

University of Southern Queensland

**An investigation of the ecology and bioactive
compounds of *Pittosporum angustifolium*
endophytes**

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Abstract

Endophytes are microorganisms that reside in the internal tissue of living plants without causing any apparent negative effects to the host. Endophytes are known to produce bioactive compounds and are looked upon as a promising source of novel bioactive compounds. There is currently limited knowledge of Australian endophytes regarding the species diversity, ecological roles and their potential as producers of antimicrobial compounds. The plant *Pittosporum angustifolium* was used medicinally by Indigenous Australians to treat a variety of conditions such as eczema, coughs and colds. In this study the diversity of endophytic species, host-preference of endophytes and antimicrobial potential of the resident endophytes is investigated in *P. angustifolium*. During this study a total of 54 endophytes were cultured from leaf samples of seven different *P. angustifolium* plants. Using molecular identification methods, the ITS-rDNA and SSU-rDNA regions of fungal and bacterial endophytes respectively were sequenced and matched to species recorded in GenBank. This approach, however, could not identify all isolates to the species level. Analysing the presence/absence of identified isolates in each of the seven trees found no evidence to indicate any host-specific relationships. Screening of each isolated endophyte against four human pathogens (*Staphylococcus aureus*, *Serratia marcescens*, *Escherichia coli* and *Candida albicans*) found two species displaying antimicrobial activity. Limitations narrowed the project to focus on one species which was identified as *Pseudocercospora fuligena*. *P. fuligena* was found to inhibit *S. marcescens*. Antimicrobial testing found that a crude extract of the fungal endophyte displayed bactericidal activity with a minimum bactericidal concentration of 2.5mg/ml. Bioassay-guided fractionation of the crude extract yielded five fractions. Two fractions displayed inhibition of *S. marcescens*

both with a minimum inhibitory concentration of 125 µg/ml. The two fractions were not found to be bactericidal at any of the concentrations assayed. This study demonstrates the potential of *P. angustifolium* as a source of undiscovered endophytic species and antimicrobial compounds.

Declaration

I certify that the work reported in this thesis is entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not previously been submitted for assessment in any other course of study at any other University.

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1.0 Introduction

The need for new medically and industrially useful compounds continues to increase in order to solve problems facing society. Such problems include combatting drug resistance in bacteria, treating fungal and viral infections, treating patients with conditions such as cancer and diabetes and providing alternatives to synthetic agricultural pesticides.

Endophytes are a significant source of new bioactive compounds that have attracted the attention of researchers and may provide solutions to various problems that society faces. Endophytes are described as the microorganisms residing in the internal tissue of living plants without causing any apparent negative effects (Tran et al., 2010). Endophytes have been classified into two groups, the clavicipitalean and the non-clavicipitalean (Sieber, 2007). The clavicipitalean endophytes are those that form symbioses with grasses and tend to colonize the host shoot system (Sieber, 2007).

It is believed that each individual plant on earth is host to one or more endophytes (Strobel and Daisy, 2003), and these spend all or part of their life cycle residing asymptotically within the host plants tissues (Debbab et al., 2012). When inside the host tissue, fungal endophytes enter a quiescent (latent) state either for the whole of the infected plant tissues lifetime or for an extended period of time, which may be until environmental conditions are favourable for the fungus or the phase disposition of the host changes to the advantage of the fungus (Sieber, 2007). Plants seem to have been associated with endophytic fungi for over 400 million years as indicated by fossil records (Rodriguez et al., 2009).

Endophytes are transmitted between plants by both vertical (through host seeds) or horizontal transmission (through spores or mycelium) or a combination of both (Gundel et al., 2012, Bihon et al., 2011). Some factors that endophytes confer to their host includes higher antioxidant levels, plant hormone production and anti-herbivore alkaloids as well as enhanced photosynthesis, which is likely to increase the fitness of the host plant (Gundel et al., 2012, Sanchez-Azofeifa et al., 2011). However, the ecological roles of most endophytes are still unclear and are yet to be studied.

1.1 Techniques for endophyte isolation

The plants that are chosen for study of their endophytes usually have properties which make them of interest to researchers including unique biology, age, endemism, ethnobotanical history, and/or environmental setting (Strobel, 2003). Using such criteria removes the random aspect of the selection process and thus allows researchers to narrow down the selection to only those plants they believe will be useful to their current study.

As endophytes reside within plant tissue it is essential to use techniques that allow the isolation of the endophyte from the host plant for identification or biochemical analysis. After a plant is selected, sections of the plant are removed and are later processed in the laboratory. Plant samples are then surface sterilized to ensure only endophytic microbes are cultured. Techniques for isolating fungal endophytes and not epiphytes commonly utilize 70% (v/v) ethanol immersion which is followed by rinsing in sterile water, and in some cases using other sterilants such as sodium hypochlorite followed by sterile water. The plant samples are then sectioned with scalpel blades or hole punching devices and commonly

placed on nutrient agar plates and incubated. Control plates may also be made using plant samples which have not been surface sterilized to check for epiphytic contaminants (Puri et al., 2006, Liu et al., 2008, Kusari et al., 2009a, Kjer et al., 2009). The length of time and strength of sterilant required differs depending on the leaf thickness, host species or type of organ being sterilized. Long sterilization times may reduce the number of endophytes isolated by damaging the endophytes within while short times may not remove all epiphytes present (Hyde and Soyong, 2008). The amount of time determined to be too long or too short for sterilization may differ between leaves of different species.

Researchers interested in only obtaining fungal endophytes and not bacterial endophytes, typically carry out initial isolations on agar plates containing antibiotics that suppress bacterial growth (Giridharan et al., 2012, Kjer et al., 2009, Kusari et al., 2009a). Once fungal growth has begun, the growing tips of the fungal mycelium are removed and placed onto plates with fresh potato dextrose agar (Liu et al., 2008, Kusari et al., 2009a, Giridharan et al., 2012) or other media such as malt agar (Kjer et al., 2009) in order to obtain pure cultures of each endophyte.

Bacterial endophytes are isolated in a similar manner, using 70% ethanol and/or sodium hypochlorite (% varies around 1 – 5%) for surface sterilization (Rashid et al., 2012, Reiter and Sessitsch, 2006, West et al., 2010). The sterilized plant tissue can then be placed onto media such as nutrient agar and incubated to allow growth of bacterial colonies, similar to fungal endophyte isolation. The bacteria are then aseptically streaked onto new individual nutrient agar plates (West et al., 2010) to grow pure colonies.

Bacterial endophytic isolation may involve the homogenization of the plant tissue instead of placement of intact tissue on an agar medium (Andreote et al., 2009). Homogenization often involves a mortar and pestle and may occur in various solutions such as phosphate buffered saline or Ringer's solution. The resulting homogenate is then diluted and plated on various media such as R2A agar, tryptic soy agar, Luria agar or tryptone soya broth agar (Andreote et al., 2009, Rashid et al., 2012, Chen et al., 2010) depending on the type of bacteria that is selected for. After incubation, the resulting bacterial colonies are then aseptically streaked onto the respective media until pure cultures are obtained (Andreote et al., 2009, Chen et al., 2010, Rashid et al., 2012).

1.2 Biodiversity of endophytes

The biodiversity of organisms throughout the planet varies along with the variation in ecosystem. Endophytic species diversity appears to be greater in ecosystems which have overall high biodiversity (Strobel et al., 2004). Studying the endophytes of plants located in ecosystems which display a high diversity of overall species could potentially increase the odds of researchers discovering new endophytic species. Endophytes of such areas may also produce novel compounds as biological diversity can lead to chemical diversity (Strobel et al., 2004)

In order to identify the extent of a plant's endophytic species diversity, successful isolation of endophytes is necessary. However, culture-dependent methods only favour fast-growing microbes while the unculturable or slow-growing microbes may not be isolated (Duong et al.,

2006). Therefore the diversity of endophytes found in a study may not accurately represent the true diversity of the host plant's endophytic community.

Ascomycetes have been the dominant fungal endophytes isolated with few basidiomycetes being reported as endophytes (Pinruan et al., 2010) where most research has relied on culture methods. Pinruan et al. (2010) found that the majority of endophytes isolated from the oil palm *Elaeis guineensis* were ascomycetes or their anamorphs (320 strains) while only 20 strains were basidiomycetes. Basidiomycetes have equal ability to colonize diverse habitats as other fungi so it is unclear why so few basidiomycetous endophytes have been found (Pinruan et al., 2010).

The composition of endophytic communities has been found to vary depending on the ecosystem their host plant is found in. For example, a study found that there was an increase observed in the incidence, diversity and host breadth of endophyte communities when moving from arctic to tropical sites (Arnold and Lutzoni, 2007). Arnold and Lutzoni (2007) also found that identification of 1403 endophytic strains isolated over arctic/boreal, temperate and tropical sites revealed that the majority of species found in each area were specific to that area. This study shows how species richness can vary depending on the ecosystem.

Plant endophytic richness also seems to be affected by the age of the plant (Asraful Islam et al., 2010). It was suggested that in the plant *Coccoloba cereifera* that the variation in diversity due to age may be caused by nutritional or defence properties that occur with different stages of leaf development (Sanchez-Azofeifa et al., 2011). For tropical plants,

there are often high levels of anthocyanins in the leaves of young plants, which may act as antifungals. Chemical defences against fungi decline in older and mature leaves where more species of endophytes are found (Sanchez-Azofeifa et al., 2011).

The species richness of the host plant's endophytic community is also affected by the water content of the plant tissue due to its effect on the endophytes growth, frequency of emergence and interaction with other symbiotic fungi (Sanchez-Azofeifa et al., 2011).

Growth of endophytes can also be stimulated by production of flavonols, CO₂, volatile substances and other substances by the host plant (Sanchez-Azofeifa et al., 2011).

Endophytic richness may change depending on plant tissue type. For example Sun et al. (2012) found that twigs of *Betula platyphylla* (Betulaceae), *Quercus liaotungensis* (Fagaceae) and *Ulmus macrocarpa* (Ulmaceae) harboured more endophytic fungal taxa than the leaves.

The vast majority of plants have undocumented endophyte communities. As interest in this area grows, more plant species are likely to be studied which may bring about discoveries of new fungal and bacterial species along with novel bioactive compounds of potential benefit to society.

1.3 Host preference and specificity

Although most endophytic fungi associate with a wide variety of host plants some species are host specific, only associating with a single host plant species (Liu et al., 2012). To a lesser degree, endophytes may show a host preference where they are not entirely restricted to a particular plant species but have significant differences in their frequency of occurrence in individual plants (Cannon and Simmons, 2002). It may be the case that

coevolution plays a role in the association of endophytes and their host plant species. Evidence for this is found with the lack of plant defences against endophytes in some species as well as the connection between the reproductive systems of both symbiotic partners with vertical transmission of endophytes (Aly et al., 2011).

Fungal endophytes seem to have a preference for certain tissue types such as branch, bark or leaf. This preference may be due to the endophyte's capacity for utilizing or surviving in the conditions of the tissue (Wu et al., 2013). As discussed previously, tissue water content and the host plant's chemical products affect the growth of endophytes; the difference of these factors between tissue types may affect the preferences of endophytes. In a study on *Betula platyphylla* (Japanese White Birch), *Quercus liaotungensis* (Oak) and *Ulmus macrocarpa* (Elm), the host species was found to have a greater effect on endophytic community composition (caused 30.1% of variance in community composition) than tissue specificity (15.1% of variance in community composition) (Sun et al., 2012). Research needs to be undertaken to explain why host species had a greater impact than tissue type. Indeed host preference or specificity may be more affected by the environment the plant lives in rather than the environment within the plant itself.

Nissinen et al. (2012) found endophytic communities to be host-plant specific among plants from the Arctic and found that *Sphingomonas* spp. displayed host preference with *Oxyria digyna* and *Diapensia lapponica*. Differences in the endophytic communities between the three plants studied (*Oxyria digyna*, *Diapensia lapponica* and *Juncus trifidus*) could be attributed to the habitats in which they grow, the main factors being differences in snow cover and pH which result in different soil microbes. However, as similar endophytic taxa

were found in each plant species growing in wide pH ranges, it is likely that host species is of higher importance than the host plants habitat (Nissinen et al., 2012).

Endophytes isolated from the hosts *Heisteria concinna* and *Ouratea lucens* have also provided evidence of host preference. Arnold et al. (2001) found that of the endophytes sampled from their first site, 62% of non-singleton fungal DNA sequences occurred in either *H. concinna* or in *O. lucens*, but not in both species. However, they also found that spatial heterogeneity of endophytes may be the cause of the presence of certain endophytes within *H. concinna* as when the host plant was sampled from two different sites; they found that 48% of non-singletons occurred in only one of two sites but not in both (Arnold et al., 2001). This suggests that location can have an effect on the presence of endophytes within a host as well as the host plant itself.

With the small amount of research into host preferences and specificity of endophytes, it cannot be definitively confirmed that such relationships exists. More research into the ecology of host-endophyte relationships may allow us to understand the role these relationships played in the evolution of both organisms.

1.4 Secondary metabolites

Secondary metabolites are described as metabolic products of an organism that are not essential for the normal growth, development or reproduction but may have other roles in areas such as interspecies competition and providing defensive mechanisms (Vaishnav and Demain, 2011). Secondary metabolites are chemically and taxonomically diverse low molecular weight (MW < 3000) compounds and many have shown promise as antibacterial

or antifungal agents, anticancer drugs, cholesterol-lowering agents, immunosuppressants, antiparasitic agents, herbicides, diagnostics, and tools for research (Bérdy, 2005, Vaishnav and Demain, 2011). Use of secondary metabolites by humans spans many areas including medicine, veterinary science, agriculture and pure scientific research among others (Bérdy, 2005). Secondary metabolites are produced by most types of living organisms. Both prokaryotes and eukaryotes produce these compounds, however, some organisms produce secondary metabolites more frequently and with greater variety than others (Bérdy, 2005). The organisms with the most frequent and versatile production are often found to be bacteria, fungi and filamentous actinomycetes (Bérdy, 2005).

Many secondary metabolites are produced by pathogenic fungi. These metabolites may be crucial to allow the fungus to establish disease in the host, especially host-specific toxins which may allow the fungus to overcome a specific resistance mechanism of the host. However, nonspecific toxins contribute only partially to virulence and some mycotoxins only take effect after the death of the fungus, which is not beneficial to the fungal producer of the metabolite (Fox and Howlett, 2008).

Generally the set of genes that code for the successive steps of antibiotic secondary metabolite production are clustered together, along with other related genes coding for gene regulators and resistance against the antibiotic produced. This clustering implies that at least some of the evolution of the genes has occurred as a group (Stone and Williams, 2006).

Production of secondary metabolites is often associated with sporulation of the microorganism. Some metabolites activate sporulation, others may be pigments of sporulation structures (e.g. melanins (Yu and Keller, 2005)) and others may be toxic metabolites secreted upon sporulation (Calvo et al., 2002). The sporulation-associated pigment melanin is required for the formation or integrity of sexual and asexual spores and overwintering bodies (Calvo et al., 2002).

The nuclear protein LaeA is a global regulator of secondary metabolism in *Aspergillus* spp. regulating multiple genes. In one study, deletion of the *laeA* gene blocked the expression of genes including sterigmatocystin (carcinogen), penicillin (antibiotic), and lovastatin (an antihypercholesterolemic agent) gene clusters (Bok and Keller, 2004). Penicillin and lovastatin production was increased with overexpression of *laeA* (Bok and Keller, 2004). The *veA* gene is a regulator of secondary metabolism in many fungal species. In *Aspergillus nidulans*, *veA* is involved in regulation of genes for the synthesis of the mycotoxins sterigmatocystin and aflatoxins (Calvo, 2008).

1.5 Endophytes as a source of bioactive compounds

The idea of endophytes being used as a source of natural bioactive compounds has increasingly gained the attention of biologists and chemists as the demand for new compounds continues to grow in the medical field (Aly et al., 2010). Many endophytes produce secondary metabolites that benefit the host plant by defending against pathogens and pests (Taechowisan et al., 2005). Studies have shown that some of these compounds are useful for drug development (Joseph and Priya, 2011). Endophytes are also thought to be a novel source for industrial enzymes (Zaferanloo et al., 2013). Demand for new enzymes

that can cover the thermostability and pH profiles of different applications are increasing and microbes have so far been the dominant organisms used for discovery of them (Zaferanloo et al., 2013).

As previously mentioned it is important to select plants suspected to have a high likelihood of isolating endophytes capable of producing novel bioactive compounds. Such plant species include those living in unique environments and having novel strategies for survival; having an ethnobotanical history and which the traditional use relates to the interest of the study; plants that have occupied an ancient land mass or are an endemic species with unusual longevity as well as plants growing in areas of high biodiversity (Strobel and Daisy, 2003).

Throughout history various plants have been used for medical purposes and the traditional medicinal plants of various cultures may be an important source of endophytes to study (Kaul et al., 2012). Traditional Chinese medicine (TCM) utilizes many plant species, some of which have been used in the discovery of modern drugs, and are now being used to isolate endophytes which produce bioactive compounds (Miller et al., 2012).

Indigenous Australians have traditionally used a variety of plants medicinally prompting researchers to investigate the endophytes within these plants (Miller et al., 2010). In a study on Snakevine (*Kennedia nigricans*), a novel class of antibiotic called munumbicins were isolated. The munumbicins were isolated from *Streptomyces* - NRRL 3052, a bacterial endophyte of the plant. The compounds showed activity against many human as well as plant pathogenic fungi and bacteria, and a *Plasmodium* sp. (Castillo et al., 2002).

Endophytic fungi have been found to produce a number of plant secondary metabolites. Taxol (paclitaxel) is one such example of an important anticancer drug that is found to be produced by multiple endophytes. Originally found in the bark of the pacific yew tree (*Taxus brevifolia*), taxol is known to be a potent chemotherapeutic agent, used for a variety of cancers including ovarian and breast cancers (Lin et al., 1996). The first case of taxol production by endophytic sources was *Taxomyces andreanae*, showing that organisms other than *Taxus* spp. could produce taxol (Strobel et al., 1996). Since this discovery, other endophytes have been found to produce taxol, including *Pestalotiopsis microspora* (Strobel et al., 1996), *Ozonium* spp., *Mucor* spp., *Alternaria* spp. (Zhou et al., 2007).

Podophyllotoxin is a lignin produced by *Podophyllum* species and has been found to be produced from the fungal endophytes *Trametes hirsuta* and *Phialocephala fortinii* which were isolated from *Podophyllum hexandrum* and *Podophyllum peltatum* (Puri et al., 2006, Eyberger et al., 2006). Podophyllotoxin is an important compound being a precursor to three anticancer drugs: etoposide, teniposide and etoposide phosphate. These compounds inhibit the enzyme topoisomerase II, thus disrupting the cell cycle due to the cell's inability to replicate DNA (Eyberger et al., 2006). The endophytic fungus *Aspergillus fumigatus* was found to produce the compound deoxypodophyllotoxin, also produced by the host plant *Juniperus communis*. Deoxypodophyllotoxin is a lignin with anticancer, antiproliferative and broad spectrum insecticidal activity (Kusari et al., 2009a).

Camptothecin is a pentacyclic quinoline alkaloid first found in the plant *Camptotheca acuminata* which inhibits topoisomerase I and has two semisynthetic derivatives; topotecan and irinotecan. Camptothecin is also produced by the endophytes *Fusarium solani* and

Entrophospora infrequens from the tree *Apodytes dimidiata* and the twigs of *Nothapodytes foetida* respectively (Shweta et al., 2010, Kusari et al., 2009b, Amna et al., 2006).

In addition to the production of plant secondary compounds, some endophytes have been found to produce unique bioactive secondary compounds which are not produced by plants. Such bioactive compounds isolated have been found to have a range of properties including antimicrobial, antiparasitic, antiviral, anticancer, insecticidal, cytotoxic, neuroprotective, antioxidant, insulin mimetic, and immunosuppressant properties (Aly et al., 2011, Strobel et al., 2004).

The prospects for finding novel bioactive compounds from endophytic sources are high. Many compounds have so far been discovered and with only a small portion of the earth's plants having been studied, many more compounds may be found. Below are further examples of bioactive compounds derived from endophytes grouped into those with antimicrobial, antiviral and anticancer activity.

1.5.1 Antimicrobial bioactive compounds

Antimicrobial bioactive compounds produced by fungal endophytes include terpenoids, alkaloids, phenylpropanoids, aliphatic compounds, polyketides, and peptides (Mousa and Raizada, 2013). Antibiotics are defined as being low-molecular-weight organic natural products made by microorganisms that are active at low concentration against other microorganisms (Guo et al., 2011).

Phomopsis sp. strain E02018, isolated from a dead twig of *Erythrina crista-galli* synthesized the polyketide lactone named phomol (Weber et al., 2004). Phomol exhibited antibacterial and antifungal activity, inhibiting a variety of bacteria and fungi including *Arthrobacter citreus*, *Pseudomonas fluorescens*, *Aspergillus ochraceus* and *Fusarium fujikuroi*. Phomol showed cytotoxic effects with proliferation of the cell lines used (L1210, Colo-320, MDA-MB-231) being reduced 50% between 20 µg/ml (L1210) and 50 µg/ml (Colo-320, MDA-MB-231) (Weber et al., 2004).

A *Monochaetia* sp. endophyte isolated from *Taxus wallichiana* as well as the endophyte *Pestalotiopsis microspora*, isolated from the rainforest plants; *Taxus baccata*, *Torreya taxifolia*, *Wollemia nobelis* and *Dendrobium speciosum* were found to produce the cyclohexenone called ambuic acid. Ambuic acid exhibited antifungal activity against *Diplodia natelensis*, and *Cephalosporium gramineum*. Ambuic acid was also active against *Pythium ultimum* with a minimum inhibitory concentration (MIC) of 7.5 µg/ml. The cyclohexenone moiety of the compound is similar to tetracycline (Li et al., 2001).

Pestalotiopsis jesteri is an endophyte isolated from *Fragaria bodenii* which synthesizes the cyclohexenone epoxides jesterone and hydroxy-jesterone. Both compounds possess antifungal activity. Jesterone had relatively low MIC values when tested against the oomyceteous fungi *Pythium ultimum*, *Aphanomyces* sp., *Phytophthora citrophthora* and *P. cinnamomi* compared to the high MIC values of hydroxyl-jesterone (Li and Strobel, 2001).

The endophyte *Xylaria* sp.YX-28 of the host plant *Ginkgo biloba* L. synthesized the compound 7-amino-4-methylcoumarin which inhibited the growth of the 13

microorganisms tested in a study including *S. aureus*, *E. coli*, *S. typhia*, *S. typhimurium*, *S. enteritidis*, *A. hydrophila*, *Yersinia sp.*, *V. anguillarum*, *Shigella sp.*, *V. parahaemolyticus*, *C. albicans*, *P. expansum*, and *A. niger*. Due to the broad spectrum activity the compound may be effective as a natural preservative in food (Liu et al., 2008).

The endophytic fungus *Alternaria sp.*, isolated from the mangrove plant *Sonneratia alba* from China yielded two new compounds called xanalteric acids I and II. The two compounds were tested against a variety of multiresistant bacterial and fungal strains and showed weak antibacterial activity against *Staphylococcus aureus* with MIC values of 250 -125 µg/ml (Kjer et al., 2009).

Acremonium zeae, isolated from the maize kernels of *Zea mays* produced two antimicrobial compounds pyrrocidines A and B. These two compounds were found to have antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides* with both compounds inhibiting *F. verticillioides* more than *A. flavus*. Pyrrocidine A also showed high inhibition against most Gram-positive bacteria (Wicklow et al., 2005).

Ecomycins are a family of antimycotic lipopeptides produced by the bacterium *Pseudomonas viridiflava* found in the leaves of lettuce (*Lactuca sativa*) and many grass species. The ecomycins affect a wide range of human and plant pathogens. Ecomycin B had a MIC of 4 µg/ml against *Cryptococcus neoformans* and 31 µg/ml against *Candida albicans* (Miller et al., 1998).

1.5.2 Antiviral bioactive compounds

Novel drugs are also needed to treat the viral diseases that affect humanity and endophytes may be a source for new antiviral drugs. There is scant literature on the effects of endophytic bioactive compounds on viruses compared to the effects on bacterial pathogens, though some compounds with antiviral activity have been discovered.

Xiamycin is a novel pentacyclic indolosesquiterpene found to be produced by *Streptomyces* sp. GT2002/1503, an endophyte isolated from the mangrove plant *Bruguiera gymnorhiza*. Xiamycin was found to have moderate antiviral activities against HIV. It specifically blocked CCR5 (R5) tropic HIV-1 while it had no effect on CXCR4 (X4) tropic HIV-1 (Ding et al., 2010).

A solid state fermentation extract of the endophytic fungus *Cytonaema* sp. revealed two novel compounds called cytonic acids A and B. These compounds are inhibitors of human cytomegalovirus (hCMV) protease. MS and NMR methods revealed their structures as *p*-tridepsides (Guo et al., 2000).

The endophyte *Alternaria tenuissima* isolated from a stem of the Sonoran desert plant *Quercus emoryi* was found to produce four novel compounds. These secondary products, called compounds DK, DL, DM and DP, inhibited HIV-1 replication almost completely at the highest non-cytotoxic dose possible (0.5 µg/ml for compound DL and 1.5 µg/ml for compounds DK, DM and DP) (Wellensiek et al., 2013).

1.5.3 Anticancer bioactive compounds

Cancer is currently one of the leading causes of death worldwide and it has been estimated that there will be more than 1.6 million new cases of invasive cancer throughout the year 2013 (Siegel et al., 2013). As such, it is critical to find new drugs or technologies capable of treating the disease. There have been cases of anticancer compounds being produced by endophytes, most notably taxol (mentioned previously). Other cases of endophytic anticancer products are mentioned below.

Anticancer effects were found in compounds produced by two endophytic strains of *Fusarium oxysporum* isolated from the root tissue of host plant *Ephedra fasciculata*. The compounds were identified as beauvericin and bikaverin by NMR and were found to be cytotoxic when evaluated against four sentinel human cancer cell lines, NCI-H460 (non-small-cell lung), MIA Pa Ca-2 (pancreatic), MCF-7 (breast), and SF-268 (CNS glioma). The concentrations resulting in 50% inhibition of cell proliferation/survival were found to range between 0.01 and 1.81 μ M (Zhan et al., 2007).

Cajanol is an isoflavone produced by *Cajanus cajan* that has been described as a novel anticancer agent. It has also demonstrated other properties including antiplasmodial, antifungal and antimicrobial activity. It has been found to be produced by the endophytic fungus *Hypocrea lixii* isolated from the roots of the host plant pigeon pea (*Cajanus cajan*). The level of cytotoxic activity towards A549 cell lines is greater for fungal-produced cajanol than plant-produced cajanol (Zhao et al., 2013).

Santos et al. (2012) isolated many compounds (not yet identified) with anticancer activity from endophytes of the Brazilian medicinal plant *Combretum leprosum*. Extracts of the

fungus *Aspergillus oryzae* CFE108a showed significant cytotoxic effects against cell lines causing histiocytic sarcoma (J774) with IC_{50} of 0.80 and Leukemic T-cell lymphoblast (Jurkat) with IC_{50} of 0.89. The greatest inhibition was against bladder carcinoma (ECV304) with IC_{50} of 3.08 and cervical cancer cells (HeLa) with IC_{50} of 2.97. Extracts from *Fusarium oxysporum* had high rates of inhibition of cell lines causing lymphoid leukemia (P388) with IC_{50} of 2.14 and histiocytic sarcoma (J744) with IC_{50} of 2.98 (Santos et al., 2012).

Fourteen anthracenedione derivatives were isolated from the mangrove endophytic fungus *Halorosellinia* sp. (No. 1403) and *Guignardia* sp. (No. 4382). Growth of KB and KBv200 cells were strongly inhibited with the strongest of the fourteen compounds displaying cytotoxicity with IC_{50} values of 3.17 and 3.21 μ M to KB and KBv200 cells, respectively. Each compound possessed varying R groups which suggest the cause for the varying levels of cytotoxicity found for each compound is the structure and R groups (Zhang et al., 2010).

Ergoflavin is a compound of the class ergochromes with anticancer and anti-inflammatory properties. Ergoflavin was originally reported as the major secondary metabolite of *Claviceps purpurea* but has since been isolated from the endophyte designated PM0651480, found in the Indian medicinal plant *Mimosops elengi* (*bakul*). Ergoflavin significantly inhibited human TNF- α and IL-6 with IC_{50} values of 1.9 ± 0.1 and 1.2 ± 0.3 μ M respectively and induced cytotoxicity in ACHN, H460, Panc1, HCT116, and Calu1 cancer cell lines with IC_{50} values of 1.2 ± 0.20 , 4.0 ± 0.08 , 2.4 ± 0.02 , 8.0 ± 0.45 , and 1.5 ± 0.21 μ M, respectively (Deshmukh et al., 2009).

Sclerotiorin is a potent anti-proliferative compound effective against different cancer cells which was isolated from the endophytic fungus *Cephalotheca faveolata* found in the leaves of *Eugenia jambolana* (Giridharan et al., 2012). Incubating the cancer cells at 37°C along with sclerotiorin demonstrated that sclerotiorin displays effects of time dependent down regulation of the anti-apoptotic protein BCL-2 whereas it showed time dependent up regulation of the pro-apoptotic protein BAX, both within the range of 6 to 24 hours. Sclerotiorin also promoted over expression of caspase-3 from 12 to 24 hours after treatment (elevated caspase-3 expression being an indicator of apoptosis) (Giridharan et al., 2012).

1.6 *Pittosporum angustifolium* (Pittosporaceae)

The tree species *Pittosporum angustifolium* belongs to the *Pittosporaceae* family which consists of 9 genera and approximately 250 species (Linnek et al., 2012). Species of *Pittosporum* have been found in Australia, New Zealand, Norfolk Island, the Society and Sandwich Islands, the Moluccas, China, Japan, Madeira and Africa. *Pittosporum* was introduced into Europe and America last century for horticultural purposes (Cayzer et al., 2000). Seven of the nine genera are entirely endemic to Australia, although one may extend into Malesia (Chandler et al., 2007).

Figure 1. *Pittosporum angustifolium* (centre) from Pittsworth, QLD



Although not a common species, *P. angustifolium* is widespread throughout Australia. It was previously wrongly named as *Pittosporum phillyreoides*. It is found in habitats of open eucalypt woodlands and moister areas near inland lakes and drainage lines on sandy soils in arid zones (Cayzer et al., 2000). *P. angustifolium* can be described as having pendulous branches, falcate and glabrous leaves in a weeping canopy with yellow flowers (Cayzer et al., 2000).

The Australian Aboriginals are known to have used various parts of the plant for different purposes. A drink was made using the seeds, fruit pulp, leaves or wood in order to relieve

pain and cramps while a decoction of the fruit was used to treat eczema and pruritus (Cayzer et al., 2000). In some areas of Australia, the Aboriginals utilized the fruits to prepare a concoction that was drunk for coughs, colds or as a lactagogue, however, it is noted that not all Aboriginals utilized the fruits, as the Pitjantjatjara tribe did not consume the fruits of the plant at all (Sadgrove and Jones, 2013).

A recent study by Sadgrove and Jones, (2013) extracted the essential oils of *Pittosporum angustifolium* and assessed their inhibitory activity against various microbial species. The oils from the fruits and leaves of two *P. angustifolium* plants showed moderate antimicrobial activity against the three microbes that were tested (*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Candida albicans*), while the fruit of another *P. angustifolium* plant showed relatively high inhibition. The difference in antimicrobial activity of each plant may be due to different chemical compositions found in plants of different geographical locations. Analysis of the chemical composition of *P. angustifolium* essential oil extracts revealed 51 different chemicals, the composition of which differed with each geographically distinct sub species as well as between the leaves and fruits of the plants. For example, leaf essential oils showed greater quantities of esters and sesquiterpenols than the oils of the fruit. Chemical screening of the extracts revealed the presence of saponins, phenols (both soluble and insoluble), flavonoids (pre- dominantly in the methanol and hexane extracts), triterpenoids and tannins (Sadgrove and Jones, 2013). Sadgrove and Jones (2013) suggested that the essential oil components limonene, sabinene, terpinenes, α -pinene and bicyclogermacrene may be the cause of the antimicrobial activity in the study.

1.7 Research questions and objectives

There is currently limited knowledge of Australian endophytes regarding species diversity, ecological roles as well as their potentials as producers of antimicrobial compounds. This project seeks to expand this knowledge by examining the endophytes of the plant species *P. angustifolium* and asks the questions: 'What are the endophytes of *P. angustifolium*', 'Do endophytes of *P. angustifolium* exhibit a host-specific relationship' and 'Do endophytes of *P. angustifolium* produce bioactive compounds capable of inhibiting strains of human pathogenic bacteria and fungi (*Staphylococcus aureus*, *Serratia marcescens*, *Escherichia coli* and *Candida albicans*).'

There were three main objectives in this project:

- 1) To identify both fungal and bacterial endophytes in the leaves of *P. angustifolium*.
- 2) To determine if the fungal and or bacterial endophytes of *P. angustifolium* display host preference, that is if the same endophytes are present in hosts at multiple plant locations.
- 3) To detect and isolate bioactive compounds produced by the endophytes of *P. angustifolium*.

2.0 Materials and Methods

2.1 Sample collection

Leaves of *P. angustifolium* were sampled across seven sites located in South East

Queensland in 2013 (Table 1). One plant was sampled from each site and the leaf samples were taken from three different heights on each plant to gain a better representation of the overall endophyte community within the plants leaves. Samples were placed in a plastic bag and stored on ice until they could be processed within the laboratory. Processing of samples occurred within three hours of collection.

Table 1. Sampling sites of *Pittosporum angustifolium* in Southeast QLD

Site	Location
A	Felton
B	Biddeston
C	Mount Tyson
D	Oakey
E	Gowrie Junction
F	Chinchilla
G	Pittsworth

Figure 2. Locations of sites sampled (labelled A – G) located in Southeast Queensland (Map obtained from Google Maps)



2.2 Endophyte isolation

Leaf samples were washed by partially filling each sample bag with tap water and shaking vigorously. This was repeated twice. Samples were then moved to a biohazard safety cabinet. Leaves were surface sterilized to eliminate any epiphytic microbes and ensure isolation of only the leaf endophytes. This involved first soaking each leaf for 5 minutes in sterile water. The leaves were then transferred into 95% ethanol (EtOH). Samples from sites

A, B and C were submerged in 95% EtOH for 70 seconds, however, later samples were submerged in 95% EtOH for 60 seconds in order to reduce over-sterilizing the samples. The samples were then passed through a blue flame to remove the residual EtOH. Leaves were then pressed onto a Potato Dextrose agar (PDA) petri dish which acted as a means to determine successful surface sterilization. A sterile hole punch was then used to remove sections from each leaf. Eight sections were prepared per leaf and these were placed onto a petri dish containing PDA. The procedure was repeated for each leaf sample taken. Seven plates were prepared per plant which included one control plate and duplicate plates for each of the three location samples taken per plant. Each plate was sealed with parafilm and incubated in the dark at 23°C. Plates were checked daily for growth of any bacteria or fungi growing from the edge of the leaf segments. For each fungal colony that grew, the hyphal tips were subcultured onto a separate PDA plate by cutting out a small section of the hyphae containing agar with a scalpel blade. Bacterial colonies were subcultured onto PDA plates by the use of an inoculation loop and the streak plate method. All pure culture isolates were incubated in the dark at 23°C.

2.3 Identification of endophytic isolates

2.3.1 Molecular Identification

Endophytic isolates were identified by the sequencing of important taxonomic regions within their rDNA. Fungal isolates were identified via internal transcribed spacer (ITS) sequencing while bacterial isolates were identified via small subunit (SSU) sequencing. The DNA of each isolate was extracted using a Sigma-Aldrich XNAP-1KT REExtract-N-Amp Plant

PCR Kit. Fungal ITS-rDNA and bacterial SSU-rDNA were amplified via polymerase chain reaction (PCR). The fungal isolates utilized the fungal specific primer ITS1F (Gardes and Bruns, 1993) and the primer ITS4 (White et al., 1990). Bacterial isolates utilized the primer pair 27F and 1492R (Yu et al., 2013). PCR was set up using a Sigma-Aldrich XNAP-1KT REExtract-N-Amp Plant PCR Kit. Each reaction occurred with a total volume of 20 μ l containing 4 μ l distilled water, 10 μ l PCR ReadyMix, 4 μ l extracted DNA and 1 μ l of each primer. A Thermo Hybrid PCR Express Thermal Cycler was used to perform PCR reactions. DNA was amplified with 35 cycles of 95 $^{\circ}$ C for 1 min, 50 $^{\circ}$ C for 1 min and 72 $^{\circ}$ C for 1 min, with a final incubation at 72 $^{\circ}$ C for 10 min. All reactions were performed in duplicate along with a negative control containing water instead of DNA. All PCR products were then purified using Diffinity RapidTip 2 tips as per the manufacturer's instructions. All purified products were then electrophoresed in a 1% (w/v) agarose gel with RedSafe and visualized under UV light. Molecular weight markers were electrophoresed alongside PCR products and gels were run for 30 min at 100 volts. Sequencing reactions of purified DNA were performed at the Brisbane laboratory of the Australian Genome Research Facility (AGRF) in 12 μ l volumes containing approximately 20ng/ μ l (10-11 μ l) of purified DNA and 1-2 μ l of primer. Sequencing of fungal isolates used the ITS1F primer while bacterial isolates used the 27F primer. Reactions utilized 11 μ l of purified DNA for PCR products showing a faint band after UV visualization and 10 μ l DNA for those showing a bright band. Returned sequences were analysed using the BLASTn search tool from GenBank to identify the closest match for each isolate.

Phylogenetic analysis was performed with Molecular Evolutionary Genetics Analysis (MEGA) version 6. The GenBank BLASTn closest matches (more than 97%) were included in the

sequence analysis. Sequences were aligned using the 'align by Muscle' option with default settings. The aligned sequences were modified so that each was of the same size. Gaps within the sequences which were common to all sequences were removed. Phylogenetic analysis was carried out with a neighbour-joining tree using the Maximum Composite Likelihood model and bootstrapping of 1000 replicates (Mapperson et al., 2014). The suitability of the data being analysed was checked by using the compute overall mean option. A neighbour joining tree was constructed for both the fungal endophytic isolates and the bacterial isolates. For each of the species identified to the species level, sequences of the same species were taken from GenBank and included in the analysis.

2.3.2 Morphological identification

Microscopy was used to distinguish bacterial isolates from yeast isolates. Using an inoculation loop, a sample of bacterial/yeast cells were suspended in a drop of water on a microscope slide and examined under a microscope.

2.4 Analysis for host preference

Each endophyte which was identified to the species level was analysed to determine whether they showed a host preference for *P. angustifolium*. The presence or absence of each endophyte among each of the seven sampled host plants was noted. A bar graph was constructed showing the number of host plants colonized by each of the endophytes identified to the species level.

2.5 Primary screening of endophytic isolates

Sensitest agar plates were inoculated with each endophytic isolate. For fungal isolates, approximately 0.5cm³ of pure fungal culture was removed from its original plate with a scalpel blade and transferred to the centre of the Sensitest agar plate. For bacterial isolates the bacteria was transferred to the Sensitest agar by an inoculation loop and streaked in the centre of the plate. Each isolate was cultured on two Sensitest agar plates to setup duplicate screenings. Plates were stored in the dark at 23°C. Once each culture grew to at least 2cm in diameter, the test pathogens were streaked via an inoculation loop from the margin of the fungal/bacterial colony towards the edge of the plate. Four ATCC type strain pathogens were used consisting of a gram positive bacterium: *Staphylococcus aureus* (ATCC 25923), two gram negative bacteria: *Serratia marcescens* (ATCC 14756), *Escherichia coli* (ATCC 25922) and the fungus *Candida albicans* (ATCC 14053). Plates were incubated in the dark at 37°C and checked after 24-48 hours. Endophytes which showed inhibition on both duplicate plates were recorded and used for further investigation.

2.6 Extraction of bioactive compounds

10ml of Malt Extract Broth (MEB) was prepared in McCartney bottles. The MEB was inoculated with an approximately 0.5cm³ portion of mycelia containing agar and incubated at 23°C for 1 week. The McCartney bottles were swirled by hand daily. One bottle of MEB was prepared without the inoculum as a control.

After 1 week the bottle of fungal inoculum was added into a conical flask containing 500ml of MEB. One McCartney bottle was prepared for each conical flask and multiple conical flasks were prepared in order to increase the yield of any compounds extracted from the MEB. Cotton stoppers were placed in the conical flasks and covered with alfoil to prevent contamination. All conical flasks were incubated in the dark at 23°C.

After 5 days static growth, 500µl of autoclaved pathogens prepared in saline were added to the conical flasks. The pathogen added was the same as that which the endophyte had inhibited during the primary screening. The flasks were again incubated at 23°C. Once substantial growth was observed, the temperature was increased to 25°C.

After 4-6 weeks growth, the mycelia was filtered from the broth through Chux wipes into a 1L beaker. The filtered mycelia was soaked in 100% ethyl acetate in a separate beaker and broken up with a pipette tip. 250ml of filtered broth was poured into a separatory funnel along with an equal amount of ethyl acetate. The separatory funnel was shaken and the broth layer released into a 500ml beaker. The ethyl acetate layer was filtered through Whatman filter paper into a separate 500ml beaker. The remaining broth was also put through the separatory funnel with ethyl acetate. The separatory funnel steps were repeated with the same broth to increase the yield of compound extracted from the broth. The ethyl acetate used to soak the filtered mycelia was then added to the ethyl acetate used with the separatory funnel. The ethyl acetate was then left to evaporate leaving the dried crude extract.

2.7 Bioassay-guided fractionation

2.7.1 General experimental procedures

For HPLC fractionation of the crude extract, Alltech Davisil 40–60 μm 60 Å C_{18} 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 C_{18} Onyx Monolithic semi-preparative column (10 mm \times 100 mm; Phenomenex, Torrance, CA, USA) and a Phenomenex C_{18} Onyx Monolithic analytical column (4.6 mm \times 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.

2.7.2 Analytical HPLC

A portion of the crude extract obtained from the ethyl acetate extraction (13 mg) was resuspended in methanol. Isocratic HPLC conditions of H_2O -ACN- CF_3COOH (90:10:0.1) were initially employed for the first 5 min, then a linear gradient to H_2O -ACN (0.1% CF_3COOH ; 5:95:0.1) was run over 15 min, followed by isocratic conditions of H_2O -ACN (0.1% CF_3COOH ; 5:95:0.1) for a further 5 min, all at a flow rate of 1 mL/min and at 40°C.

2.7.3 Bioassay-guided fractionation

A portion of the crude extract (67 mg) was pre-adsorbed to C_{18} -bonded silica (1 g) then packed into a stainless steel guard cartridge (10 \times 30 mm) that was subsequently attached

to a C₁₈ semi-preparative HPLC column. Isocratic HPLC conditions of H₂O-ACN-CF₃COOH (90:10:0.1) were initially employed for the first 5 min, then a linear gradient to H₂O-ACN (0.1% CF₃COOH; 5:95:0.1) was run over 15 min, followed by isocratic conditions of H₂O-ACN (0.1% CF₃COOH; 5:95:0.1) for a further 5 min, all at a flow rate of 4 mL/min. Five fractions were collected manually at appropriate intervals (Figure 9) from the start of the run, then prepared for bioassay testing.

2.8 HPLC fraction and crude extract analysis

2.8.1 Minimum Inhibitory Concentration (MIC)

The bacteria *Serratia marcescens* (ATCC 14756) and *Staphylococcus aureus* (ATCC 25923) were subcultured onto Sensitest agar plates and incubated at 37°C for 18 hours prior to antimicrobial testing. *S. marcescens* was used as the target microbe for antibacterial testing while *S. aureus* was used as a control due to it not being inhibited during the primary screening and being a Gram positive bacterium in contrast to *S. marcescens*.

HPLC fractions were weighed and dissolved in 25% EtOH/0.7% saline to a concentration of 1mg/ml. The fractions were diluted by half four times in microcentrifuge tubes to produce five different concentrations (1: 1mg/ml, 2: 500µg/ml, 3: 250µg/ml, 4: 125µg/ml and 5: 62.5µg/ml). The crude extract was also diluted into five concentrations beginning at 10mg/ml in 40%EtOH/0.7% saline (1:10mg/ml, 2: 5mg/ml, 3: 2.5mg/ml, 4: 1.25mg/ml, 5: 625µg/ml)

An antibiotic solution of ciprofloxacin (Sigma-Aldrich) was prepared as a positive control against *S. marcescens* and *S. aureus*. The solution was made at a concentration of approximately 12µg/ml in sterile H₂O.

Suspensions of both *S. marcescens* and *S. aureus* were prepared in Mueller-Hinton (MH) broth to an approximate concentration of a 0.5 McFarland standard. The suspensions were used within 15 minutes of preparation.

50µl of sterile MH broth was transferred into the wells of a 96 well microdilution tray. Aliquots of 50µl of fraction dilutions were transferred into the wells. Each fraction was tested against both *S. marcescens* and *S. aureus* with dilutions being tested in duplicate wells. 50µl of the appropriate bacteria was inoculated into all experimental wells. Columns 11 and 12 were reserved for negative, positive, contamination and solvent controls. The negative control contained 50µl of sterile water along with 50µl of bacteria. The positive control contained 50µl of antibiotic solution along with 50µl of bacteria. The contamination control contained 100µl of sterile 0.7% saline and the solvent control contained 50µl of 25% EtOH/0.7% saline and 50µl of bacteria.

The microdilution tray was incubated at 37°C, checking for bacterial growth at 18, 21 and 24 hours post incubation. The MIC was recorded as the concentrations in the first wells that showed no visible growth after incubation.

2.8.2 Minimum Bactericidal Concentration (MBC)

Non-growing samples in the microdilution tray were used to determine the minimum bactericidal concentration. 10µl was transferred from each well which showed no visible growth onto separate Sensitest agar plates and spread with an inoculation spreader. Plates were incubated at 37°C for 24 hours. Plates were checked for bacterial growth after incubation. Fractions which allowed growth of bacteria were recorded as bacteriostatic while those which displayed no bacterial growth were recorded as bactericidal.

3.0 Results

3.1 Endophyte isolation

Endophytes were successfully isolated from each of the 7 host plants sampled. Both fungal and bacterial species were isolated, with plant samples from sites A – E contributing both fungal and bacterial isolates and samples from sites F and G contributing only fungal and bacterial isolates respectively. Endophytes did not grow from all leaf sections plated. Of the sections displaying endophytic growth, some yielded only a single isolate while others yielded up to three isolates. A total of 54 isolates were obtained across the 7 plants with varying numbers of endophytes being isolated from each plant sample. There was a mean of 5.4 fungal and 2.3 bacterial isolates obtained for a single plant. The plant sample from site D had the highest number of isolates (16 fungal, 2 bacterial) (Table 2). One bacterial isolate was lost due to the agar within the petri dish drying up. As such no further analysis could be done on the isolate.

Fungal growth was observed on the sterilisation control plate of the plant samples from site A, indicating incomplete surface sterilization or contamination of the sterilisation control plate. The endophyte on the control plate was identified via DNA sequencing and the epiphytic contaminant was subsequently eliminated from further study.

Isolates were designated a code based on the site of the plant sample which they were isolated from and the order in which each was isolated (Table 4), for example A1 for the first endophyte isolated from site A.

Table 2. Total number of endophytes isolated per site.

Sample site	Number of fungal isolates obtained	Amount of Bacterial Isolates Obtained
A	7	2
B	7	1
C	1	1
D	16	2
E	2	6
F	5	0
G	0	4
Total	38	16
Mean	5.4	2.3

3.2 Identification of endophytic isolates

All isolates obtained (including the fungal isolate observed on the control plate), were identified to species or genus level via DNA sequencing. PCR amplification of the ITS regions of fungal isolates was successful for all isolates except for three (D6, D16 and E6). For the 16 bacterial isolates, PCR amplification of the SSU regions was unsuccessful for five isolates (D15, E5, E8, G3 and G4) (Figure 3).

The returned sequences from the AGRF revealed that two isolates (C2 and D5) were unable to be sequenced. After repeated sequencing failures it was decided to leave both isolates without being identified. Successfully sequenced isolates were analysed with Chromas Lite version 2.1 to check for contamination. Using the BLAST search tool 27 of the isolates were identified to the species level and six isolates identified to the genus level. Other isolates were found to belong to the Dothideomycetes class (one isolate), Sarcosomataceae family

(three isolates) and Sordariomycetes class (three isolates). Two isolates were identified to their closest match to be uncultured bacterium clones. Two isolates from site A were found to be the same species as that of the epiphytic contaminant growing on the site A control plate (Table 3). These two isolates were thus not included as endophytic isolates from site A. All of the successfully sequenced fungal isolates were found to be Ascomycetes while all of the successfully sequenced bacterial isolates belonged to the phylum Firmicutes.

Table 3. Identification of fungal contaminants.

Isolate A0 was found as a contaminant on the control plate for site A. The two isolates A1 and A2 were found by a BLAST search to be the same species as that of the fungal contaminant on the control plate.

Isolate Code	Closest match	GenBank Accession No.	Query Cover (%)	Identity (%)
A0	<i>Nigrospora oryzae</i>	JN211105.1	100	98
A1	<i>Nigrospora oryzae</i>	JN211105.1	100	99
A2	<i>Nigrospora oryzae</i>	KC937039.1	100	100

Table 4. GenBank matches of isolated endophytes.

A total of 54 endophyte isolates were obtained from seven *P. angustifolium* plants. Table 4 lists the species identified as the closest matches by the BLAST tool. Not all isolates could be successfully sequenced and matched via BLAST and are thus not shown in Table 4.

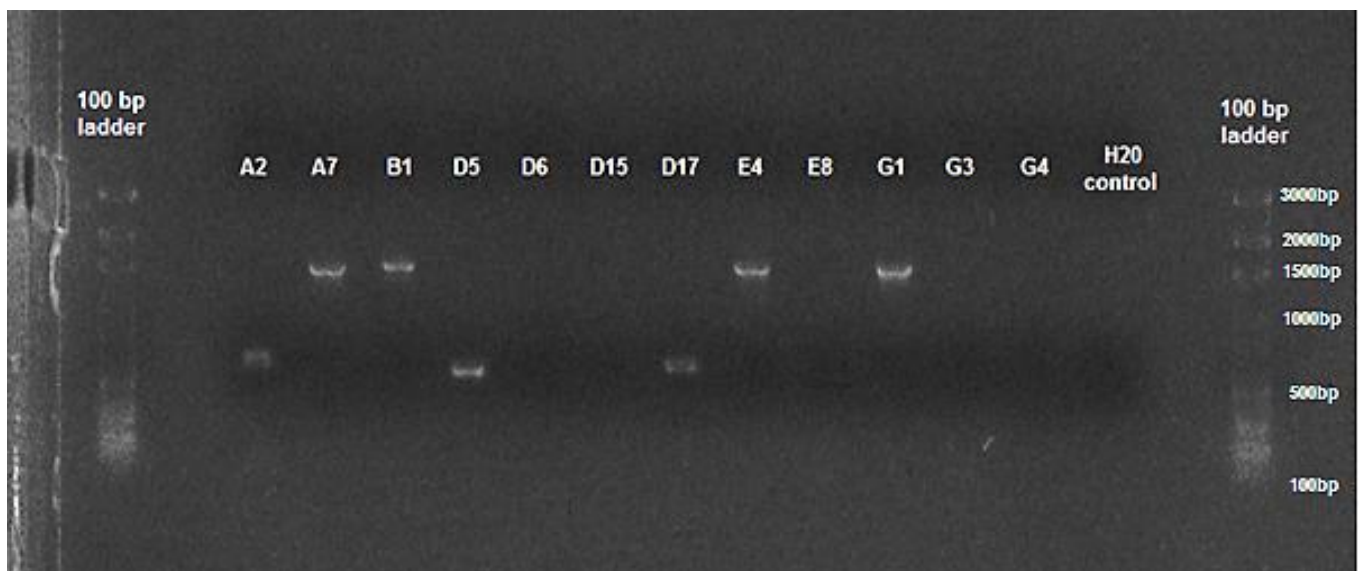
Isolate Code	Closest match	GenBank Accession No.	Query Cover (%)	Identity (%)
A3	<i>Pseudocercospora fuligena</i>	GU214675.1	100	99
A4	<i>Pseudocercospora fuligena</i>	GU214675.1	99	99
A5	<i>Guignardia mangiferae</i>	AY816311.1	100	100
A6	<i>Guignardia mangiferae</i>	EU677814.1	100	94
A7	<i>Uncultured bacterium clone</i>	HM676109.1	100	99
A8	<i>Dothideomycetes sp.</i>	JQ760353.1	98	98
A9	<i>Uncultured bacterium clone</i>	HM332406.1	100	99
B1	<i>Bacillus subtilis</i>	JN366795.1	100	87
B2	<i>Guignardia mangiferae</i>	KF381072.1	99	99
B3	<i>Xylaria sp.</i>	AB512404.1	100	100
B4	<i>Sarcosomataceae sp.</i>	KF128806.1	98	96
B5	<i>Preussia minima</i>	AY510425.1	95	96
B6	<i>Sarcosomataceae sp.</i>	KF128803.1	100	100
B7	<i>Coniochaeta sp.</i>	KF128810.1	100	99
B8	<i>Xylaria sp.</i>	JN225909.1	99	95
D1	<i>Pseudocercospora fuligena</i>	GU214675.1	100	99
D2	<i>Pseudocercospora fuligena</i>	GU214675.1	100	99
D3	<i>Pseudocercospora fuligena</i>	GU214675.1	100	99
D4	<i>Pseudocercospora fuligena</i>	GU214675.1	99	99
D7	<i>Pseudocercospora fuligena</i>	GU214675.1	100	99
D8	<i>Pseudocercospora fuligena</i>	GU214675.1	99	99
D9	<i>Pseudocercospora fuligena</i>	GU214675.1	99	99
D10	<i>Pseudocercospora fuligena</i>	GU214675.1	100	99
D11	<i>Pseudocercospora atromarginalis</i>	JX901780.1	100	100
D12	<i>Pseudocercospora fuligena</i>	GU214675.1	100	99
D13	<i>Pseudocercospora fuligena</i>	GU214675.1	100	99
D14	<i>Pseudocercospora fuligena</i>	GU214675.1	100	99
D17	<i>Lecytophora sp.</i>	HE863327.1	100	97
D18	<i>Xylaria hypoxylon</i>	AY327476.1	99	95
E1	<i>Bacillus pumilus</i>	JX645203.1	99	99
E2	<i>Bacillus pumilus</i>	KJ410678.1	100	99
E3	<i>Bacillus pumilus</i>	AM887694.1	99	99
E4	<i>Bacillus sp.</i>	FJ596550.1	99	93
E7	<i>Sarcosomataceae sp.</i>	KF128806.1	96	97

Table 4 continued.

Isolate Code	Closest match	GenBank Accession No.	Query Cover (%)	Identity (%)
F1	<i>Sordariomycetes sp.</i>	JQ760129.1	99	98
F2	<i>Xylaria hypoxylon</i>	AY327476.1	97	96
F3	<i>Xylaria hypoxylon</i>	AY327476.1	97	95
F4	<i>Sporormiella sp.</i>	HQ130664.1	99	99
F5	<i>Pyronema sp.</i>	KF128839.1	99	99
G1	<i>Bacillus megaterium</i>	KF933685.1	100	99
G2	<i>Bacillus megaterium</i>	HF584868.1	100	100

Figure 3. 1% (w/v) agarose gel of both successful and unsuccessful PCR products.

Bands indicate successful amplification of PCR products which include both fungal (A2, D5, D17) isolates and bacterial (A7, B1, E4, G1) isolates.

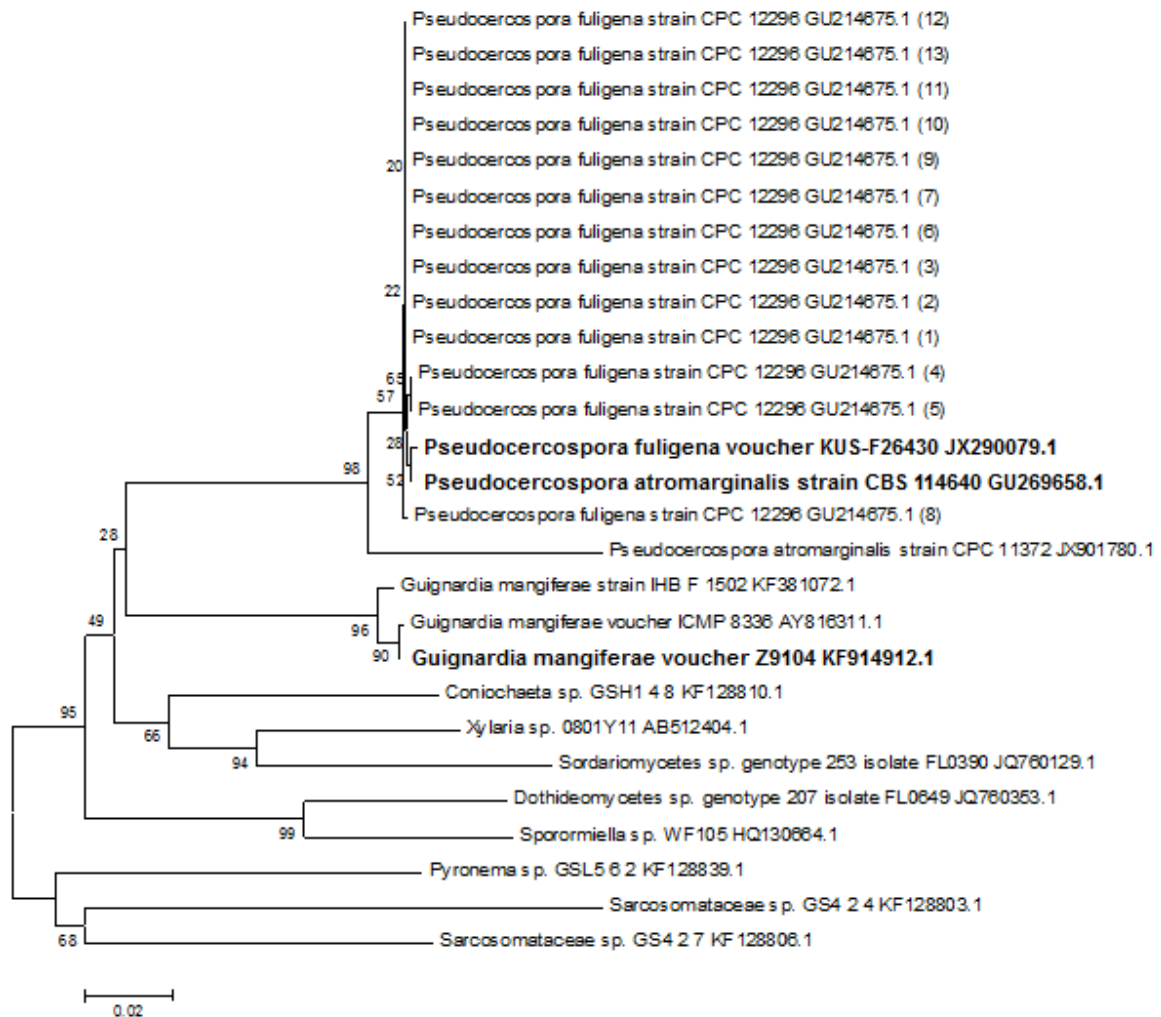


3.3 Phylogenetic Analysis

A neighbour joining tree was constructed using sequences which were identified to at least a 97% match from BLAST searches in GenBank. *P. fuligena*, *P. atromarginalis* and *G. mangiferae* were the only isolates included in the neighbour joining tree which were both matched by BLAST to 97% or higher and identified to the species level. Reference sequences representing each of these isolates were also obtained from GenBank and included in the analysis. The reference sequence of *P. fuligena* was placed in a clade clustered among the isolates of this study which were identified as *P. fuligena*. Some branching among these isolates occurs, however, low bootstrap support (values of 20, 22, 28 etc.) decreases the reliability that they should be in separate branches. The short horizontal distance of the branches indicate that these isolates are genetically similar. *P. atromarginalis* is in an adjacent clade to the *P. fuligena* isolates (98% bootstrap support) and its reference GenBank sequence. This may indicate that the isolate is incorrectly identified and may be a different but closely related species of *Pseudocercospora*. One *G. mangiferae* isolates is clustered with the GenBank reference sequence with high bootstrap support (90%), however another *G. mangiferae* isolate resides in an adjacent clade (96% bootstrap support).

Figure 4. Neighbour-joining phylogenetic tree of ITS rDNA of fungal endophytes.

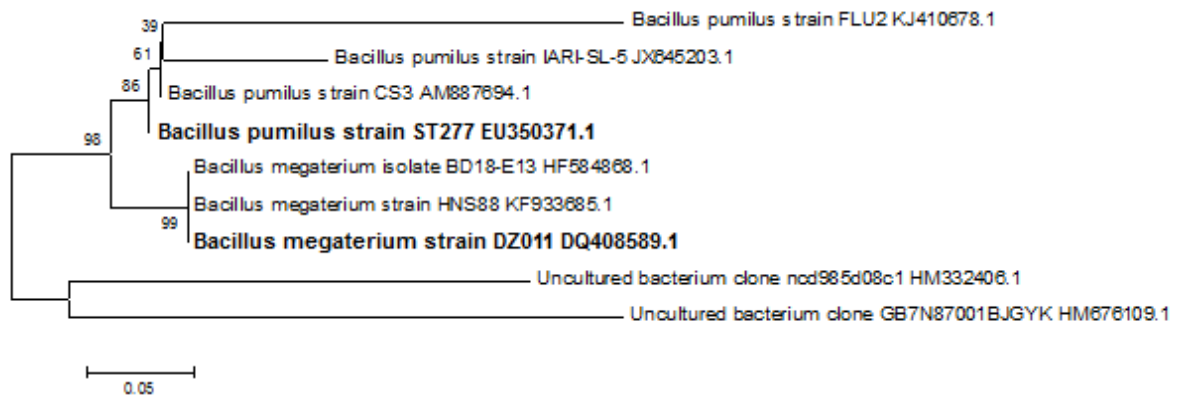
Species shown in **bold** indicate reference sequences obtained from GenBank.



A separate sequence from GenBank was included for both *B. pumilus* and *B. megaterium*.

The GenBank *B. megaterium* sequence is clustered in the same clade (99% bootstrap support) as the two isolates identified as *B. megaterium*, indicating correct identification for both isolates. The GenBank *B. pumilus* sequence is in an adjacent clade to the *B. pumilus* isolates of this study, indicating that these isolates may be a different but closely related species.

Figure 5. Neighbour-joining phylogenetic tree of SSU rDNA of bacterial isolates. Species shown in **bold** indicate sequences obtained from GenBank as a representative of that species.

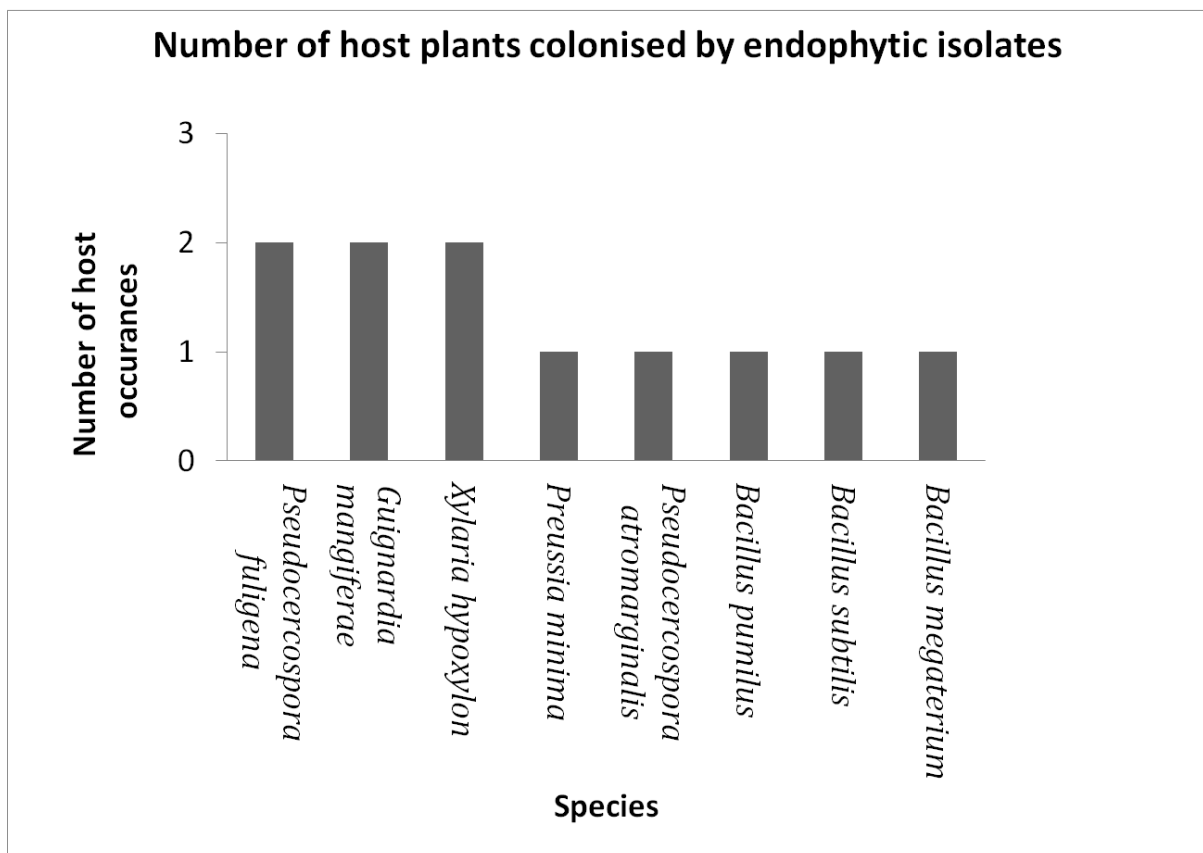


3.4 Host preference

Isolates which were successfully identified to the species level were graphed according to the number of host plants they were isolated from (Figure 6). The bar graph shows that three of the eight isolates were isolated from two plants (*Pseudocercospora fuligena*, *Guignardia mangiferae* and *Xylaria hypoxylon*). The five other isolates were only found to occur in one plant each.

Figure 6. Bar graph showing the amount of *P. angustifolium* plants that each species was isolated from.

Only those isolates which were identified to species level are included.

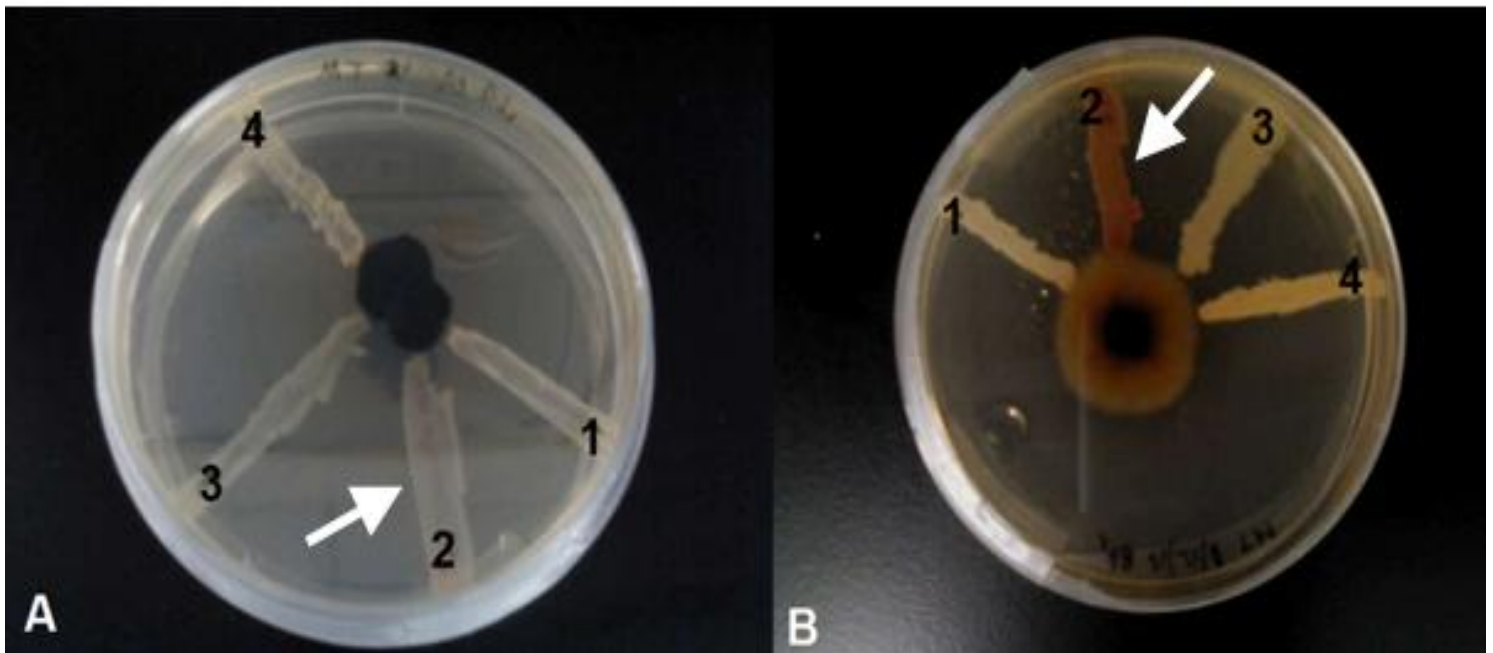


3.5 Primary Screening for Antimicrobial Activity

All but one isolate was screened for antimicrobial activity against the four test pathogens. The one isolate that was not screened was unable to be subcultured onto Sensitest agar. Four isolates appeared to show antimicrobial activity. Three of the four isolates were all found to be the species *Pseudocercospora fuligena*. The fourth isolate (bacterial) that displayed antimicrobial activity was unable to be identified and was therefore not chosen for further investigation. The *P. fuligena* isolate displayed antimicrobial activity against the test pathogen *S. marcescens* (Figure 7) and was therefore chosen for further investigation. The three *P. fuligena* isolates were compared by the BLASTn tool from GenBank, which revealed that they were each 99% similar to each other. This confirmed that the three isolates were the same species.

Figure 7. Primary screening of endophytic isolates.

A) Primary screening of *Pseudocercospora fuligena* against test microbes 1, 2, 3 and 4 showing reduced growth of 2. B) Primary screening against test microbes 1, 2, 3 and 4 showing no inhibition. Test microbes: *Staphylococcus aureus* (1), *Serratia marcescens* (2), *Escherichia coli* (3) and *Candida albicans* (4).



3.6 Bioassay-Guided Fractionation

Upon the completion of the ethyl acetate extraction, the total yield of the fungal crude extract was 105mg. 25mg of this extract was retained and analysed later to identify whether the compound/s responsible for the antibacterial activity was successfully extracted from *P. fuligena*. Approximately 13mg of crude extract underwent an analytical HPLC run (Figure 8). A range of peaks at 210nm spectra are observed from 1.5min to 4min over 500mAU and one further peak at 15min over 500mAU. Another noticeable peak occurred at 12min with various other smaller peaks occurring throughout.

Approximately 67mg of crude extract pre-adsorbed to C₁₈-bonded silica then underwent HPLC fractionation (Figure 9). Five fractions were collected.

Figure 8. Analytical HPLC chromatogram for *P. fuligena* crude extract showing fractions collected for antimicrobial screening.

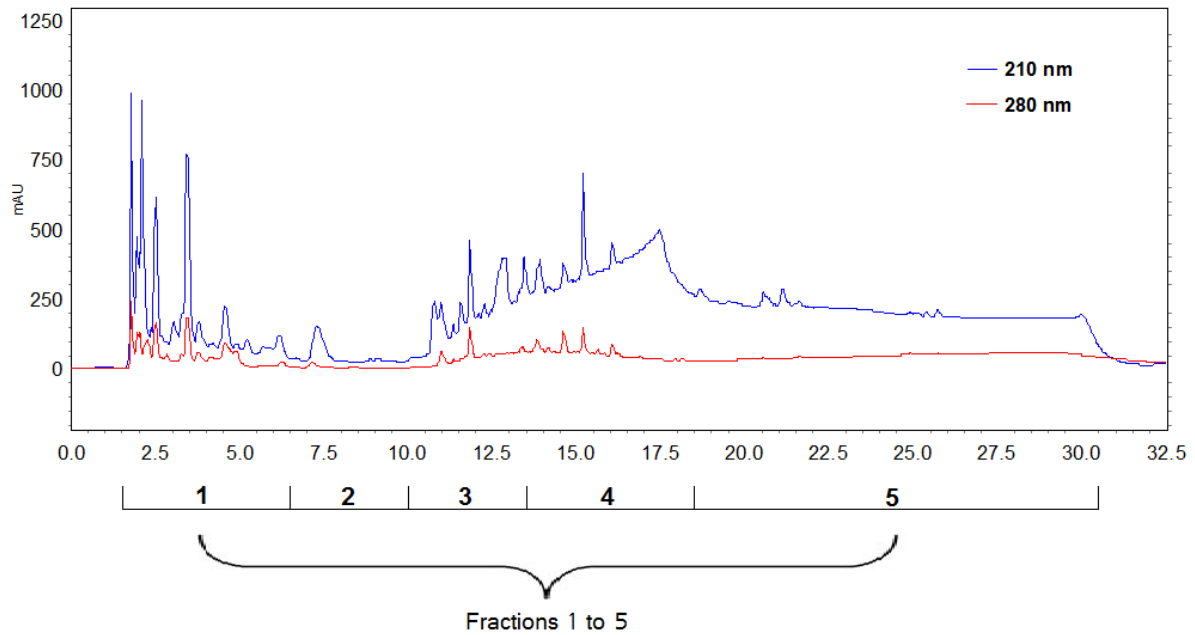
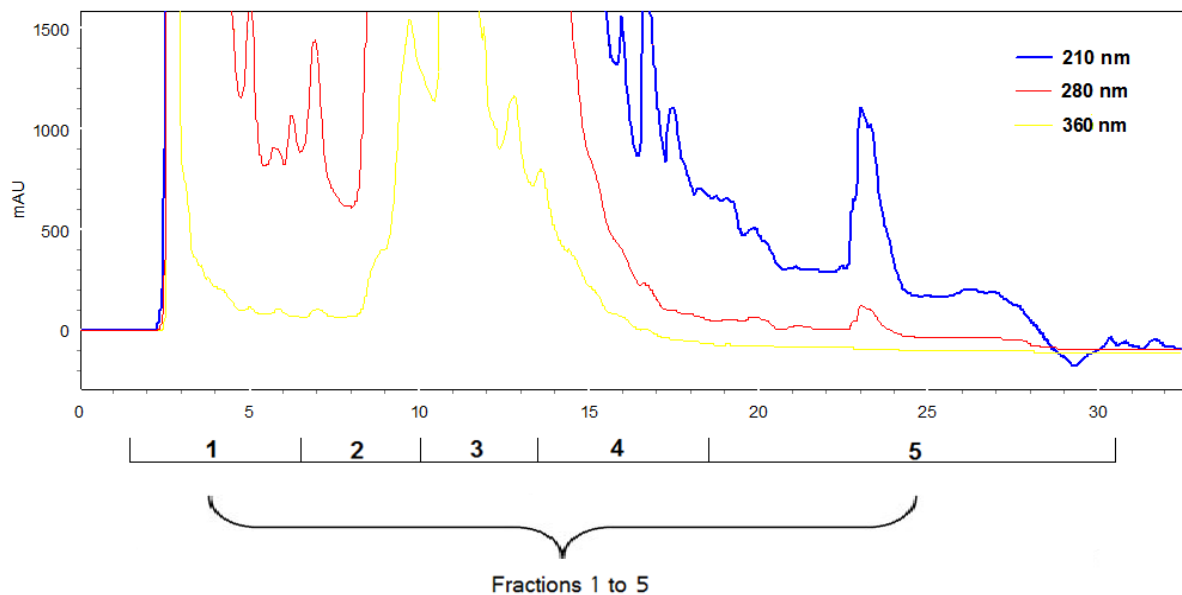


Figure 9. HPLC fractionation chromatogram of *P. fuligena* crude extract showing fractions collected for antimicrobial screening.



3.7 HPLC fraction and crude extract analysis

3.7.1 Minimum Inhibitory Concentration

Dilutions were performed for each fraction to produce five samples of different concentrations (Table 5). Dilutions were also performed in the same manner for the crude extract (Table 6).

Table 5. Dilutions of fractions for use in MIC and MBC assays.

Fraction	Initial concentration (mg/ml)	Dilution 1 (µg/ml)	Dilution 2 (µg/ml)	Dilution 3 (µg/ml)	Dilution 4 (µg/ml)
1	1	500	250	125	62.5
2	1	500	250	125	62.5
3	1	500	250	125	62.5
4	1	500	250	125	62.5
5	1	500	250	125	62.5

Table 6. Dilutions of crude extract for use in MIC and MBC assays.

Sample	Initial concentration (mg/ml)	Dilution 1 (mg/ml)	Dilution 2 (mg/ml)	Dilution 3 (mg/ml)	Dilution 4 (mg/ml)
Crude Extract	10	5	2.5	1.25	0.625

The wells of the microdilution tray containing the positive (antibiotic) controls as well as the contamination controls showed no visible growth while those containing the negative controls and solvent controls did contain visible growth.

All wells containing *S. aureus* displayed visible growth. Fractions 1, 4 and 5 did not inhibit the growth of *S. marcescens* for any concentration. Both fractions 2 and 3 inhibited the

growth of *S. marcescens* at all concentrations except 62.5µg/ml where it showed visible growth, therefore having a MIC of 125µg/ml (Table 7). The crude extract showed inhibition of *S. marcescens* at all concentrations assayed with the lowest concentration being 625µg/ml (Table 7).

Table 7. MIC of test samples against *S. marcescens*.

Sample	MIC against <i>S. marcescens</i>
Fraction 2	125 µg/ml
Fraction 3	125 µg/ml
Crude extract	625 µg/ml

3.7.2 Minimum Bactericidal Concentration

Fractions 2, 3 and the crude extract were tested for their MBC against *S. marcescens*. Both fractions 2 and 3 were found to be bacteriostatic rather than bactericidal, as bacterial growth was found to cover most of the STA plate used for testing. The crude extract was found to be bactericidal with a MBC of 2.5mg/ml.

4.0 Discussion

4.1 Endophyte isolation

In this investigation, all plant samples of *P. angustifolium* were found to be host to at least one endophyte. This finding supports Strobel & Daisy (2003) that each individual plant on earth is host to one or more endophytes. Some of the plants sampled had higher numbers of endophytes than others, which may be due to multiple possible reasons. The number of endophytes able to be isolated may in part be determined during the leaf tissue surface sterilization phase. The duration each leaf spent alight after passing through the Bunsen burner flame may have affected the survival of some endophytes. Other types of endophytes may also be unable to be isolated with the methods used in this investigation. It is understood that culture dependent methods favour the fast-growing microbes and that the unculturable or slow-growing microbes will not be isolated (Duong et al., 2006).

The time each leaf spent submerged in ethanol during the surface sterilization stage was modified part way through the study to attempt to increase the number of endophytes isolated. Time spent in ethanol was reduced from 70 sec to 60 sec from site D onwards. Site D had an increase in endophytes (18) over preceding samples, however, subsequent samples did not show any increases. As such, the time spent in ethanol may not have had a major impact on the endophytes of the leaf tissue. The majority of isolates from site D were of the same species (*Pseudocercospora fuligena*) which may explain the higher number of isolates. With no previous research known to have been conducted on the endophytes of *P.*

angustifolium, this project gives the first indication for what endophytes reside within the plant.

The number of endophytes isolated in this study varied between each plant sampled ranging from two isolates (site C) to eighteen isolates (site D). These results may be due to sampling plants from different locations as it has been indicated that the location a plant resides in can strongly influence the abundance of endophytes (Hoffman and Arnold, 2008). The plant's locality may also affect endophyte diversity and species composition (Hoffman and Arnold, 2008). Therefore, even though each plant sampled in this study was found in similar habitats, there may be differences in each location that affects the endophytic abundance within each plant as well as whether fungal or bacterial endophytes could colonize the plants. This project found that most plants sampled harboured both fungal and bacterial endophytes, however, only fungal endophytes were isolated from site F with only bacterial endophytes isolated from site G. It may be that unseen environmental factors differed between both locations. Further research could focus on such factors as the soil composition, rainfall, mean temperature or weather patterns of each site at the time of sampling to find if any of these affect the endophyte assemblages. Also, as sampling was undertaken over several months, changes in climate over these months may have affected the endophyte communities.

The sterilisation control plate for the site A samples displayed growth of a single endophyte identified as *Nigrospora oryzae*. This led to discounting two isolates identified by DNA sequencing of the same species due to the uncertainty that either isolate was a true endophyte.

4.2 Identification of endophytic isolates

Endophytes have been traditionally identified based on their morphological characteristics while in culture (Ko Ko et al., 2011). More recently endophytes have also been identified by molecular methods involving DNA extraction, sequencing and comparison of obtained sequences to known species via databases such as GenBank (Ko Ko et al., 2011).

Identification of endophytic isolates is an important part of ecological or bioprospecting studies. Correct identification of isolates can afford researchers the ability to use the data as a reference for future research. For example, when working with an unknown endophyte, identification then allows the researcher to search for previous research on the same endophytic species as well as allowing easier cataloguing of any results gained in their research. Identification in bioprospecting studies allows other researchers to know what species has produced what compound. It can also help to identify how isolated endophytes are related to other endophytic species. For these reasons, and others, the ability to successfully identify endophytes is an important for research in this field.

Mycological taxonomy has been developing for over 200 years and has settled on grouping fungi into four major divisions, along with one 'pseudo-division'. The four major divisions are differentiated by their modes of sexual reproduction and consist of the Chytrids, Zygomycetes, Ascomycetes and Basidiomycetes (Seifert, 2009). The pseudo-division consists of the asexually reproducing fungi, though some have known sexual states, called the Deuteromycetes or Fungi Imperfecti (Seifert, 2009). All of the fungal endophytes sequenced in this study were found to be ascomycetes. As ascomycetes are the largest phylum of fungi (approximately 64,000 species) (Schoch et al., 2009), it is not surprising that the endophytes of this project belonged to this phylum.

Molecular methods were used in this study to attempt to identify each endophyte isolated. Some isolates were ultimately unable to be identified due to either a failure to amplify the DNA sequence with PCR or failure to be sequenced at AGRF. The majority of fungal isolates were successfully amplified by PCR and as such suggests that the choice of primers for fungal rDNA amplification was not at fault. It was suspected that the concentration of DNA may have been too high in the PCR reactions. Some isolates DNA were able to be amplified after reducing the volume of DNA extract in the PCR reaction. The isolates which were again not amplified may have succeeded with further decreases to volume of DNA extract. The failure of DNA sequencing of some isolates may have been due to the concentration of DNA being too high and may have been overcome by decreasing the concentration. It is also possible that the water used for the sequencing contained a sequencing inhibitor and that if fresh pure water were used, would allow successful sequencing.

Of the isolates identified, *P. fuligena*, *P. atromarginalis*, *G. mangiferae*, *B. pumilus* and *B. megaterium* all had $\geq 97\%$ identity to their matched species by BLAST. 97% was used as the threshold for correct identification. *Pseudocercospora* is a large genus of plant pathogenic fungi which are commonly associated with leaf and fruit spots as well as blights on a wide range of plant hosts (Crous et al., 2013). They are found in many areas including cool temperate, sub-tropical and tropical regions (Crous et al., 2013). *P. fuligena* is known to be the causal pathogen of black leaf mould, which is a major fungal disease of tomato in Asia (Mersha et al., 2014). *P. atromarginalis* has been found to cause leaf spots on the plant *Lycianthes biflora* (Phengsintham et al., 2013). Neither species appears to have been identified as an endophyte in the literature, however, Crous et al. (2013) states that species of *Pseudocercospora* are recognized as endophytes.

Guignardia is a genus of fungi which contains around 330 known species, with many of these species being considered as endophytic fungi (Wickert et al., 2014). The species *Guignardia mangiferae* has been previously identified as a ubiquitous endophyte and has been confused with the citrus black spot pathogen *Guignardia citricarpa* (Romao et al., 2011). Despite being endophytic in a wide range of hosts, it causes foliar spots in *Mangifera indica* (mango) (Wickert et al., 2014).

Bacillus is a genus of gram positive bacteria and has members that are capable of producing antibiotics, such as *B. subtilis* (Stein, 2005, Ouoba et al., 2007) and *B. amyloliquefaciens* (Yuan et al., 2012). Members of the genus may also have potential agricultural uses due to their ability to produce antimicrobial metabolites to control plant pathogens as well as to fix nitrogen (Liu et al., 2006). Studies have shown that strains of the isolate *B. pumilus*, which was isolated in this study, have displayed antimicrobial activity (Aunpad and Na-Bangchang, 2007, Ouoba et al., 2007). A strain of *B. pumilus* produced the compound pumilicin 4 which was found to be active against two drug-resistant pathogenic bacteria, Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (Aunpad and Na-Bangchang, 2007). The second *Bacillus* species isolated in this study, *B. megaterium*, has demonstrated nitrogen fixing abilities in some strains (Liu et al., 2006) and has also shown potential to control the fungal caused disease septoria tritici blotch (Kildea et al., 2008). Both *B. pumilus* and *B. megaterium* have previously been isolated as endophytes (Rai et al., 2007, Moore et al., 2006).

Various isolates that were matched by BLAST had a closest match below 97%. This introduces uncertainty to whether those endophytes are the same as their matched species.

As such, their species identities cannot be confirmed. It may be that these endophytes are new species of known fungal genera. With the current knowledge on Australian endophytes being sparse, it is likely that new species will be encountered during research into these microbes.

Phylogenetic analysis of the fungal species (Figure 4) showed the *P. fuligena* isolates grouped in adjacent clades near the GenBank *P. fuligena* sequence. While there is some branching into separate clades among the isolates, the bootstrap values for these clades are low which decreases the reliability of the branching. The horizontal distances of each branch is minimal which suggests little genetic variation. The BLAST results for each *P. fuligena* isolate also show low variation as each isolate was matched to *P. fuligena* with a % identity of 99%. This suggests that the *P. fuligena* isolates have been correctly identified. The *P. atromarginalis* isolate from this study resides in a clade adjacent to its GenBank reference sequence with strong bootstrap support (98%). This suggests that the isolate was incorrectly identified. The phylogenetic analysis suggests unreliability of the BLAST analysis as the isolate matched with *P. atromarginalis* was matched with a % identity of 100% from BLAST. This may suggest the isolate is a closely related species to *P. atromarginalis*. One of the *G. mangiferae* isolates appears to be identified correctly as it was grouped together with the GenBank reference sequence with a bootstrap support of 90%. The other isolate identified by BLAST as *G. mangiferae*, however, is in an adjacent clade (96% bootstrap support) which indicates it was incorrectly identified. It may be a closely related species to *G. mangiferae*.

The neighbour-joining phylogenetic tree of the bacterial species (Figure 5) indicates that the *B. pumilus* isolates are closely related to the *B. pumilus* GenBank sequence, however the low

bootstrap support decreases the reliability of the branching. The isolates may be closely related species rather than the same species. The *B. megaterium* isolates are clustered in the same clade (99% bootstrap support), which suggests that both isolates have been identified correctly. Arnold and Lutzoni (2007) note the limitations of using BLAST for species identification which are that BLAST matches are based on non-evolutionary matching criteria, are subject to error due to mis-identified sequences, can be difficult to interpret when all top matches are unidentified isolates or environmental samples, and are limited to those fungi present in GenBank.

The results of this study have shown that *P. angustifolium* is host to a variety of endophytes and is a potential source for identifying new species.

4.3 Host preference

From the data gained in this study, it cannot be concluded that any host preferences or host specific relationships exist with any of the endophytes isolated from *P. angustifolium*. Out of the 54 endophyte isolates, only 8 were identified to the species level and of these only 3 isolates were found in multiple plants. The low frequency of occurrence (2 plants) observed for the 3 isolates suggested no host preference.

There is the possibility that the endophyte community of a plant may vary from leaf to leaf. As such, sampling of more leaves from each plant may have revealed endophytes which occur frequently in different plants. Also, as mentioned previously, some endophytes may not be able to be isolated with the methods used here and it may be these endophytes that show a host preference.

The endophytic community of *P. angustifolium* may be affected by location more than host preference. This appeared to be the case in a study by Arnold et al. (2001) which found that when *Heisteria concinna* was sampled from two different sites, many of its endophytes were found only at one of the sites, despite similar conditions. This corresponds to the current study, where most endophyte species were found in only one plant. There may be unknown environmental factors at each site that determine the ability of certain species to colonize the plant, such as changes in soil nutrients, the amount of water available or the temperature of each area.

The endophytic communities in tissue other than leaves may vary and potentially show host preference. Studies have shown that endophytes are able to colonize plant tissue other than the leaves, such as the roots and xylem (Macia-Vicente et al., 2008, Oses et al., 2008). While previous research has indicated that there seems to be no host specificity among root endophytes (Girlanda et al., 2002), a study has shown host preference of root endophytic *Fusarium* spp. (Macia-Vicente et al., 2008). Host preference is similar to specificity, however, the relationship is not exclusive to one species (Kernaghan and Patriquin, 2011). The literature on root endophyte host preference or specificity is sparse so there is not enough evidence to suggest whether host specific relationships exist. The possibility exists that endophytes occupying the tissues of *P. angustifolium* other than the leaves, may show a host preference.

The isolation of *P. fuligena*, *G. mangiferae* and *X. hypoxylon* from two different plants may be an indication of host preference. If more *P. angustifolium* plants were sampled, the three endophytes mentioned may be found to be commonly present. Also, if more leaves were

processed from the plants sampled in this project, it may be found that *P. fuligena*, *G. mangiferae*, *X. hypoxylon* or others are found in each of the plants.

4.4 Primary Screening for Antimicrobial Activity

In this study, each endophyte isolated from *P. angustifolium* underwent a primary screening for antimicrobial activity. Each of the four pathogens chosen to be screened against are clinically significant pathogens. Within this study, two gram negative bacteria were chosen to be screened against: *Escherichia coli* and *Serratia marcescens*. *E. coli* is one of the most frequent and best studied bacterial organisms and is the most abundant facultative anaerobic bacteria of the human intestinal flora (Dobrindt, 2005, Jaureguy et al., 2008). *E. coli* has non-pathogenic commensal variants of the normal human gut flora, as well as pathogenic variants which can cause intestinal or extraintestinal infection in humans (Dobrindt, 2005). Extraintestinal *E. coli* infections typically include urinary tract infections (UTI), meningitis (mostly in neonates and after neurosurgery), diverse intraabdominal infections, pneumonia (particularly in hospitalized and institutionalized patients), intravascular-device infections, osteomyelitis and soft-tissue infections (Russo and Johnson, 2000). *E. coli* is also a leading cause of bacteraemia and because of an increase in β -lactam resistant strains, it can be difficult to treat (Courpon-Claudinon et al., 2010). Third-generation cephalosporin (3GC) resistance has especially become problematic as 3GCs are a common part of empirical antimicrobial chemotherapy in severe infections (Courpon-Claudinon et al., 2010). *S. marcescens* is an opportunistic enteric pathogen and is responsible for a significant proportion of hospital-acquired infections (Murdoch et al., 2011). It was thought to be a nonpathogenic saprophytic water organism until late in the 20th century (Su et al., 2003). Many strains of *S. marcescens* are also resistant to multiple

antibiotics (Kurz et al., 2003). It is known to be an opportunistic pathogen which has caused outbreaks of nosocomial infections of varying severity including urinary tract infections (UTIs), respiratory tract infections, bacteraemia, conjunctivitis, endocarditis, meningitis, and wound infections (Su et al., 2003).

Staphylococcus aureus is a gram positive bacterium which was chosen as one of the pathogens to be screened against in this study. *S. aureus* is the most common cause of nosocomial pneumonia and surgical site infections and the second most common cause of bloodstream, cardiovascular, and eye, ear, nose, and throat infections in the United States (Noskin et al., 2005). A major concern has been the emergence of methicillin resistant *S. aureus* strains due to their resistance to nearly all β -lactam antibiotics (Arede et al., 2013).

The fourth pathogen screened against in this study is the fungus *Candida albicans*. *C. albicans* is a commensal fungus which occurs in the gastrointestinal tract and the oral and vaginal mucosa of many, if not all, healthy individuals but is also the most common human fungal pathogen (Kim and Sudbery, 2011). *C. albicans* has the ability to grow either as a unicellular budding yeast or in filamentous pseudohyphal and hyphal forms and it is the hyphal form that, during mucosal infections, invades epithelial and endothelial cells and causes damage (Sudbery, 2011). In immunocompromised individuals *C. albicans* can cause a range of mucosal and systemic infections, including acute pseudomembranous oral candidiasis (thrush), the most common opportunistic infection of HIV-infected patients (Cheng et al., 2003). Immunocompromised individuals may also develop blood stream infections called candidemia which can lead to colonization of internal organs, known as disseminated candidiasis (Kim and Sudbery, 2011).

The aforementioned diseases caused by each of the test microbes chosen in this study along with the increase in antibiotic resistance, gives finding novel antimicrobial compounds a great importance. In this study, the endophyte *P. fuligena* inhibited the growth of *S. marcescens* in the primary screening stage and therefore was chosen to undergo liquid broth fermentation and extraction of any produced metabolites. Along with *P. fuligena*, a bacterial endophyte was found to display inhibitory effects against the test microbes. However, the endophyte was unable to be identified after multiple attempts and in order to save time it was decided not to proceed with further experimentation with the isolate.

4.5 Bioassay-Guided Fractionation

In this study, the antimicrobial activity of *P. fuligena* was assessed using a bioassay-guided fractionation approach. Previous studies have shown success with this method for isolating fungal metabolites (Rosa et al., 2013, Zhao et al., 2012, Ratnaweera et al., 2014). Rosa et al. (2013) reported the identification of the antifungal fatty acids caproic, caprylic, myristic, palmitic, heptadecanoic, stearic, oleic, linoleic and stearic acids from a crude extract of the endophytic fungus *Coniochaeta ligniaria*. Zhao et al. (2012) isolated two antimicrobial compounds from the crude extract of the endophytic fungus *Gliomastix murorum* Ppf8 using a bioassay-guided fractionation approach. They identified the compounds as ergosta-5,7,22-trien-3-ol and 2,3-dihydro-5-hydroxy- α,α -dimethyl-2-benzofuranmethanol which displayed antibacterial activity against five Gram negative and two Gram positive bacteria, and antifungal activity against one fungus. Ratnaweera et al. (2014) also successfully isolated an antibacterial compound with this approach. They isolated the antibacterial compound helvolic acid from an endophytic *Xylaria* sp. which had activity against the Gram-positive bacteria, *Bacillus subtilis* and methicillin-resistant *Staphylococcus aureus*. These studies

reinforce the worth of the bioassay-guided fractionation method as means to isolate bioactive compounds.

In this study, extracts of *P. fuligena* afforded five fractions after HPLC fractionation. Testing of each fraction found two fractions which had bacteriostatic activity against *S. marcescens*. This confirms the finding from the primary screening that *P. fuligena* can inhibit the growth of *S. marcescens*. Fraction 2 (Figure 9) which eluted between 6.5 to 10 minutes displayed bacteriostatic inhibition against *S. marcescens*. Fraction 3 (Figure 9) which eluted between 10 to 13.5 minutes also displayed bacteriostatic inhibition against *S. marcescens*. Neither fraction was bactericidal against the bacterium which coincides with the results from the primary screening. As seen in Figure 7, the growth of *S. marcescens* was reduced rather than prevented by the endophyte. This study appears to be the first report of the fungus *P. fuligena* displaying antimicrobial activity.

The crude extract from the ethyl acetate extraction displayed bactericidal activity against *S. marcescens*. The MBC for the crude extract was 2.5mg/ml which is 20-fold greater concentration than the MIC for the two bacteriostatic fractions (125 µg/ml). This may suggest that at higher concentrations, the two fractions may also be bactericidal. In a study by Radhakrishnan et al. (2011) it was found that the MBC of the compound embelin was at a higher concentration than the MIC was against all microbes tested. For example, embelin had an MIC of 50µg/ml and an MBC of 400µg/ml against the bacteria *Klebsiella pneumoniae* (Radhakrishnan et al., 2011). The compound responsible for the bactericidal activity of the crude extract in this study most likely makes up only a small fraction of the mixture. As a

pure compound it may need less than the MBC concentration of the crude extract in order to be bactericidal by itself.

P. fuligena did not display bactericidal activity during primary screening as no clear zone of inhibition around the endophyte was observed. Instead, the growth of *S. marcescens* only appeared to be reduced. It is possible that the endophyte did not produce sufficient amounts of the bioactive compound that is needed to cause bactericidal activity. The bioactivity displayed by *P. fuligena* supports the notion that endophytes are a promising source of bioactive compounds and shows the potential of Australian plants as sources of these endophytes. Further research of the bioactive compounds of *P. fuligena* could potentially impact on future control of disease caused by *S. marcescens*.

4.6 Future Directions

This study could be continued in many directions. More *P. angustifolium* plants could be sampled in order to gain access to a potentially greater variety of endophytic species which would allow further investigation into the host-endophyte relationship as well as the bioactive potential of isolated endophytes. All of the endophytes isolated in this project could be identified morphologically as well as molecularly which would help to confirm the species matches from BLAST. Other plant species could be sampled in the same area as the *P. angustifolium* plants sampled in this study to observe whether the same endophytes are present in both species. This would help to identify whether the endophytes identified in this study are specific to the host plant rather than the habitat of the host. Further research could be conducted on comparing the endophytes of other parts of the plant (such as the roots or bark) with that of the leaves. Furthermore, roots may have a different variety of

endophytic species which may produce antimicrobial compounds. This would give a better assessment of the overall endophyte species diversity of the plant.

P. fuligena could be regrown in bulk in order to achieve a greater yield of crude extract.

During HPLC a greater number of fractions could be generated within the time that fractions 2 and 3 from this study were eluted in order to isolate a pure compound. Any pure compounds isolated could be identified via NMR spectroscopy or X-ray crystallography. Pure compounds could be tested for MIC and MBC to identify the compound responsible for the results found in this study.

The endophytes in this study could also be screened against other microbes such as MRSA and *Bacillus cereus*. The fractions isolated from *P. fuligena* could also be tested against other pathogens. Any antimicrobial pure compounds isolated from *P. fuligena* could be analysed with cytotoxicity assays to gain a better idea of their potential use in medicine. The bacterial endophyte displaying antimicrobial activity which was not further analysed could be further attempted to be identified and its antimicrobial activity analysed. Research into *P. angustifolium* endophytes could also aim to identify the endophytes anticancer activity.

5.0 Conclusion

Little is known about the endophyte diversity of Australian plants and even less is known about their antimicrobial properties. As such, the results of this study have added to the current pool of knowledge on Australian endophytes.

Many of the isolated endophytes were successfully sequenced in this study which resulted in matches with sequences from GenBank. However, only eight of the 54 isolates were identified down to the species level. This demonstrates the limitations of molecular identification techniques. The isolates not matched to the species level may have been new species of known genera. *P. angustifolium* was found to have more fungal endophytes than bacterial, however, this may not be a good representation of the whole endophytic diversity of the plant.

No host specific relationships were shown to exist between any of the isolated endophytes and their host. However, this study only investigated the leaves of seven plants. Sampling of more plants, leaves or plant tissue type may give different results than this study.

This study also adds to the growing evidence that endophytes are capable of producing valuable antimicrobial compounds. A crude extract from one fungal endophyte (*P. fuligena*) displayed bactericidal activity against the human pathogen *S. marcescens* while two fractions of the crude action displayed bacteriostatic activity. The compounds causing the antibacterial activity could be potential targets for further investigation to determine whether they are of use in the medical arena.

This study demonstrates the potential of *P. angustifolium* as a source of undiscovered endophytic species and antimicrobial compounds. The study may encourage further research into the endophytes of *P. angustifolium* or other Australian plants as sources of undiscovered species and novel antimicrobial compounds. Continued research into endophytes may help to alleviate the problem of antibiotic resistant microbial pathogens.

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Appendices

Appendix A

Media recipes for fungal and bacterial endophytes

Potato Dextrose Agar (PDA)

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

Sensitest Agar (STA)

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

Malt Extract Broth (MEB)

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

Mueller-Hinton Broth (MHB)

Dissolve 8.4g of MHB in 400ml of distilled water. Autoclave at 121°C for 15 minutes.

Appendix B

MIC data

The following tables represent the microdilution trays used for MIC assay. Five fractions collected from HPLC were tested along with the crude extract from the ethyl acetate extraction. Column 11/rows A-D contained the negative control. Column 11/rows E-H contained the contamination control. Column 12/rows A-D contained the positive control. Column 12/rows E-H contained the solvent control.

+ is shown to indicate growth of the bacteria.

- is shown to indicate no growth of the bacteria.

Microdilution tray 1

HPLC fractions	Row	Column	1	2	3	4	5	6	7	8	9	10	11	12
			Conc. µg/ml		1000	500	250	125	62.5	Controls				
1	A	<i>(S. marcescens)</i>	+	+	+	+	+	+	+	+	+	+	+	-
	B	<i>(S. aureus)</i>	+	+	+	+	+	+	+	+	+	+	+	-
2	C	<i>(S. marcescens)</i>	-	-	-	-	-	-	-	-	+	+	+	-
	D	<i>(S. aureus)</i>	+	+	+	+	+	+	+	+	+	+	+	-
3	E	<i>(S. marcescens)</i>	-	-	-	-	-	-	-	-	+	+	-	+
	F	<i>(S. aureus)</i>	+	+	+	+	+	+	+	+	+	+	-	+
4	G	<i>(S. marcescens)</i>	+	+	+	+	+	+	+	+	+	+	-	+
	H	<i>(S. aureus)</i>	+	+	+	+	+	+	+	+	+	+	-	+

Microdilution tray 2

HPLC fractions	Row	Column	1	2	3	4	5	6	7	8	9	10	11	12
		Conc. µg/ml	1000		500		250		125		62.5		Controls	
5	A	<i>(S. marcescens)</i>	+	+	+	+	+	+	+	+	+	+	+	-
	B	<i>(S. aureus)</i>	+	+	+	+	+	+	+	+	+	+	+	-
		Conc. mg/ml	10		5		2.5		1.25		0.625		Controls	
Crude extract	C	<i>(S. marcescens)</i>	-	-	-	-	-	-	-	-	-	-	+	-
	D	<i>(S. aureus)</i>	+	+	+	+	+	+	+	+	+	+	+	-
	E												-	+
	F												-	+
	G												-	+
	H												-	+