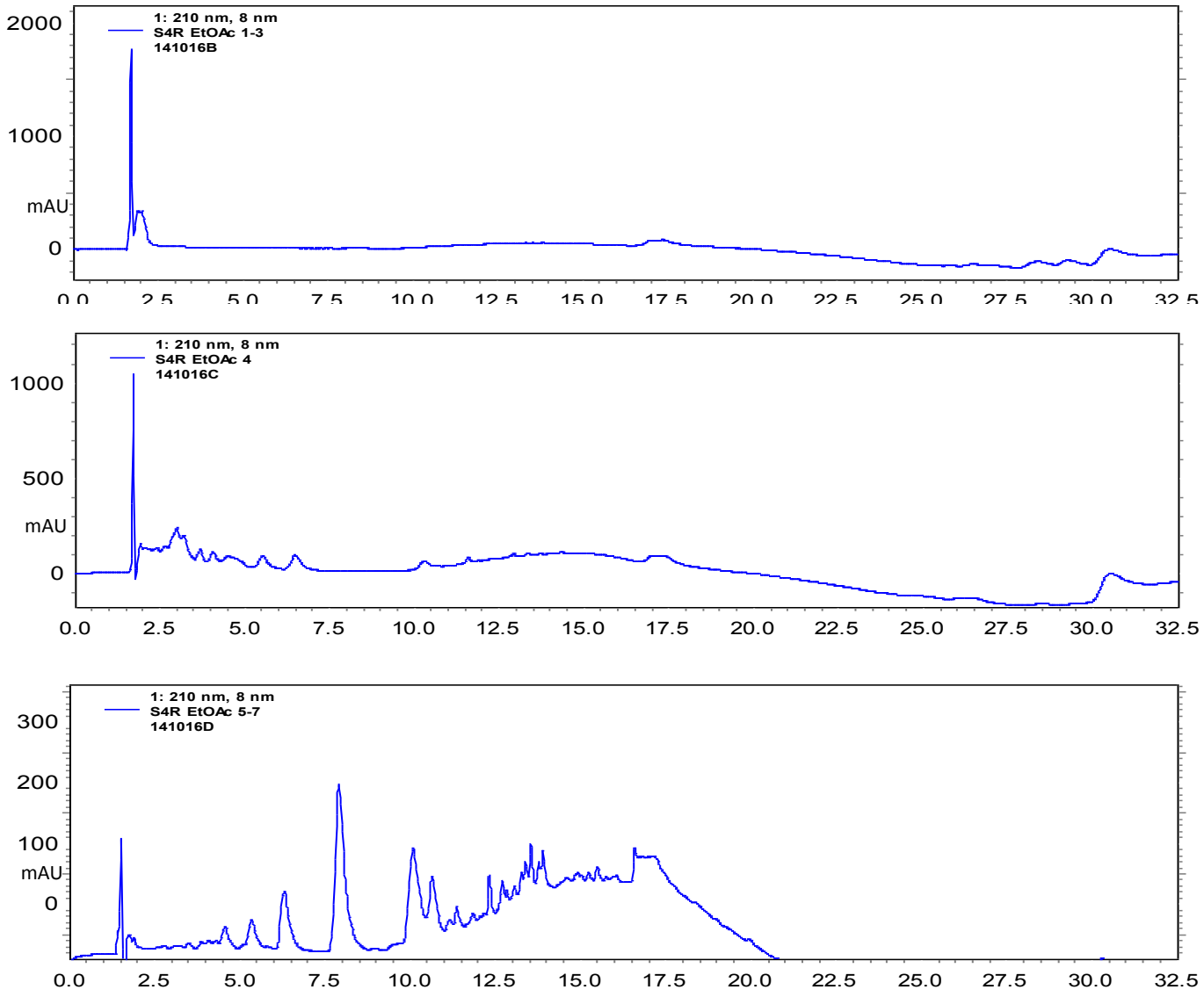

Figure 21 Analytical HPLC of S4R extracts. Top-Bottom: S4R 1-3, S4R 4, S4R 5-7.

smaller peaks between 2.5min and 5min. S5F fraction 5 and fraction 6-7 both showed large ~200 mAU peaks between 12.5min and 20min, these peaks observed correlate with those observed in the crude extract (Figure 20).

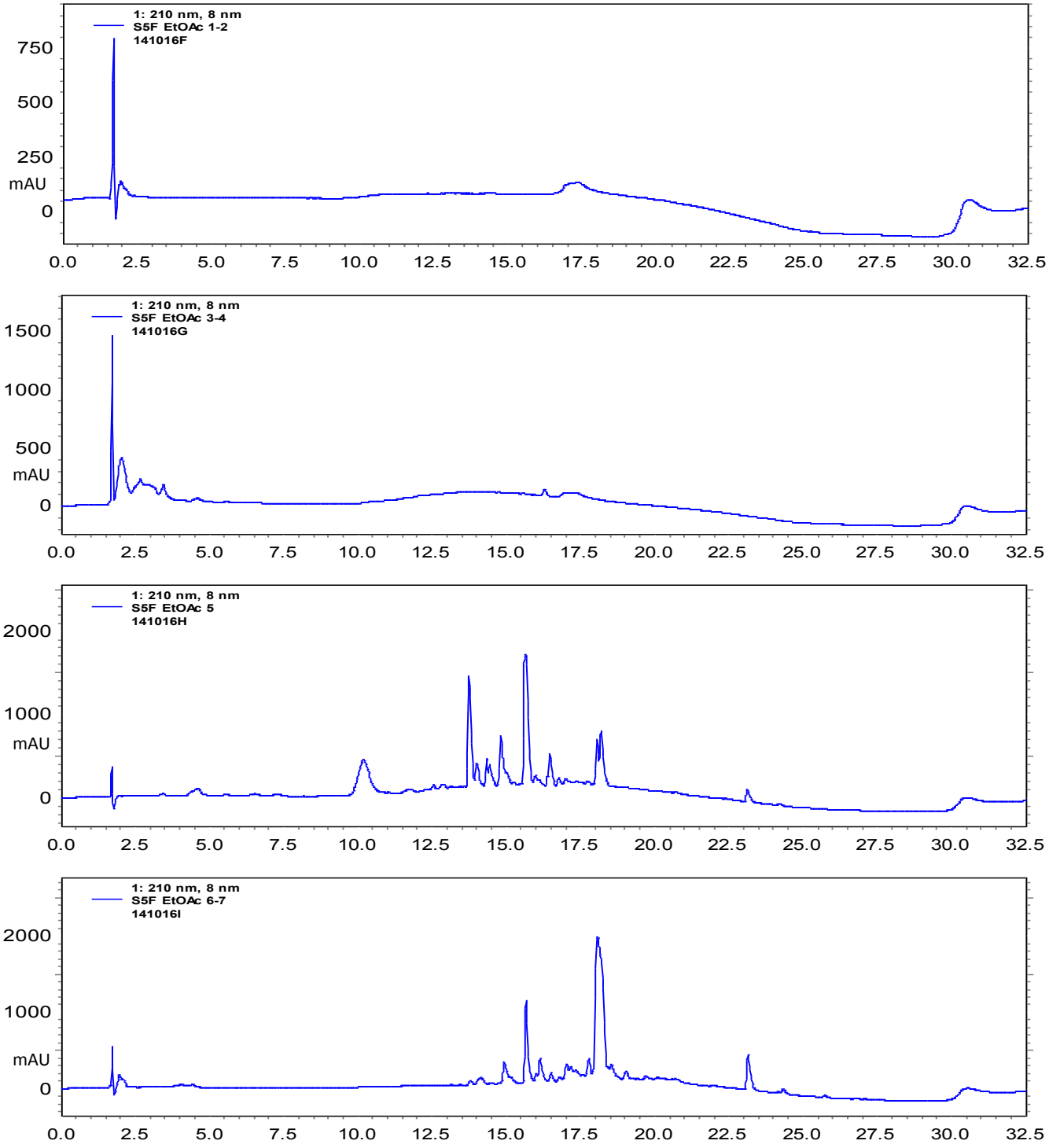

Figure 22 Analytical HPLC of S5F extracts. Top-Bottom: S5F 1-2, S5F 3-4, S5F 5, S5F 6-7.

3.5.4 Testing of Crude Extract and Fractions

Fungal fractions 1, 2 and 3 from S4R were combined, as were 5, 6 and 7 to final weights of 12.5mg and 8.8mg respectively. Fractions 1 and 2, 3 and 4, and 6 and 7 of S5F were combined to final weights of 41.8mg, 41.5mg and 24.5mg respectively.

Table 6 Fraction and crude extract concentrations used in assay tests.

Sample	Initial Concentration (mg/ml)	Dilution 1 (mg/ml)	Dilution 2 (mg/ml)	Dilution 3 (mg/ml)
S4R 1-3	1	0.5	0.25	0.125
S4R 4	1	0.5	0.25	0.125
S4R 5-7	1	0.5	0.25	0.125
S5F 1-2	1	0.5	0.25	0.125
S5F 3-4	1	0.5	0.25	0.125
S5F 5	1	0.5	0.25	0.125
S5F 6-7	1	0.5	0.25	0.125
S4R crude	10	5	2.5	1.25
S5F crude	10	5	2.5	1.25

Wells of the microdilution tray containing the antibiotic and the contamination controls showed no growth, while the wells containing the solvent controls and negative controls all showed growth.

All fractions and crude extracts showed varying degrees of inhibition of *S. marcescens* in the microdilution assay (Table 7). Two fractions (S4R 4, S4R 5-7) also showed varying degrees of inhibition of *S. aureus* (Table 8).

Table 7 MIC of fractions and crude extracts against S. marcescens.

Sample	**MIC against *S. marcescens***
S4R 1-3	0.25mg/ml
S4R 4	0.25mg/ml
S4R 5-7	0.125mg/ml
S5F 1-2	0.25mg/ml
S5F 3-4	0.125mg/ml
S5F 5	0.125mg/ml
S5F 6-7	0.25mg/ml
S4R crude	1.25mg/ml
S5F crude	1.25mg/ml

Table 8 MIC of fractions against S. aureus.

Sample	**MIC against *S. aureus***
S4R 4	1mg/ml
S4R 5-7	0.125mg/ml

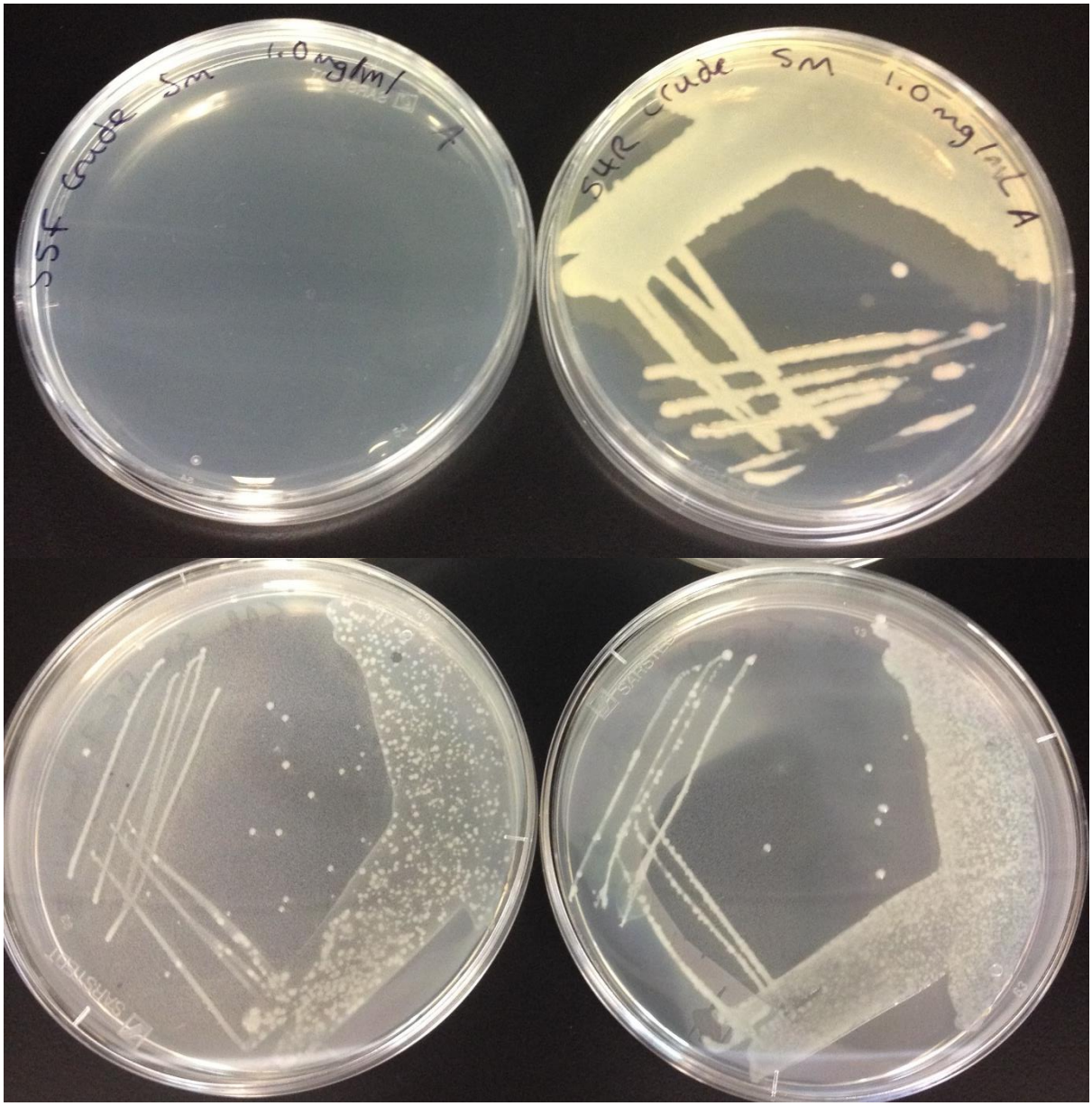

Figure 23 Top: Crude extract STA plates of S5F and S4R at 10mg/ml. No growth of S. marcescens was observed on S5F (left) while growth was observed on S4R (right). Bottom: S4R 5-7 at 1mg/ml and 0.5mg/ml. Reduced growth of S. aureus can be seen at 1mg/ml (left) when compared to 0.5mg/ml (right).

Inhibition of *S. marcescens* by S4R 5-7, S5F 3-4, S5F 5, S4R crude and S5F crude was seen at the lowest concentrations tested, actual minimum inhibitory concentration (MIC) may be lower than was observed due to fractions only going to a low value of 0.125mg/ml. Inhibition of *S. aureus* by S4R 5-7 was seen at the lowest concentration tested, so actual MIC value may also be less than was observed.

All fractions and crude extracts which were found to have inhibitory action in the microdilution assay were plated onto STA. Growth was observed on all *S. marcescens* plates except for S5F crude extract plates. This indicates that against *S. marcescens,* all fractions as well as S4R crude extract are bacteriostatic, and that S5F crude extract is bactericidal (Figure 23). Growth was seen on all the *S. aureus* test plates, indicating that S4R 4 and S4R 5-7 are also bacteriostatic against *S. aureus*. However, it should be noted that there was reduced growth of *S. aureus* seen on both the 1mg/ml test plates of S4R 5-7 when compared to other fraction concentrations (Figure 23).

3.6 Plant Analysis

3.6.1 Leaf Extraction

The extracts from the leaf matter produced total dry weight yields of 0.0214g, 0.1901g and 0.3779g for EtOAc, MeOH and H_2O respectively. These values equate to approximately 0.0238g, 0.2112g and 0.4198g of extracted material per gram of dried leaf matter for each respective solvent. The 17 fold difference in extract amount from EtOAc and H_2O is possibly indicative of reduced permeability of the plant matter to EtOAc, increased solubility of organic sugars and proteins in H_2O not present in EtOAc.

3.6.2 Leaf Analysis HPLC

Regarding the *S. lanceolatum* leaf extract HPLC results, H_2O extract, MeOH extract and EtOAc extract analytical HPLC traces can be seen in Figure 24. Both H_2O and MeOH show high absorbance at 10-20min (as indicated by the blue line), while EtOAc has little peak activity. Most peaks also have high yellow absorbance as indicated by the red line. The lack of peaks in the EtOAc trace correlate with a reduced dry weight yield. Note the H_2O trace has a peak mAU of 2000, while MeOH has a peak absorbance around 1500 mAU, also correlating with there being a higher yield from the H_2O extract.

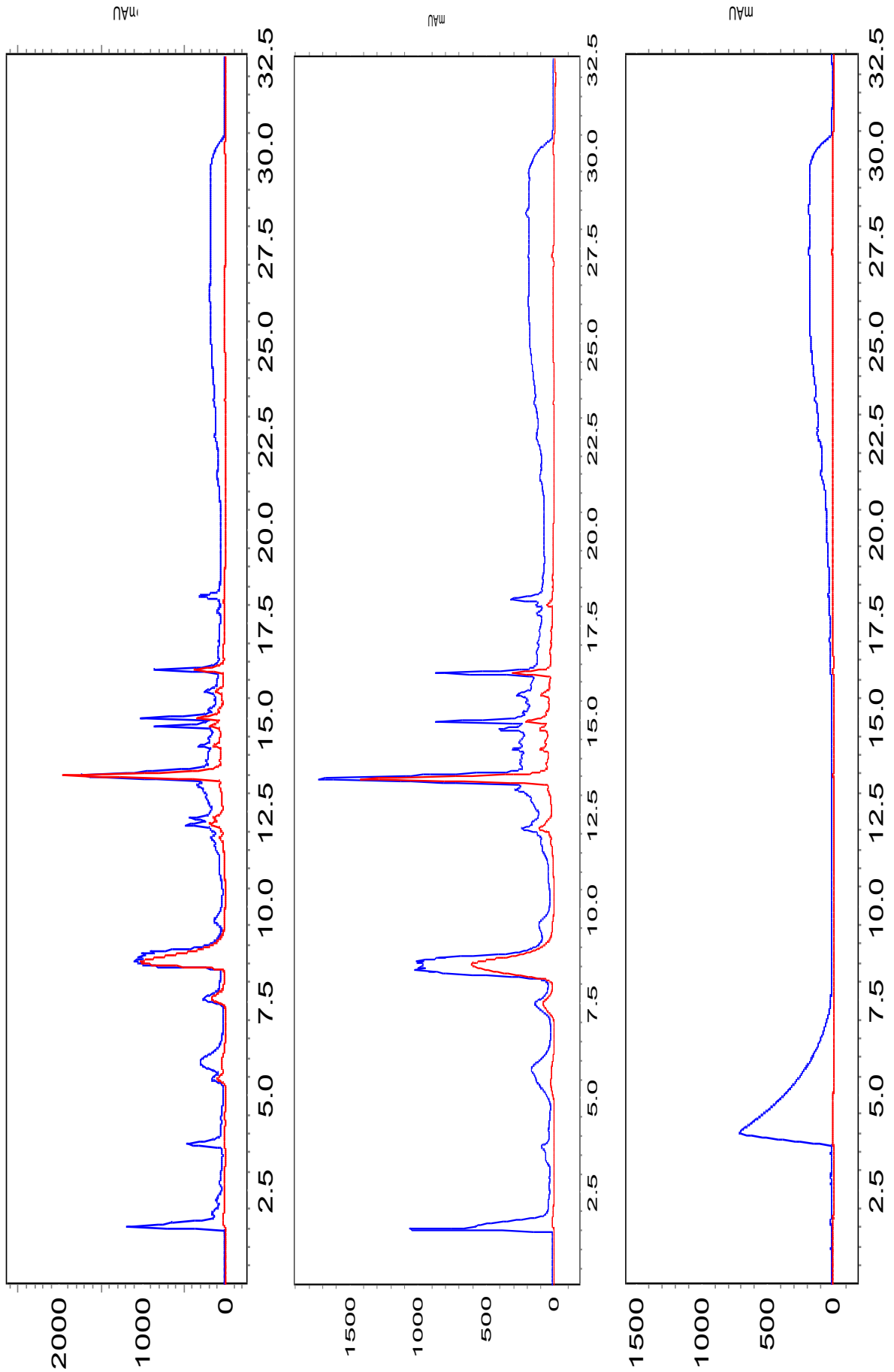

Figure 24 HPLC traces of S. lanceolatum leaf extracts. Top- Bottom; H_2O extract, MeOH extract and EtOAc extract.

3.6.3 Disc Diffusion

The purpose of this test was to see if there were any observable effects of *S. lanceolatum* leaf extracts in inhibiting the growth of select pathogens. The low yield of EtOAc extract meant it was excluded from testing. It is possible that the low yield of EtOAc extract was due to the leaf matter not being permeable to the solvent, or that few compounds are soluble in 100% EtOAc and 5-10% MeOH or H_2O needed to be added to increase yield. Fractional HPLC could have been utilised in extracting fractions along a gradient from the 100% solvent extracts, and these fractions tested rather than exclusively evaluating the 100% solvent extracts.

The disc diffusion tests of the MeOH and H_2O leaf extracts against *E. coli*, *S. aureus* and *S. marcescens* did not indicate that there was any measurable antimicrobial activity in terms of zones of inhibition (Figure 25). On all of the MeOH test plates there was disturbed growth of the pathogens around the disc, but nothing >1mm. Disturbed growth was also seen on the H_2O extract plates for *E. coli* and *S. aureus*. There was no disturbance of growth of *S. marcescens* around the negative control discs or H_2O extract discs. Distinct zones of inhibition (~2cm diameter) were observable around the gentamycin positive control.

The patchy growth seen on the *S. aureus* plates (Figure 25) may have influenced how the extract present on the disc interacted with the pathogen. However, as the growth around the gentamycin and blank disc controls was comparable to that seen on the other two pathogen plates, the effect of the patchy growth on the results obtained should be minimal.

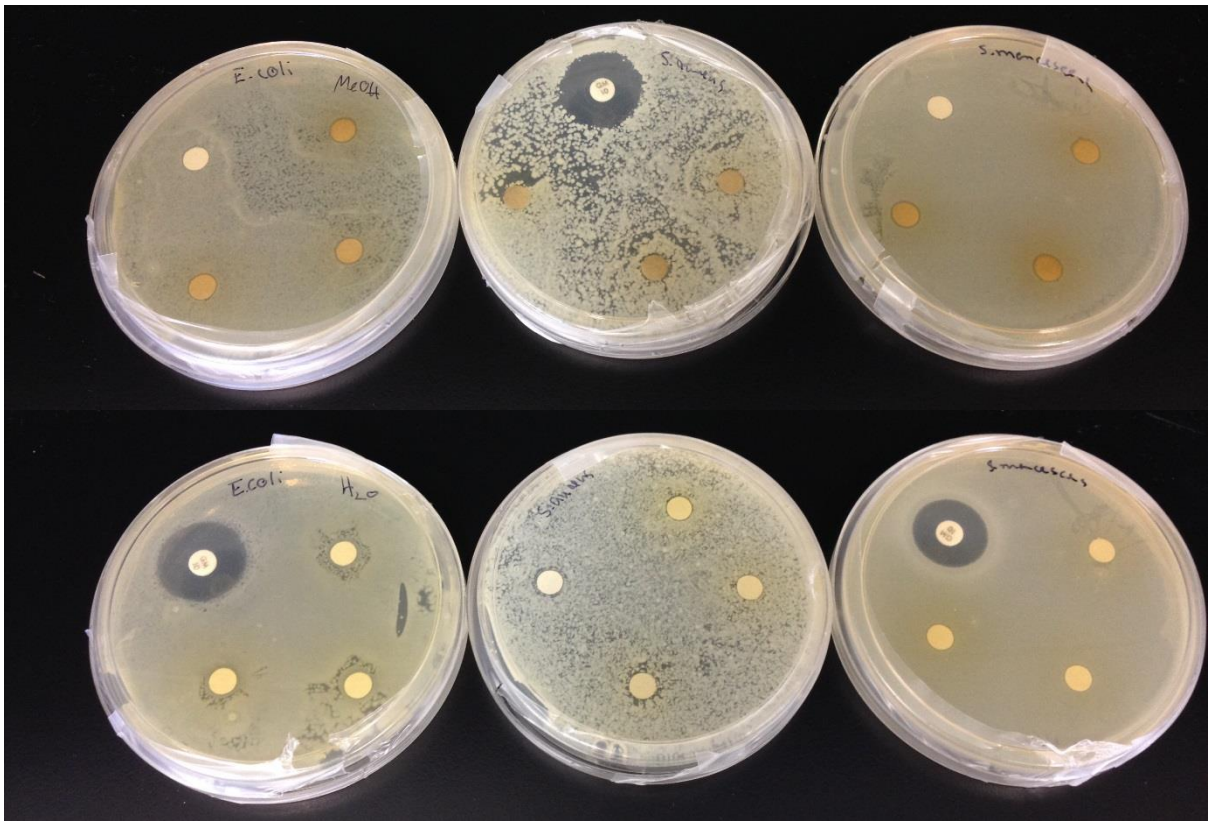

Figure 25 Disc diffusion plates. Top row: MeOH extract discs on E.coli, S. aureus and S. marcescens. Bottom row: H_2O extract discs on E.coli, S. aureus and S. marcescens. Gentamycin control discs are on MeOH S. aureus, H_2O E. coli, and H_2O S. marcescens.

4.0 Discussion

With the knowledge that there are a number of chemotherapeutic agents – eg. penicillin (Chain et al. 1940), cephalosporins (Zaffiri, Gardner & Toledo-Pereyra 2012) – which are produced by fungi, it is logical to again look in that direction to find novel compounds to combat increasing global antimicrobial resistance. The estimated number of fungal species worldwide is 1.5 million and there is a great proportion of these, approximately 95%, which are undescribed (Hawksworth & Rossman 1997). Areas in which fungi are in competition for space or resources with other organisms are likely to be locations of high fungal biochemical activity. Plants which are colonised by endophytes, when challenged by pathogens and herbivores, are better defended than their counterparts without symbiotic relationships (Redman et al. 1999; Rodriguez et al. 2009). Of the estimated 300,000 plant species on Earth, each individual plant of each species is home to at least one endophyte (Strobel & Daisy 2003). It is the stimulation of the production of endophytic secondary metabolites which is the focus of this project.

Strobel and Daisy (2003) in their paper on bioprospecting, described four criteria to determine suitable host plants for likely endophyte bioactivity. (1) Plants which come from novel environmental settings, may also have unusual biological activity and have different survival strategies to the norm; (2) plants with historical medicinal use by indigenous peoples; (3) plants that are able to reach great age or have occupied ancient land masses; (4) plants growing in areas of great biodiversity, such as rainforests. *Santalum lanceolatum* has been noted for its use by the Indigenous Australian population as a treatment for Upper Respiratory Tract Infections (URTI) and minor skin infections (Palombo & Semple 2001). This project has

focussed on the isolation, identification and antimicrobial properties of endophytic fungi from *Santalum lanceolatum*.

This study yielded 71 isolated endophytes from the tissues of *S. lanceolatum*, with 54 of these being obtained from 240 leaf samples. The number of isolates obtained from the leaves of each site were; site 1 – 9, site 2 – 9, site 3 – 14, site 4 – 14; and site 5 – 8. All these values are within one standard deviation (6.96) of the mean (10.8) and as such there was no major impact of site, and plant location within the site, on the number of endophytes obtained. The frequency of endophyte colonisation was 22.5% for the leaves, 100% for bark (10/10) and 37.5% for fruit (6/16), for a whole plant colonisation rate of 26.32%. This is a low result when compared to Huang et al. (2008) who showed colonisation rates ranging from 36.7% – 100% depending on the plant species. Douanla-Meli, Langer and Talontsi Mouafo (2013) also had high colonisation at 82.3%. Factors which may have influenced the number of isolates obtained include; over-sterilisation of the sample, competition with other endophytes and the specific nutrient requirements of the isolate. The sterilisation of the leaf involved soaking in 95% EtOH and then passing through the flame of a Bunsen burner. The length of time spent in the EtOH may have caused the death of some of the endophytes living close to the surface. Once passed through the flame, the remaining EtOH on the leaf caught alight to kill any remaining epiphytes. This process may have also affected the growth of, or killed entirely some of the endophytes close to the surfaces of the leaf as well as any epiphytes. On some plates it was noted that some endophytes grew much faster than others. This may have prevented the growth of endophytes which are slower to establish, meaning they were not able to be separated from the faster species and sub-cultured. On some plates where there was little endophyte activity, it was noted that hyphal tips could be seen to emerge from the leaf sections, however these

hyphae did not grow longer than 1-2mm. This may have been due to the unavailability of a required nutrient or the presence of a substance toxic to that specific species.

The bacterial endophytes S3D and DP1 were both isolated from Duggan Park (site 3). Both of these bacteria grew in similar manners; producing a sticky biofilm; covering the surface of a Petri dish in 16 hours at 37°C; presenting as both pink and purple on a Gram stain – possibly due to safranin becoming stuck in the biofilm; and developing small wavy peaks in the bacterial biomass. Sequencing of the SSU of both bacteria was unsuccessful, even though a strong bands were seen on the electrophoresis gel for both isolates. This indicates that an identifying sequence site other than 16S should be used. Sequencing may have been unsuccessful due to some bacteria carrying multiple copies of the 16S gene, resulting in a chromatogram with no dominant peak. An alternate sequencing site could be the *rpoB* gene (Case et al. 2007). In a study by Case et al. (2007), 111 bacterial genomes were sampled, finding 460 copies of 16S and 111 copies of *rpoB*. Phylogenetic analysis using rpoB was found to be of equal, if not greater resolution in a majority of the tests conducted, indicating it may be a viable alternate sequencing option (Case et al. 2007). Both S3D and DP1 displayed zones of inhibition to all fungal isolates from the same leaf sections as well as to all the pathogens tested. As DP1 showed a slightly larger zone of inhibition to fungi from the same leaf segments, it was subject to bulking over S3D. When transferred from the McCartney bottle to the Erlenmeyer flask in the bulking process, DP1 did not continue to grow and due to time constraints work on it was not continued.

Fungal endophytic growth patterns *in planta* vary greatly; some taxa occur prolifically throughout the tissues of the plant, while others only colonise certain areas and show tissue-specificity (Suryanarayanan et al. 2009). Extensive tissues colonisation was observed in *S.*

lanceolatum, particularly in regards to the *Nigrospora isolates*. Six endophytes were identified as *Nigrospora* spp. (which formed their own clade in the phylogenetic tree), these endophytes were from 3 separate sites, S1, S4 and S5. S4E, S4F, S4K and S4P had identical ITS sequences, yet were isolated from all three canopy heights sampled. This is indicative of systemic colonisation of a plant with the one species of endophyte. The presence of unique endophytes within the bark of *S. lanceolatum* could be due to penetration of the bark by epiphytic fungi, or fungal preference for possible different environmental conditions.

Nyctanthes arbor-tristis (Night-Flowering Jasmine), generally found in tropical areas of the Asia region, has historical use by the Indian population as a medicinal plant. In a study by Gond et al. (2012), *N. arbor-tristis* was colonised by a few of the genera of endophytic fungi found in *S. lanceolatum*. These genera being; *Aspergillus*, *Fusarium* and *Nigrospora*. The presence of these genera indicates that these are wideperead endophytic taxa. In Gond et al. (2012), *Nigrospora oryzae* was found to have activity against S*almonella enterica* subsp. *enterica*, serovar Paratyphi, *Shigella flexnerii*, *Shigella boydii* and *Pseudomonas aeruginosa*. This is in contrast to the results recorded here where none of the *Nigrospora* showed any antimicrobial activity against the pathogens tested. Several other studies have also found that *Nigrospora* spp. display activity against pathogenic bacteria and fungi (Kim et al. 2001; Kumar & Kaushik 2013; Raviraja, Maria & Sridhar 2006; Rosa et al. 2012; Suryanarayanan et al. 2009; Wu et al. 2009). This indicates that either additional microbial pathogens need to be tested, the *Nigrospora* isolates differed in secondary metabolite production from those of other studies or that the methods used here may require some optimisation. For example, the streaking technique used here may not consistently deliver the same amount of pathogen, and thus it is difficult to determine if reduced growth is due to fungal bioactivity or uneven streaking. Due to the *Nigrospora* isolates growing in a sparse formation and reaching the 2cm

diameter size required for streaking by 36-48hr, the immaturity of the fungi when exposed to the pathogens and lack of hyphal density in the centre of the plate, may have meant that the subsequent strength of any produced bioactive compounds may have been insufficient and resulted in a false negative.

Chaeprasert et al. (2010) conducted a study of fungal endophytes in mangroves in Thailand. The fungal genera identified included *Alternaria*, *Cladosporium*, *Nigrospora*, *Phomopsis*, *Pestalotiopsis*, *Colletotrichum*, *Phyllosticta* and *Xylaria*, again indicating that species identified in this project are mostly cosmopolitan species. The bacteria against which the mangrove endophytes were tested were; *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923). Testing of the fungal crude extracts was conducted at 1mg/ml using the agar diffusion method, rather than the 96 well microtitre assay conducted in this study. Both methods appear to be comparably effective in determining activity of fungal extracts. The advantage of the microtitre method is that it can be followed by plating of the well onto agar, a method that allows for differentiation of bactericidal and bacteriostatic compounds.

Molecular identification of fungi and bacteria which were not able to be identified by morphological methods meant that only 7 fungi and 3 bacteria went unidentified – a total identification rate of 85.92%. Only sequences on GenBank with full species name, ≥ 97% identity and an E value of 0 were used for phylogenetic analysis. Molecular identification of all endophytes would provide the possibility for more diverse and structured phylogenetic trees and given greater understanding of the scope of colonisation. All plants were inhabited by *Nigrospora* spp. which may indicate a host preference. The isolation of both *Pestalotiopsis* spp. and *Diaporthe* spp. from two sites each may also be indicative of a host preference.

However more plants would need to be sampled to determine if this is the case. Of the 15 isolates obtained which were identified as not being a *Nigrospora* spp., 8 were isolated from the bark, 5 from the fruit and 2 from the upper canopy. This could be indicative of fungal site specificity within the tree itself. To determine if this is the case, more samples of the bark and fruit would need to be taken and endophytic isolates grown out and identified.

The method of histological preparation used in this study was an adaptation of the formalin histology preparation method of Poudyal, Panchal and Brown (2010). An alternate method of sample preparation could have been to embed samples in Optimal Cutting Temperature (OCT) medium and sectioning them with a cryotome (Cox et al. 2006). While not being able to further assist with fungal species identification, histological analysis showed that there was extensive colonisation throughout the leaf possibly explaining the speed with which hyphae grew out from the leaf sections onto PDA. In Figures 17 and 18 the fungal hyphae appear to have hyphal clamp connections, this may indicate the presence of Basidiomycota, as Ascomycota do not display this trait (Prillinger et al. 2002). All identified fungal isolates obtained were of genera belonging to Ascomycota, which may indicate the growth medium was not optimal for the growth of Basidiomycetes. More extensive observation of endophytes throughout the full profile of the leaf were not able to be conducted, due to the leaf sections being cut by hand, and the inconsistencies in thickness that resulted. In this study, staining of leaf tissue with toluidine blue and lacto-fuchsin assisted with locating fungal hyphae as has been shown by Sánchez and Moore (1999), Carmichael (1955) and Waqas et al. (2012). The staining technique used in both Knight and Sutherland (2011) and Knight and Sutherland (2013), used fluorescent dyes to differentiate between fungal and plant tissues, first staining the tissue with safranin and then with solophenyl flavine 7GFE. Additional staining using tryptophan or safranin and solophenyl flavine 7GFE could have provided more contrast between fungal and plant tissues.

Pyronema spp. are macro-Ascomycetes that are typically reported as free-living saprophytes on timber, particularly after fire (Fuhrer 2005). Botella and Diez (2010) recently identified, an endophytic *Pyronema* sp. from *Pinus halepensis* (Aleppo Pine) in Spain. The identification code for the species is BLE17 and GenBank accession number for the ITS sequence isolated is FN868474.1. The ITS sequence for BLE17 has been included in the *Pyronema* phylogenetic tree (Figure 26). As can be seen, BLE17 aligns with the two isolates from site 4. Pairwise difference of the three sequences (BLE17, S4R, S4U) returned values of 0, indicating that the ITS sequences of the three isolates are identical. Antimicrobial testing was not conducted on BLE17 as the study focused on the decline of *Pinus halepensis*, rather than anti-pathogenic activity. Of interest would be to see if extracts of BLE17 contain antimicrobial activity at a similar concentration to S4R.

Haddadderafshi et al. (2011) also isolated *Pyronema* spp. as endophytes from *Prunus avium* (Cherry) in Hungary. However sequences were not available on GenBank for inclusion in the phylogenetic analysis. Antimicrobial activity tests were not conducted on the isolated *Pyronema* endophytes as the study conducted was a diversity analysis. Hakizimana et al. (2011) found *Pyronema* spp. in the roots of *Persea americana* (avocado), again the study was ecological so did not involve human bacterial pathogens, sequences were also not available on GenBank.

In Mapperson (2014), four *Pyronema* endophytic isolates were sequenced, with the sequences being uploaded to GenBank. These sequences, when included into the *Pyronema* phylogenetic tree (Figure 26), all formed part of the clade including S4R and S4U. Three isolates,

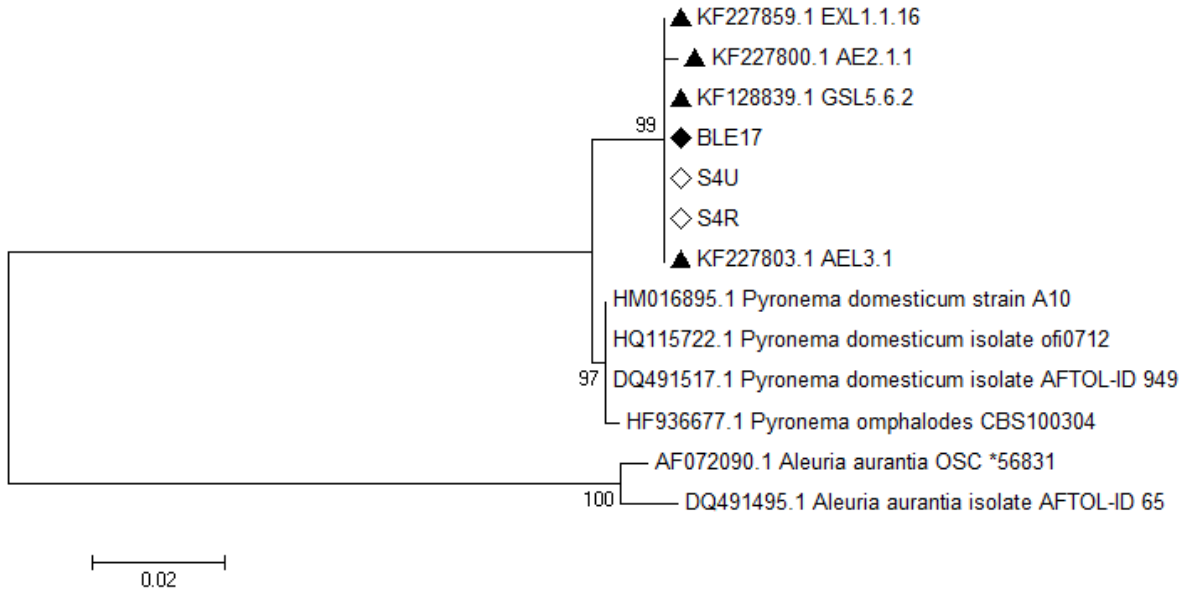

Figure 26 Pyronema phylogenetic tree with BLE17 (Botella & Diez 2010) – black diamond, and EXH1 16, AE3 1, GSL5 6 2 and AE2 1 (Mapperson 2014) – black triangles, included.

KF227859.1 EXL1.1.16, KF227803.1 AE3.1 and KF128839.1 GSL5.6.2 had a pairwise difference of 0 to S4R and S4U, as well as BLE17. This indicates that all isolates had identical ITS sequences. The fourth sequence, KF227800.1 AE2.1.1, from Mapperson (2014) had a pairwise difference of 1, and can be seen on a separate small branch of the same clade. The percent identity to *Pyronema domesticum* strain A10 and *Pyronema domesticum* isolate ofi0712 was 98% for S4R and 99% for S4U for both identified test sequences. This would indicate that *Pyronema* spp. isolated by Mapperson (2014) and Botella and Diez (2010) are closely related. There are very few *Pyronema* spp. listed on GenBank – all listings with full species name and an E-value of 0 were included in the phylogenetic analysis. The question of whether the clade of S4R and S4U is a single new species or belongs as a strain of *Pyronema domesticum* then arises. There is currently only one other named and sequenced *Pyronema* species, *Pyronema omphalodes* (Moore & Korf 1963), to which S4R and S4U did not return identity, which may also indicate that, even if the S4R-S4U clade is not a separate species, that it should be considered a distinctively different strain of *Pyronema domesticum*.

Of the *Nigrospora* isolates obtained, many were able to be identified by morphological means and as such were not sequenced. Of the 13 sequenced isolates, when analysed phylogenetically, 7 matched at 99% or 100% and formed clades with named GenBank sequences. The six isolates (S1E, S4E, S4F, S4K, S4P, S5G) which all had a closest match of 98% formed a separate clade in the phylogenetic tree. The convention for delineation of a new species is a similarity of less than 97%, although this value has been and is still disputed (Hibbett et al. 2011). In the case of the *Nigrospora* spp. isolated which formed a separate clade, it is possible to argue that in this case, a similarity of 98% is not sufficient to tie the isolates to a named species, and that they could in fact be delineated as a separate species (Nilsson et al. 2011). In this case, the species were from three different sites, Felton, Preston and Boodua, indicating a wide geographical distribution across the region sampled. Sequencing of all *Nigrospora* isolates not previously sequenced in this study could provide a greater understanding as to how prolific that particular ITS sequence is across the sites sampled and spread through the tissues of the tree.

The *Xylaria* sp. isolated was identified as a *Xylaria grammica*. *Xylaria* spp. have been widely recorded for producing bioactive metabolites (Baraban et al. 2013; Jang et al. 2009; Wang et al. 2014; Yin et al. 2011). The *X. grammica* isolated showed possible inhibition of growth in the streak plate test, and as such was included in the bioassay bulk growth and extract fractionation. In the microtitre assay, all extracts showed inhibition of growth of *S. marcescens* and when plated from the microtitre assay, the crude extract was shown as being bactericidal. This result was obtained for all concentrations of the crude extract, even 1.25mg/ml. This may be indicative of the crude extract containing a combination of the compounds that causes cell death. It could also be due to inadequate separation of the EtOAc and H_2O layers during extraction, leading to the presence of non EtOAc soluble compounds being present in the crude

extract. As insurance for no compounds being present in the EtOAc extract, the waste H_2O and dried fungal matter were both kept if further extractions with hexane or MeOH were needed. To determine if there is a bactericidal compound present in the H_2O, which is not present in the EtOAc extract, further testing would be required. To extract some compounds present in the waste H_2O, it could be re-saturated with H_2O and resin blocks added, which can be made to attract charged particles. The resin could then be removed and extracted with sterile H_2O (Liu & Fang 2002; Lores & Bradway 1977; Morasch et al. 2001). Thin-layer chromatography could also be used, all composite material of the MEB broth would have to be run on a chromatogram as well as the crude H_2O waste to eliminate broth constituents. Bands which do not correspond to a broth constituent can be scraped from the chromatogram and tested (Rouser, Siakotos & Fleischer 1966; Skipski, Peterson & Barclay 1964; Staneck & Roberts 1974; Touchstone, Chen & Beaver 1980).

The bioactivity seen in S4R fungal extracts when tested in the microtitre assay indicates that all fractions (1-3, 4, 5-7) contain a bacteriostatic compound active against *S. marcescens* and that fractions 4 and 5-7 are also active against *S. aureus*. Limited bioactivity testing of *Pyronema* spp. has been conducted using human pathogens, most studies are ecological or plant pathogen based (Hakizimana et al. 2011). Further separation of current fractions and assays on these, as well as the use of Nuclear Magnetic Resonance (NMR) imaging may allow identification of compounds responsible for inhibition of growth.

Further work needs to be conducted on the bark and fruits of *S. lanceolatum* as these had a greater fungal diversity than the leaves, and may yield further novel species. The identification of bacteriostatic compounds by NMR from all fungal extract fractions could be undertaken to determine what secondary metabolites are being produced and if any of them are already

known as antimicrobials. Sequencing and identification of the bacterial isolates S3D/DP1 needs to be conducted with different PCR primers as well as further testing of bioactivity due to the strong indication of the presence of at least one antifungal compound. The *Nigrospora* spp. identified solely by morphological means should be sequenced to give a site wide scope of these endophytes and this may also assist with more clearer phylogenetic distinction of these taxa. The *Pyronema* taxa isolated could be more fully characterised by growth on a variety of culture media and the induction of sporulation. Further characterisation might include sequencing of other taxonomically important DNA regions such as LSU, rbp2 and tubulin genes (Chen et al. 2013; Matheny et al. 2007; Robl et al. 2013). If possible, further testing of S5F crude extract and H_2O waste needs to be conducted to determine if the bactericidal agent is part of the EtOAc separation.

Comparing the yields of *S. lanceolatum* leaf extracts shows that there was quite a difference in the dry weights. The EtOAc extract was 0.0214g, while the MeOH and H_2O were 0.1901g and 0.3779g respectively. This low EtOAc yield may be indicative of the need to use multiple solvents for extraction. From these results, H_2O and MeOH appear to elicit a greater mass, however it is not possible to implement them into the fungal isolate bioactivity testing, as the fungi in these experiments were grown in a H_2O-based broth. Extraction with MeOH would not be possible as the solvents would become an emulsion and it is not possible to do a H_2O extract on a H_2O based solution. A possible alternative which was not tested, would be to use hexane as the extracting solvent as well as EtOAc, as was done by Kumar and Kaushik (2013).

Previous work on *S. lanceolatum* by Palombo and Semple (2001) showed that the plant itself only had activity against *Bacillus cereus* (ATCC 11778) and did not present activity against *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 11775), *Klebsiella*

pneumoniae (Australian Collection of Microorganisms (ACM) number 90), *Pseudomonas aeruginosa* (ATCC 10145), *Salmonella typhimurium* (ATCC 13311), *Staphylococcus aureus* (ATCC 12600) and *Streptococcus pyogenes* (ACM 17), where activity was defined as a zone of inhibition around an agar well of 5mm or more. The methodological approach of (Palombo and Semple (2001)) was similar to the experimental approach used in this study however no inhibition of any microbe was reported. Further experimentation with a higher concentration of *S. lanceolatum* extract may afford a result with defined areas of inhibition, rather than the small zones of disturbed growth as seen in Figure 25. The areas of disturbed growth observed around the test discs may be due to; minute amounts of a bacteriostatic agent; the presence of a bacteriostatic agent as well as extracted sugars, proteins, or other growth enhancing materials; the physical disturbance and displacement of the bacteria by the placement of the disc; or the minute presence of a bactericidal agent. As the test was conducted using 20µg total weight of crude extract per disc, the actual amount of possible active compound present would be much less than this and so may account for the lack of distinct zone of inhibition. The gentamycin control was a 10µg disc and had a zone of inhibition of ~ 2cm diameter for each pathogen. At 20µg total mass of extract per disc it is unlikely that any compounds present had a mass similar to that of the gentamycin positive control, and therefore similar zones of inhibition were not to be expected.

5.0 Conclusions

This project has isolated 71 endophytes from *S. lanceolatum*. Of these, 63 were able to be identified to at least the genus level – seven different genera and one bacterial genus were identified. Phylogenetic analysis has shown that some isolates may be new species of *Nigrospora* and that others may be a new species of *Pyronema*. Bioactivity testing showed 5 possible candidates for bulk growth. However only 2 of these progressed through to the end of the bulking phase. From these 2 fungi, multiple fractions were able to be obtained and tested for bioactivity against select pathogens. All fractions obtained showed some level of bacteriostatic activity against *S. marcescens*, with 2 fractions showing bacteriostatic activity against *S. aureus*. Bactericidal activity was also seen in the S5F crude extract against *S. marcescens*. Testing of the leaf matter of *S. lanceolatum* showed little activity at the concentrations used, but it is possible that at higher concentrations activity may have be seen. Histology of leaf sections successfully showed endophytes *in situ*, growing amongst the plant cells. More geographically extensive research needs to be conducted on the endophytes of *S. lanceolatum*, as this study only included five plants at 5 sites within the Toowoomba region and thus there is great potential for future discovery of useful bioactive compounds.

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Appendices

Appendix A
Growth media recipes

Potato Dextrose Agar (PDA)

Dissolve 15.6g of PDA in 400mL distilled water. Autoclave at 121°C for 15 minutes.

Sensitest Agar (STA)

Dissolve 12.8g of STA in 400mL distilled water. Autoclave at 121°C for 15 minutes.

Malt Extract Broth (MEB)

Dissolve 20g of MEB in 1L distilled water. Autoclave at 121°C for 15 minutes.

Tryptone Soy Broth (TSB)

Dissolve 30g of TSB in 1L distilled water. Autoclave at 121°C for 15 minutes.

Appendix B

Isolate Data

Plate	Type	Location	Sp.	Appearance	E. coli	E. faecalis	E. uerogenes	P. aeruginosa	S. aureus	S. marscescens	23/07	24/07	25/07	26/07	27/07	28/07
S1A	F	BA	Pestalotiopsis sp.	white	0	0	0	0	0	0						2A
S1B	F	BA	Pestalotiopsis sp.	white	0	0	0	0	0	0				2M		
S1C	F	BA	Pestalotiopsis sp.	white	0	0	0	0	0	0				2M		
S1D	F	BA	Pestalotiopsis sp.	white, concentric circles	0	0	0	0	0	0			2P			
S1E	F	BO	Nigrospora sp.	black	0	0	0	0	0	0		2P				
S1F	F	BO	Nigrospora sp.	white	0	0	0	0	0	0		2P				
S1G	F	MI	Nigrospora sp.	white	0	0	0	0	0	0		2A				
S1H	F	TO	Aspergilis sp.	soccerballs on sticks	0	0	0	0	0	0	2P					
S1I	F	BO	Nigrospora sp.	black	0	0	0	0	0	0		2A				
S1J	F	MI	Nigrospora sp.	white	0	0	0	0	0	0		2A				
S1K	F	MI	Nigrospora sp.	white	0	0	0	0	0	0		2A				
S1L	F	TO	Nigrospora sp.	white	0	0	0	0	0	0		2A				
S1M	F	MI	Nigrospora sp.	grey	0	0	0	0	0	0		2A				
S2A	F	TO	Nigrospora sp.	white	0	0	0	0	0	0		2A				
S2B	F	TO		white												
S2C	F	MI	Nigrospora sp.	white	0	0	0	0	0	0		2A				
S2D	F	MI	Nigrospora sp.	white	0	0	0	0	0	0		2A				
S2E	F	BO	Nigrospora sp.	white	0	0	0	0	0	0		2A				
S2F	F	TO	Nigrospora sp.	white/grey	0	0	0	0	0	0		1A1P				
S2G	F	TO	Nigrospora sp.	grey/black	0	0	0	0	0	0		2A				
S2H	F	BO	Nigrospora sp.	green/grey, agar yellow	0	0	0	0	0	0				2M		
S2I	F	MI	Nigrospora sp.	white												
S3A	F	FR	Nigrospora sp.	white w/ black	0	0	0	0	0	0		1A1P				
S3B	F	FR	Pestalotiopsis sp.	cream	0	0	0	0	0	0				2M		
S3C	F	TO	Nigrospora sp.	white	0	0	0	0	0	0		2P				
S3D	B															
S3E	F	MI	Nigrospora sp.	white w/ black	0	0	0	0	0	0		2A				
S3F	F	MI	Nigrospora sp.	white/grey	0	0	0	0	0	0		2A				
S3G	F	MI	Nigrospora sp.	white/grey	0	0	0	0	0	0		2P				
S3H	F	MI	Nigrospora sp.	grey	0	0	0	0	0	0		2A				
S3I	F	BO	Nigrospora sp.	grey	0	0	0	0	0	0		2A				
S3J	F	BO	Nigrospora sp.	grey	0	0	0	0	0	0		2A				
S3K	F	BO	Nigrospora sp.	grey	0	0	0	0	0	0			2A			
S3L	F	BO		white	0	0	0	0	0	0			1P			
S3M	F	C	Nigrospora sp.	white + black	0	0	0	0	0	0		2A				

S3N	F	FR	Phomopsis sp.	white	0	0	0	0	0	0				2M		
S3O	F	FR	Phomopsis sp.	white	0	0	0	0	0	0				2M		
S3P	F	FR	Phomopsis sp.	white	0	0	0	0	0	0						2A
S3Q	F	FR	Pestalotiopsis sp.	white	0	0	0	0	0	0				2M		
S3R	F	TO	Nigrospora sp.	grey	0	0	0	0	0	0			2A			
S3S	F	TO	Nigrospora sp.	grey	0	0	0	0	0	0			2A			
S3T	F	BO	Nigrospora sp.	white/grey	0	0	0	0	0	0		2A				
S4A	F	BA	Phomopsis sp.	white	0	0	0	0	0	0			2P			
S4B	F	BA	Nigrospora sp.	white + black	0	0	0	0	0	0		2A				
S4C	B	BA		yellow												
S4D	F	TO	Nigrospora sp.	white + black	0	0	0	0	0	0			2A			
S4E	F	TO	Nigrospora sp.	black	0	0	0	0	0	0						
S4F	F	TO	Nigrospora sp.	black	0	0	0	0	0	0			2A			
S4G	F	TO	Nigrospora sp.	white + grey	0	0	0	0	0	0						2A
S4H	B	TO	Bacillus sp.	white	0	0	0	0	0	0						2A
S4I	F	TO	Nigrospora sp.	black	0	0	0	0	0	0		1P		1M		
S4J	F	MI	Nigrospora sp.	white	0	0	0	0	0	0		2A				
S4K	F	MI	Nigrospora sp.	black	0	0	0	0	0	0		1P	1A			
S4L	F	MI	Nigrospora sp.	black	0	0	0	0	0	0		2P				
S4M	F	MI	Nigrospora sp.	black + grey	0	0	0	0	0	0		2A				
S4N	F	BO	Nigrospora sp.	black/grey	0	0	0	0	0	0		2A				
S4O	F	BO	Nigrospora sp.	black/grey	0	0	0	0	0	0		1A	1A			
S4P	F	BO	Nigrospora sp.	black	0	0	0	0	0	0			2A			
S4Q	F	BO	Nigrospora sp.	white	0	0	0	0	0	0			2P			
S4R	F	BA	Pyronema sp.	cream	1	1	1	0	1	1		2A				
S4S	F	BA	Fusarium sp.	white + pink	0	0	0	0	0	0				2M		
S4T	F	BA		white	0	0	0	0	0	0				2M		
S4U	F	BA	Pyronema sp.	cream	1	1	1	0	1	1		2A				
S5A	F	TO		white + grey	0	0	0	0	0	0		2A				
S5B	F	MI		white	0	0	0	0	0	0		2A				
S5C	F	BO	Nigrospora sp.	grey	0	0	0	0	0	0		2A				
S5D	F	TO	Nigrospora sp.	white + black	0	0	0	0	0	0		2A				
S5E	F	TO	Nigrospora sp.	black	0	0	0	0	0	0		2P				
S5F	F	TO	Xylaria sp.	white + cream + green	-	-	-	-	-	-						
S5G	F	MI	Nigrospora sp.	black	0	0	0	0	0	0		1P		1M		
S5H	F	TO		white/brown	0	0	0	0	0	0						
DP1	B	BO		pink on TPDA	1	1	1	1	1	1						