Molecular Interactions of Endophytic Actinobacteria in Wheat and Arabidopsis

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Declarations

I certify that this thesis does not contain material which had been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis or in the notes.

Vanessa Michelle Conn

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Abbreviations

µl; ml; l: microlitre; millilitre; litre

ρM; µM; mM; M: picomolar; micromolar; millimolar; molar

½ MS salts: half strength Murashige and Skoog salt medium

ACC: 1-aminocyclopropane-1-carboxylic acid

AM fungi: arbuscular mycorrhizal fungi

Ap: abundance percentage

AUD: Australian dollar

bp: base pairs

CFU: colony forming units

CR: crown rot

CTAB: cetryltrimethylammonium bromide

DGGE: denaturing gradient gel electrophoresis

DNA: deoxyribonucleic acid

dNTPs: dinucleotide triphosphates

Ecc: *Erwinia carotovora* subsp. *carotovora*

EDTA: ethylenediamine tetraacetic acid

ET: ethylene

eGFP: enhanced green fluorescent protein

FAME: fatty acid methyl ester

FHB: Fusarium head blight

Ggt: *Gaeumannomyces graminis* var. *tritici*

GRDC: Grains Research and Development Corporation

HEX: hexachlorofluorescein phosphoramidite

hr: hour

hrs: hours

HR: hypersensitive response

IPTG: isopropyl β-D-thiogalactoside

ISR: induced systemic resistance

JA: jasmonic acid

LB: Luria broth

MeJA: methyl jasmonate

min: minutes

MS: mannitol soy agar

NCBI: National Centre for Biotechnology Information

ng; μg; mg; kg: nanograms; micrograms; milligrams; kilograms

NSW: New South Wales

PCR: polymerase chain reaction

PDA: potato dextrose agar

PGPR: plant growth promoting rhizobacteria

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

RO: reverse osmosis

RT: room temperature

SA: salicylic acid

SAR: systemic acquired resistance

SDS: sodium dodecyl sulphate

sp.: species (singular)

spp.: species (plural)

TBE: tris-borate EDTA

TET: 6-carboxy-2',4,7,7'-tetrachlorofluorescein

TGGE: temperature gradient gel electrophoresis

T-RFLP: terminal restriction fragment length polymorphism

TRF: terminal restriction fragment

UV: ultraviolet

YME: yeast malt extract agar

Abstract

Wheat is the most economically important crop forming one quarter of Australian farm production. The wheat industry is severely affected by diseases, with fungal pathogens causing the most important economic losses in Australia. The application of fungicides and chemicals can control crop diseases to a certain extent, however, it is expensive and public concern for the environment has led to alternative methods of disease control to be sought, including the use of microorganisms as biological control agents. Microorganisms are abundant in the soil adjacent to plant roots (rhizosphere) and within healthy plant tissue (endophytic) and a proportion possess plant growth promotion and disease resistance properties.

Actinobacteria are gram-positive, filamentous bacteria capable of secondary metabolite production such as antibiotics and antifungal compounds. A number of the biologically active endophytes belonging to the Actinobacteria phylum were isolated in our laboratory. A number of these isolates were capable of suppressing the wheat fungal pathogens *Rhizoctonia solani*, *Pythium* sp. and *Gaeumannomyces graminis* var. *tritici*, both *in vitro* and *in planta* indicating the potential for the actinobacteria to be used as biocontrol agents. The aim of this research was to investigate the molecular mechanisms underlying this plant-microbe interaction.

The indigenous microbial populations present in the rhizosphere and endophytic environment are critical to plant health and disruptions of these populations are detrimental. The culture-independent technique Terminal Restriction Fragment Length Polymorphism (T-RFLP) was used to characterise the endophytic actinobacteria population of wheat roots under different conditions. Soils which support a higher number of indigenous microorganisms result in wheat roots with higher endophytic actinobacterial diversity and level of colonisation. Sequencing of 16S rRNA gene clones, obtained using the same actinobacteria-biased PCR primers that were used in the T-RFLP analysis, confirmed the presence of the actinobacterial diversity, and identified a number of *Mycobacterium* and *Streptomyces* species. It was found that the endophytic actinobacterial population of the wheat plants contained a higher diversity of endophytic actinobacteria than reported previously, and that this diversity varied significantly among different field soils.

The endophytic actinobacteria have previously been shown to protect wheat from disease and enhance growth when coated onto the seed before sowing. As the endophytes isolated were recognised as potential biocontrol agents, the impact on the indigenous endophytic microbial population was investigated. Utilising the T-RFLP technique it was established that the use of a commercial microbial inoculant, containing a large number of soil bacterial and fungal strains applied to the soil, disrupts the indigenous endophyte population present in the wheat roots. The hypothesis is that non-indigenous microbes proliferate and dominate in the soil preventing a number of endophytic-competent actinobacterial genera from access to the seed and ultimately endophytic colonisation of the wheat roots. This dramatically reduces diversity of endophytes and level of colonisation. In contrast the use of a single endophytic actinobacteria endophyte inoculant results in a 3-fold increase in colonisation by the added inoculant, but does not significantly affect this indigenous

population.

Colonisation of healthy plant tissues with fungal endophytes has been shown to improve the competitive fitness with enhanced tolerance to abiotic and biotic stress and improved resistance to pathogens and herbivores. In this study the fungal endophyte population of wheat plants grown in four different soils was analysed using partial sequencing of 18S rRNA gene sequences. Sequence anlaysis of clones revealed a diverse range of fungal endophytes. In this diverse range of fungal endophytes a number sequences were highly similar to those of previously known fungal phytopathogens. A number of sequences detected were similar to fungal species previously identified in soil or plant material but not as endophytes. The remaining sequences were similar to fungal species without a known relationship with plants.

Plants have developed an inducible mechanism of defence against pathogens. In addition to local responses plants have developed a mechanism to protect uninfected tissue through a signal that spreads systemically inducing changes in gene expression. In the model plant *Arabidopsis thaliana* activation of the Systemic Acquired Resistance (SAR) pathway and the Jasmonate (JA)/Ethylene (ET) pathway is characterised by the production of pathogenesis-related (PR) and antimicrobial proteins resulting in systemic pathogen resistance. Endophytic actinobacteria, isolated from healthy wheat roots in our laboratory, have been shown to enhance disease resistance to multiple pathogens in wheat when coated onto the seed before sowing. Real Time RT-PCR was used to determine if key genes in the SAR and JA/ET pathways were induced in response to inoculation with endophytic actinobacteria.

Inoculation of wild-type *Arabidopsis thaliana* with selected strains of endophytic actinobacteria was able to ‘prime’ the defence pathways by inducing low level expression of SAR and JA/ET genes. Upon pathogen infection the defence-genes are strongly up-regulated and the endophyte coated plants had significantly higher expression of these genes compared to un-inoculated plants. Resistance to the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* was mediated by the JA/ET pathway whereas the fungal pathogen *Fusarium oxysporum* triggered primarily the SAR pathway.

Further analysis of the endophytic actinobacteria-mediated resistance was performed using the *Streptomyces* sp. EN27 and Arabidopsis defence-compromised mutants. It was found that resistance to *E. carotovora* subsp. *carotovora* mediated by *Streptomyces* sp. EN27 occurred via a NPR1-independent pathway and required salicylic acid whereas the jasmonic acid and ethylene signalling molecules were not essential. In contrast resistance to *F. oxysporum* mediated by *Streptomyces* sp. EN27 occurred via a NPR1-dependent pathway but also required salicylic acid and was JA- and ET-independent.

This research demonstrated that inoculating wheat with endophytic actinobacteria does not disrupt the indigenous endophytic population and may be inducing systemic resistance by activating defence pathways which lead to the expression of antimicrobial genes and resistance to a broad range of pathogens.

Chapter One: Introduction and Literature Review

1.1 The Wheat Industry

1. 1.1 The Australian Wheat Industry

The wheat industry contributes significantly to the Australian economy. Wheat is the dominant grain crop forming one quarter of all Australian farm production. The wheat growing regions of Australia are shown in Figure 1.1. In the 2000 to 2001 season, wheat production covered 12.08 million hectares with 21.2 million tonnes of grain produced (Rathmell, 2001). Wheat accounts for 16% of total farm exports and contributes AUD$8 billion to the Australian economy annually based on 1996 to 1999 figures. Domestic consumption is low due to Australia's low population, leading to approximately 80% of Australian wheat being exported (Rathmell, 2001).

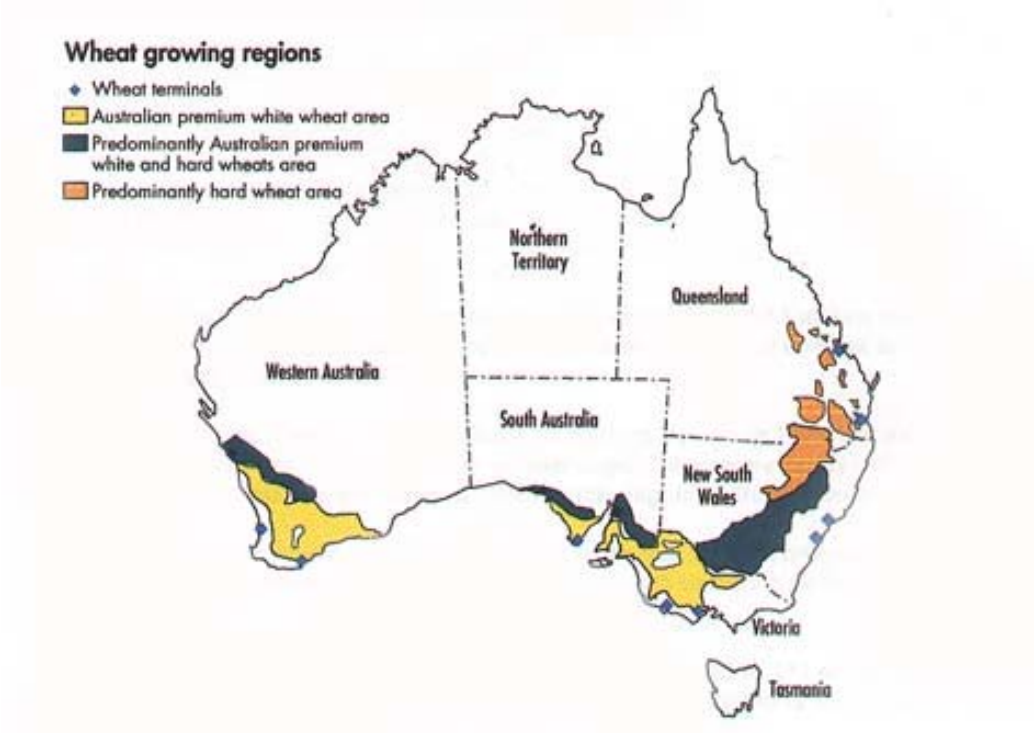


Figure 1.1: Wheat growing regions of Australia (Hogan *et al.*, 2004)

The world population is growing by 160 people per minute and wheat is predicted to be the most important cereal crop in the world (Hoisington *et al.,* 1999). To meet future cereal production demands it is imperative crop yields are increased. A large amount of research and resources are being invested in plant breeding programs to improve disease resistance as the Australian wheat industry suffers serious economic losses due to disease. Six major diseases of wheat in Australia cause an average loss of AUD$288 million annually. Crown rot is the most economically significant disease in Queensland, with an average loss of AUD$23 million annually (CRCTP, 2002). Cereal diseases can be controlled by chemical fungicides and chemicals in some cases, but it is expensive and public concern for the environment and health issues has led to alternative methods of disease control to be sought, such as biological control.

1.1.2 Wheat Growth and Development

The wheat seed germinates when sown in moist soil with the correct temperature conditions. The grain absorbs water through the opening of the hilum, diffuses through the pericarp and seed coat, increasing the size and weight of the grain. The material and energy for germination of the embryo is derived from the scutellum and endosperm. Figure 1.2 shows the anatomy of the seed.

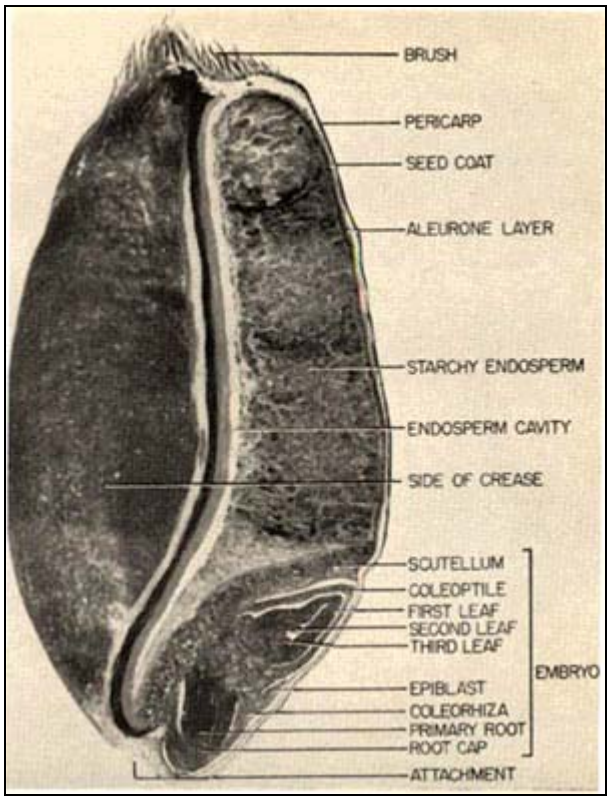


Figure 1.2: Anatomy of a wheat seed (Peterson, 1965).

There are five stages of germination. Firstly the root-sheath (coleorhiza) breaks the pericarp then approximately one day later the primary root breaks through the end of the root sheath. A pair of roots covered by the root-sheath appears next to the primary root and soon after a second pair of seminal roots grow giving rise to the familial pattern of the five seminal roots. At the tip of each root, cell division occurs and the tip is protected by a root-cap. The outer cells of the root-cap are sloughed off and renewed as the root elongates. After the roots have reached several inches in length they develop branches. As the roots are developing the stem is growing

upwards. The stem at this stage consists of a series of nodes. The stem has a leaf attached at each node, and at the lower nodes a small bud develops within the angle formed by the stem and leaf. Each bud is a potential branch.

In the mature plant the seminal roots are slender and have fine branches. Seminal roots can extend downward from one to seven feet into the soil, but four to six feet is common. The adventitious roots are heavier and coarser than the seminal roots but do not grow as deep into the soil. There are six stages of growth and development of the wheat plant after germination, emergence, tillering, and stem extension, heading, flowering and ripening (Figure 1.3).

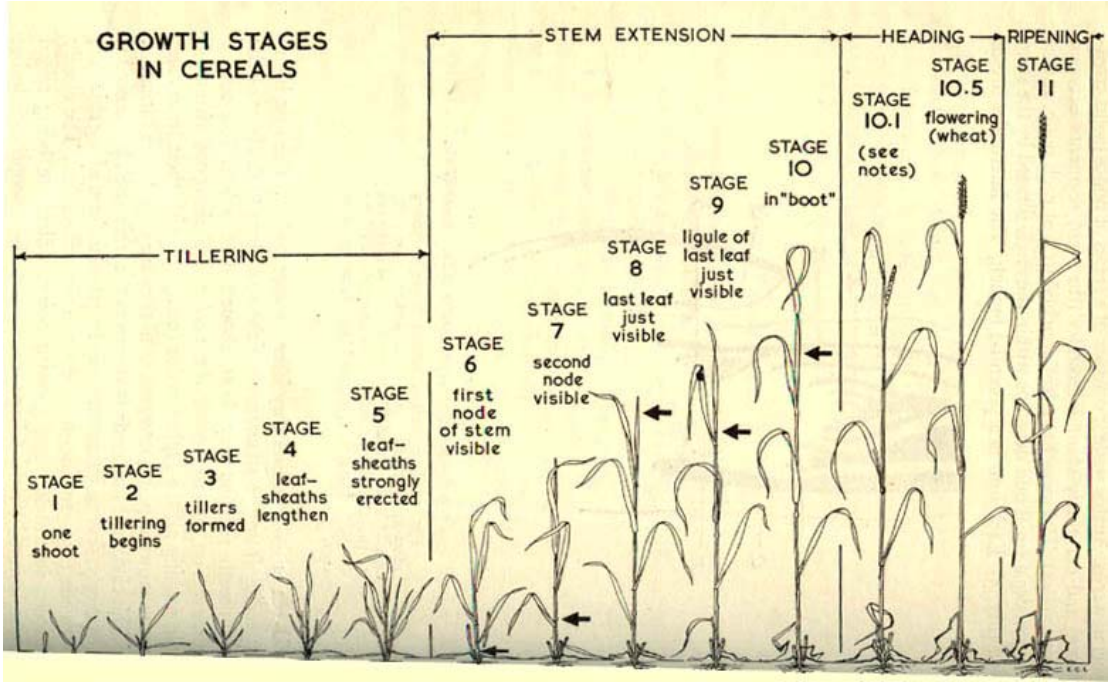


Figure 1.3: The eleven stages of wheat growth (Peterson, 1965)

1.1.3 Avenues of Infection in the Developing Wheat Plant

Fungal, viral and bacterial pathogens can cause serious disease in wheat. Understanding the germination, growth and development of wheat is important as it provides information on the possible routes that pathogens can use to invade the developing wheat plant.

The first avenue for infection occurs when the seed germinates. The seed-coat and a semi-permeable membrane protect the seed from infection; if breaks occur it provides a route for fungi, viruses or bacteria to invade the seed and cause infection (Figure 1.4) (Wiese, 1977).

Figure 1.4: Fungi emerging from wheat seeds (Wiese, 1977).

Microorganisms are present on and within the pericarp, some of which may be disease-causing organisms infecting the seed when the opportunity arises. A break in the protective seed coating is not the only avenue for infection. As the seed germinates the primary roots break through the coleorhiza and root-sheath, providing a route for invading microorganisms. The coleoptile is susceptible to pathogenic

fungi for a short time after which it becomes resistant. The base of the young shoot at the scutellar node and first internode are susceptible to infection also.

Other avenues of infection include the stomata, hydathodes of the leaves, glumes, awns and the developing grains. Stomata and hydathodes are important entry-points for the rusts and bacterial pathogens.

The main infection avenues occur as the wheat seedling is developing. Therefore, a seedling that grows quickly and vigorously is less susceptible to infection than a seed that is slow to germinate, as there is more time for microorganisms to infect the growing seedling.

1.1.4 Cropping Practices

Genetic resistance to root diseases is rare and the agricultural industry has to rely on practices such as crop rotation and soil fumigation to control plant soilborne diseases (Cook *et al.,* 1995). Crop rotation and tillage management have been shown to influence specific soil microbial populations (Sturz and Nowak, 2000). Reduced tillage unlike conventional soil cultivation causes minimum disruption to the soil. Combining reduced tillage along with stubble retention has been shown to increase soil organic matter, decrease soil erosion and improve soil structure (Simpfendorfer *et al.*, 1999).

Cultural practices such as reduced tillage can encourage some pathogens such as *Fusarium graminearum, F. culmorum* and *F. avenaceum,* which cause common root rot and *Cephalosporium gramineum,* Ggt, *Pythium* spp., and *Rhizoctonia solani*

(Roget and Rovira, 1991; Bockus and Shroyer, 1998). This is due to the crop residues in soil maintaining the inoculum of the pathogens while the fields are left fallow or sown with a non-host break crop. Whereas the incidicence of disease is reduced with tillage as it disrupts the fungal hyphae in the soil.

It has also been shown that after long periods (10 or more growing seasons) stubble retention can induce disease suppression of pathogens such as *Rhizoctonia solani* and *Gaeumannomyces graminis* var. *tritici* (Ggt) (Raajimakers and Weller, 1998). The induced disease suppression is thought to occur from the proliferation of indigenous microorganisms in the soil, some of which are antibiotic and antifungal producers that prevent the outbreak of single group of organisms, such as pathogenic fungi.

1.1.5 Diseases of Wheat

Wheat is susceptible to a large number of fungal, bacterial, viral and nematode pathogens that can cause serious disease and dramatically reduce crop yields. Fungal pathogens cause many of the most serious crop diseases (Tucker and Talbot, 2001) and this literature review will focus on three fungal diseases prevalent among Australian wheat crops.

1.1.5.1. Fungal Pathogens

1.1.5.1.1 Take-All Disease

The most economically damaging disease to wheat in Australia is caused by

the fungus *Gaeumannomyces graminis* var. *tritici* (Ggt), which has been given the name ‘take-all’. The term was used in Australia over 100 years ago to describe a severe seedling blight that destroyed entire fields (Lipps, 1996). Take-all, also known as root rot, can cost the Australian farmers up to $100 million a year when the disease outbreak is severe. It is the most-studied root disease of wheat, yet it is still the most damaging disease of wheat world wide (Cook, 2003).

Ggt is a poor saprophyte and does not survive without a plant host. In the soil, native microorganisms decompose the root and basal stem residues of the wheat plant effectively killing the fungus. The reduced tillage method favours survival of the take-all fungus as it leaves large infested plant fragments in the soil (Bockus and Shroyer, 1998). Wheat plants become infected when the roots encounter residues or living plants infected with the take-all fungus (Lipps, 1996). The fungus spreads out from an infected root surface and spreads to another root through the soil by ‘runner hyphae’. The fungus proceeds to block the xylem causing the root to blacken and die (Figure 1.5). The vascular blockage causes the plant to become water-stressed soon after heading and produce empty bleached heads (Cook, 2003).

Take-all is more severe in lighter soils that have a higher pH and low fertility (Lipps, 1996). As a rule the earlier the infection occurs, the worse the disease and yield loss (Lipps, 1996). Take-all is reduced by maintaining adequate levels of nitrogen, phosphorus and potassium in the soil for crop growth. Environmental factors also affect the persistence of the take-all fungus; a wet spring will favour fungal persistence and a dry summer can reduce the fungal load (GRDC, 2002).

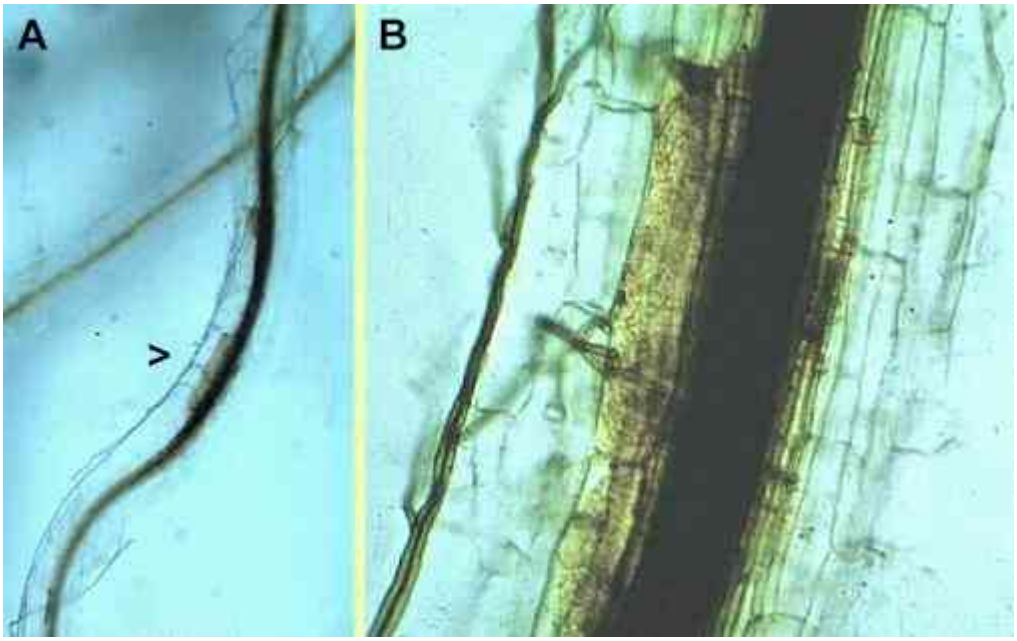


Figure 1.5: (A). Low power micrograph of a wheat root infected by *G. graminis*, showing conspicuous vascular discolouration and dark runner hyphae of the fungus on the root surface. (B) close-up of the region shown as arrowhead in Fig. A, showing invasion of the root cortex, some browning reactions in the inner cortex and phloem, and intense blockage of the xylem by darkly pigmented vascular gels (Deacon, 2001).

Currently there are no cultivars resistant to take-all and fungicides are ineffective or not economical (Cook, 2003). Crop rotation is the most successful method of controlling take-all with a 72% increase in wheat yield compared to the application of fungicide (8%), microbial antagonists (6%), chloride (4%) and fumigation (7%) (Gardner *et al.,* 1998). Crop rotation with two years between susceptible crops is recommended as are practices such as tillage, nitrogen fertilisers and chemical and biological seed treatments (Weller *et al.,* 2002).

Interestingly ‘induced suppression’ in take-all soils can occur. ‘Induced

suppression' is a natural phenomenon where the soil converts from a favourable environment for take-all to a suppressive one due to the microbial activity. Spontaneous take-all decline has been known to occur when a severe outbreak of take-all is followed by continuous cropping of wheat or barley (Cook *et al.*, 1995). Studies have indicated that the fluorescent *Pseudomonas* spp. present in the rhizosphere inhibit the take-all pathogen. Some *Pseudomonas* spp. have been shown to produce the broad spectrum antibiotic 2-4-diacetylphloroglucinol (2-4-DAPG) which has been implicated in take-all inhibition (Weller *et al.*, 2002). However, Cook *et al.* (1995) have shown that *Pseudomonas fluorescens* can account for 50-90% of the take-all suppression by the production of the antibiotic phenazine-1-carboxylate (PCA). This was shown by the inability of Phenazine-deficient (Phz-) mutants to inhibit Ggt *in vitro*, but, when phenazine production was restored so was the take-all inhibition (Cook *et al.*, 1995). The 'induced suppression', however, probably occurs from a number of antibiotic producing *Pseudomonas* spp. and possibly other microorganisms.

1.1.5.1.2 *Rhizoctonia solani*

Rhizoctonia solani and *R. oryzae* cause root rot disease in wheat. *R. solani* has been estimated to cost the southern Australian wheat industry AUD$30 million annually (Harvey and Hawke, 2002). This fungal pathogen can survive in soil and crop residues and is favoured by reduced tillage systems (Bockus and Shroyer, 1998; Cook, 2001). The fungi cause plants to become stunted, maturity is delayed, roots rot, seedlings usually include a dark brown root terminus that tapers to a fine point two to

three inches from the crown or seed. The mycelium of the fungus is present in the rotted tissues and root stubs near the crown (Dickson, 1956). Chemicals such as the general toxins mercurous chloride and organic mercury have been used to control the disease. Cultural control of root rot includes crop rotation with legumes, rapeseed, potatoes and crops other than grass species to reduce disease severity (Dickson, 1956). Severity can also be reduced greatly by soil disturbance methods such as conventional tillage (Rovira, 1986). Crop rotation is limited by the fact that *Rhizoctonia solani* infects over 500 plant species, making it one of the most common plant pathogens. Rhizoctonia disease in sugar beet roots has also been controlled using isolates of *Candida valida*, *Rhodotorula glutinis* and *Trichosporon asahii* that were able to colonise the sugar beet roots, promote growth and protect the plant from *R. solani* (El-Tarabily, 2004). This indicates the potential of biocontrol agents to be used in the control of Rhizoctonia disease in wheat crops.

1.1.5.1.3 *Fusarium* spp.

Fusarium head blight (FHB) and crown rot (CR) cause browning and rotting of the roots and crown in wheat. FHB is predominantly caused by *Fusarium graminearum* while CR is caused by *Fusarium pseudograminearum* (Akinsanmi *et al.*, 2004). Outbreaks of FHB can lead to enormous losses. A severe outbreak of FHB was seen in NSW in 1999 with disease incidence in individual crops ranging from 2 to 100% (Manning *et al.*, 2000). The disease often occurs when low tillage and stubble retention practices are in place (Burgess and Swan, 2001). The disease symptoms include small brown to black lesions on the roots and browning of the

crowns. Plants with severe disease on the crown often do not survive whereas plants with moderate disease on the roots and crown tiller sparsely (Watkins, 2003). Crop rotation is used to control the disease, but the fungus can survive in the soil and on crop residues (Burgess and Swan, 2001). Some cultivars such as Sunvale, Sunco and Baxter are tolerant to *Fusarium pseudograminearum*; however there are no resistant cultivars (Burgess and Swan, 2001).

1.1.6 Infection Mechanisms of Soil-Borne Fungal Diseases

Approximately 10% of all known fungal species can cause plant diseases (Kahmann and Basse, 2001). As stated previously, fungal pathogens are responsible for the most damaging crop diseases. This can be attributed to the fungi being able successfully compete in the highly populated rhizosphere, being able to locate and recognise the host plant root surface then penetrate the root and colonise internal plant tissue. Understanding the underlying molecular mechanisms of fungal infection will help in understanding how to control debilitating fungal diseases but also may provide clues to how beneficial microorganisms internally colonise healthy plants.

For fungal pathogens to effectively parasitise a host plant, penetration of the root is critical. Morphogenetic events preceding infection often depend on specific plant chemical and physical signals, and are a prerequisite for a particular mode of penetration (Mendgen *et al.,* 1996). Different methods can be used by fungi to penetrate the root surface including the formation of appressoria, which are

specialised infection structures that penetrate the plant cuticle and epidermal layer by generating high internal turgor (Kahmann and Basse, 2001). The pathogen *Rhizoctonia solani* can penetrate the root directly without a discrete appressorium but from an infection cushion (Dean, 1997). Fungal pathogens can also gain entry into the plant through natural openings like the stomata (Dean, 1997; Kahmann and Basse, 2001). *Fusarium oxysporum* and *R. solani* accumulate hyphae that may form infection cushions before individual hyphae penetrate with minor modifications of their morphology (Mendgen *et al.,* 1996). *F. oxysporum* f. sp. *vasinfectum* produces a net-like mycelium on the surface of the root tip from which penetration hyphae develop. These hyphae penetrate epidermal walls directly and subsequently colonise the tissue by intra- and intercellular growth (Mendgen *et al.,* 1996).

Colletotrichum spp. have a range of strategies that are used to colonise plants. Some species penetrate the host tissue through wounds and stomatal pores, destroying the host tissue immediately (Horowitz *et al.,* 2002). Other species form appressoria which penetrate the cuticle and grow in the subcuticular spaces asymptomatically for several days after which necrotrophic hyphae develop and invade the host cells destroying them (Horowitz *et al.,* 2002). One other mode of infection involves the fungi breaking the cuticle and epidermal cell wall establishing biotrophic hyphae that can interact with the host cell but do not kill the cell (Horowitz *et al.,* 2002).

Localised production of cell wall degrading enzymes by the fungal pathogens often assists in the penetration of the root. Melanin has been shown to be important for penetration as melanin deficient mutants are both unable to form melanized appressoria and are apathogenic (Mendgen *et al.,* 1996). A number of pathogen genes

expressed during pathogenesis-related development have also been identified and are involved in initial host contact, response to plant defense, acquiring nutrition and in the necrotic phase (Kahmann and Basse, 2001).

Chemical signals such as potassium and calcium ions, simple sugars, arolein, pH gradients and temperature shifts are involved in the formation of appressorium. Other major signals provided by the host are hydrophobicity, hardness, components of the plant surface and topographical properties (Mendgen *et al.*, 1996).

Preventing an invading pathogen from recognising plant signals that initiate the formation of appressorium or other infection mechanisms may prevent the pathogen entering the plant and causing disease.

1.2 Plant-Microbe Populations

Microorganisms can have serious effects on plant health. While some microorganisms are pathogenic, a subset of microorganisms are beneficial to plant health and live in a symbiotic relationship. Such beneficial microorganisms include the mycorrhizal fungi which enable plants to acquire phosphorous and other minerals from the soil and nitrogen-fixing Rhizobia bacteria found within leguminous plants.

1.2.1 Rhizosphere Microorganisms

The rhizosphere is the zone immediately adjacent to the plant root where root exudates are released. This is a nutrient-rich environment due to the release of sugars,

amino acids, organic acids, isoflavanoids, plant hormones and enzymes (Pierson and Pierson, 2000). This high concentration of nutrients makes the rhizosphere a site of intense and complex microbial activity. Rhizosphere microorganisms are involved in the decomposition process and in the cycling of nutrients in soil plant systems (Germida *et al.,* 1998). Rhizosphere microorganisms also play a significant role in plant health with some being deleterious and some beneficial while others seem to have no effect at all.

Deleterious rhizobacteria can cause growth retardation, wilting, necrotic reactions, distortions of leaves and roots or stunting of plants (Schippers *et al.,* 1987). Beneficial rhizosphere microorganisms include symbionts (rhizobia, some actinobacteria and mycorrhizal fungi) and free-living saprophytes that increase the availability of nutrients or plant growth substances to the plant and/or suppress pathogens. A group of these beneficial rhizosphere microorganisms have been classed as plant growth promoting rhizobacteria (PGPR). Many rhizosphere microorganisms produce auxin, ethylene, cytokinins, vitamins and other plant growth substances which can have negative or positive effects on crop production (Schippers *et al.,* 1987).

The population dynamics of the rhizosphere microorganisms can change as the root structure and patterns of root exudation alter during development and as environmental conditions such as water availability and temperature alter. Adding to the complexity of the rhizosphere are the interactions among the members that take place including the competition for nutrients, colonisation sites, scavenging and the production of antibiotics and bacteriocins that inhibit growth. When multiple

bacterial species co-exist they do not colonise in distinct areas as pure cultures but as complex communities known as biofilms and this is thought to be the case also for rhizosphere bacteria living on plant roots (Pierson and Pierson, 2000). Rhizosphere microorganisms may also depend on other members of the community to provide nutrient sources as one bacterium may convert a plant exudate into a form that can be used by another.

Germida *et al.* (1998) conducted a study to isolate and identify bacteria from rhizosphere soil. In this study over 300 rhizobacteria were identified using fatty acid methyl ester (FAME), of the 18 bacterial genera identified, 73% belonged to four genera, *Bacillus* 29%, *Flavobacterium* 12%, *Micrococcus* 20% and *Rathayibacter* 12%. As the community composition differed between field-grown canola and wheat rhizospheres it suggests that a plant-specific relationship for the rhizobacteria exists.

Signalling between rhizosphere microorganisms is known to occur and can affect biosynthetic pathways. A good example of this is the regulation of the phenazine biosynthetic pathway in *Pseudomonas aureofaciens* strain 30-84. Strain 30-84 produces three phenazine antibiotics, phenazine-1-carboxylic acid (PCA), 2-hydroxy-phenazine-1-carboxylic acid (2-OH-PCA) and 2-hydroxy-phenazine (2-OH-PZ) (Wood *et al.,* 1997), which inhibit the take-all pathogen *Gaeumannomyces graminis* var. *tritici*, with 50% to 90% of the inhibition shown to be from the production of the antibiotics (Cook *et al.,* 1995; Pierson and Pierson, 2000). The phenazine biosynthetic pathway is regulated by three distinct systems in strain 30-84. The product of the genes, *phzR* and *phzI*, which are situated next to the phz biosynthetic operon, belong to the LuxR/LuxI family of ‘quorum sensing’ regulating

proteins (Wood *et al.*, 1997; Pierson and Pierson, 2000). The *phzR* gene encodes a transcriptional regulator, which activates *phz* expression in response to accumulation of a diffusible *N*-acylated homoserine lactone (AHL) signal (Pierson and Weller, 1994). The phzI gene encodes an AHL synthase that produces a diffusible signal hexanoyl-homoserine lactone (HHL). Gram-negative bacteria have been shown to produce AHL signals which *P. aurofaciens* can recognise. Pierson and Pierson (2000), tested 700 rhizobacteria for AHL signal production and found that 8% of the isolates produced AHL signals that restored phenazine gene expression in mutant phzI strain, 30-84I. Pierson and Pierson (2000) also found that 6% of the rhizobacteria were able to inhibit phenazine biosynthesis. This demonstrates the complex nature of the rhizosphere where members of the community can have both a positive and negative influence on bacterial gene expression. If successful manipulation of rhizosphere microorganisms to improve plant growth and health is to occur it is important to determine the dominant species interacting with different plant species or cultivars.

1.2.1.1 Plant Growth Promoting Rhizobacteria

Plant growth promoting rhizobacteria (PGPR) increase plant growth indirectly either by the suppression of diseases caused by major or minor pathogens, by associative nitrogen fixation, solubilising nutrients such as phosphorous, promoting mycorrhizal function, regulating ethylene production in roots, releasing phytohormones and decreasing heavy metal toxicity (Whipps, 2001). A key feature of all PGPR, irrespective of mode of action, is the colonisation of plant root to some

extent. Soilborne pathogens inhibited to some degree by PGPR include *F. oxysporum*, *Gaeumannomyces graminis*, *Phytophthora* spp., *Pythium* spp., *R. solani*, *Sclerotium rolfsii*, *Thielaviopsis basicola* and *Verticillium* spp. (Kloepper *et al.*, 1999).

The supplementation of crops or soils with PGPR was first reported in the 1950s (Zehnder *et al.*, 2001). PGPR were first used to improve crop fertility by increasing the amount of nitrogen available to the plant. The most efficient nitrogen-fixing strains belong to the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Allorhizobium* (Bloemberg and Lugtenberg, 2001). PGPR are now used as biological control agents for the suppression of soilborne pathogens (Zehnder *et al.*, 2001).

1.2.1.2 Biological Control

Biological control refers to the suppression of phytopathogens with non-pathogenic microorganisms. Over the last century research has shown that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens. Biological control agents have been intensely investigated due to the commercial applications and the environmental concern over use of chemical pesticides.

1.2.1.3 Mode of Action

Biological control can occur through different modes of action including antibiosis, competition for iron through the production of siderophores, competition for colonisation sites and nutrients, induced systemic resistance, inactivation of

pathogen germination factors, degradation of pathogen toxins and parasitism that may involve the production of cell-wall degrading enzymes (Whipps, 2001).

Antibiosis involves production of antifungal metabolites including ammonia, butyrolactones, 2-4-diacetylphloroglucinol, HCN, kanosamine, Oligomycin A, Oomycin A, phenazine-1-carboxylic acid, pyoluterin, pyrrolnitrin, viscosinamide, xanthbaccin and zwittermycin A as well as several uncharacterised moieties (Whipps, 2001). These act as antibiotics killing non-resistant bacteria and fungi.

Biological control can be achieved through competition for iron. When bacteria are growing in conditions where iron is limited they produce a wide range of siderophores. Siderophores are iron-chelating compounds which have a very high affinity for ferric iron. In the rhizosphere siderophores produced by biocontrol agents are able to sequester the limited supply of iron thereby making it unavailable to pathogenic fungi, restricting their growth. Iron competition as a mechanism of biological control can be complicated as some siderophores can only be used by the bacteria that produce them whereas others siderophores can be used by many different bacteria. Iron competition is further complicated by the fact that pyoverdine and salicylate may act as elicitors for inducing systemic resistance (Whipps, 2001).

Parasitism of pathogenic fungal spores by bacteria and especially actinobacteria has been established (El-Tarabily *et al.,* 1997). The production of extracellular enzymes has also been implicated in biological control. The production of β-1-3, β-1-4 and β-1-6 glucanases from a number of actinobacteria isolates had the ability to suppress the fungal pathogens *Phytophora fragariae* var. *rubi*, the cause of raspberry root rot (Valois *et al.,* 1996). Chitinolytic enzymes produced by *Bacillus*

cereus and *Pantoea agglomerans* appear to be involved in the biological control of *R. solani* (Chernin *et al.,* 1995). Insertion of the chitinase gene from *Trichoderma harzianum* into tobacco plants rendered the plants resistant to fungal pathogens demonstrating the effectiveness of chitinase in reducing fungal growth (El-Tarabily *et al.,* 2000). While there are distinct modes of action which result in the biological control of phytopathogens some biocontrol agents work by several modes of action.

1.2.1.4 Commercial Products

In 1985 the first commercial rhizobacteria biological control product became available in the U.S. using the *Bacillis subtilis* A-13 strain and related strains GB03 and DB07 sold under the names Quantum®, Kodiak® and Epic®, respectively (Zehnder *et al.,* 2001). The products are used in combination with seed treatment fungicides to protect the seed against fungal soil pathogens. In China, PGPR biocontrol agents have been in commercial development for over 20 years and referred to as 'yield increasing bacteria' that are applied to over 20 million hectares of crops (Chen *et al.,* 1996).

The first successful field trials with PGPR were conducted in cucumber and showed that seed treatment followed by soil drench application resulted in reduction of bacterial wilt disease symptoms and the control of bacterial angular leaf spot and anthracnose. In Brazil, the strain *Paenibacillus macerans* is being used to increase yield, germination and protect seeds against seed-borne pathogens for wheat and corn crops (Glick *et al.,* 2001). BioYield®, manufactured by Compton, a commercial PGPR inoculant of *P. macerans,* has been applied to a variety of plants where it has

given significant increases in both size and yield of tomatoes and peppers as well as reduction in disease incidence (Glick *et al.*, 2001). In 2001 there were over 80 commercial biocontrol products world-wide (Paulitz and Belanger, 2001). The majority of the products are formulations of either the fungi *Gliocladium-Trichoderma* or the bacterial agents *Pseudomonas* and *Bacillus* (Paulitz and Belagner, 2001). Table 1.1 shows some of the commercial products available. A more comprehensive list is available at the website for Appropriate Technology Transfers for Rural Areas (Dufour, 2001).

The use of rhizobacteria as biocontrol agents has been met with varying degrees of failure which has been attributed mainly to the difficulties of incorporating non-resident bacteria into established and acclimatised microbial communities (Sturz and Nowak, 2000). Limitations of rhizobacteria as biocontrol agents include the instability of the bacterial agents in long-term culture, storage, effectiveness, range of pathogens targeted, ease of use, distribution and cost in comparison to chemical agents and the effects of positive and negative signalling among bacteria (Hoitink and Boehm, 1999; Sturz and Nowak, 2000).

Table 1.1: Commercial biological control agents available (Table adapted from Dufour, 2001).

Beneficial Organism	Trade Name	Manufacturers and Suppliers	Pests Controlled	Type of Action	Country Registered
Agrobacterium radiobacter	Norbac 84-C™ Nogall	New BioProducts	Crown gall caused by *A. tumefaciens*	Antagonist	U.S.
	Galltrol-A™	AgBioChem			
Ampelomyces quisqualis	AQ-10™	Ecogen	Powdery mildew	Hyperparasite	U.S.
Bacillus popilliae	Doom™	Fairfax Biological Laboratory	Larvae of Japanese beetles, Oriental beetles, chafers, some May & June beetles	Gut toxin	
	Agree™ (Turex U.S.)	Certis			
	Mattch™	Ecogen			
	Koni™	Bioved, Ltd			
Fusarium oxysporum nonpathogenic	Biofox C™	SIAPA	*Fusarium oxysporum, Fusarium moniliforme* on basil, carnation, cyclamen, tomato		Italy
	Fusaclean™	Natural Plant Protection			France
Gliocladium spp.	GlioMix™	Kemira Agro Oy	Soil pathogens		Finland
Gliocladium virens	Soil Guard12G™	Certis	Soil pathogens that cause damping off and root rot, esp. *Rhizoctonia solani & Pythium* spp.	Antagonist	U.S.
Pseudomonas cepacia	Intercept™	Soil Technologies	Soil pathogens: *Rhizoctonia, Fusarium, Pythium*		U.S.
Pseudomonas chlororaphis	Cedomon™	BioAgri AB	Leaf stripe, net blotch, *Fusarium* spp., spot blotch, leaf spot, and others on barley and oats		Sweden
Pseudomonas fluorescens	Conquer™	Mauri Foods Sylvan Spawn	*P. tolasii* on mushrooms		Europe, Australia

1.2.2 Endophytes

The definition of an endophyte has evolved over time, the most recent definition is “fungi or bacteria, which for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease” (Wilson, 1995). In an experimental sense endophytes are defined as microbes isolated from surface-disinfected plant tissue or extracted from within the plant, and do not visibly harm the plant (Hallmann *et al.,* 1997).

1.2.2.1 Fungal Endophytes

1.2.2.1.1 AM Fungi

Endophytic fungi have been identified in woody plants, trees, shrubs, ferns and grasses (Saikkonen *et al.,* 1998). The best studied plant-fungal symbiosis is the mycorrhizal association. The most common of these associations is the arbuscular mycorrhizal (AM) association, which is formed between the roots of higher plants and Zygomycete fungi (Harrier, 2001). The types of plants forming this association are quite diverse, including mosses, liverworts, pteridophytes, gymnosperms and angiosperms (Provorov *et al.,* 2002). Approximately 15 species of AM fungi can colonise 225,000 species of plants (Gadkar *et al.,* 2001). This indicates AM fungi have a wide host range. The symbiosis of AM fungi with plants creates an intimate link between the plants and the rhizosphere.

Colonisation of the root system by AM fungi benefits the growth and development of the host plant as the fungi mobilise phosphorous and other minerals from the soil. In return the plant the plant provides fixed carbon to the fungi (Hirsch and Kapulnik, 1998; Smith and Goodman, 1999; Harrier, 2001). This symbiosis also enhances the plant resistance to biotic and abiotic stresses (Hirsch and Kapulnik, 1998; Harrier, 2001).

The process of AM fungal colonisation of host plant roots is characterised by distinct stages involving a series of complex morphogenetic changes in the fungus including spore germination, hyphal differentiation, appressorium formation, root penetration, intercellular growth, arbuscule formation and nutrient transport (Harrier, 2001). The pre-infection stage of AM development involves the germination of fungal spores and growth of the hyphal germ tubes (Harrison, 1999; Provorov *et al.*, 2002). While this can occur in the absence of the plant root, root exudates and volatiles such as CO_2 can stimulate both processes (Harrison, 1999). Root exudates from a host plant can elicit rapid and extensive branching of the hyphae and once the hyphae reach the root surface, attachment structures called appressoria are formed. Appressoria will only form on a host plant, as a signal from the epidermal cell wall is required (Harrison, 1999). Following attachment to the root surface, penetration hyphae develop and enter the root. The penetration of the root can occur by mechanical force in combination with the localised production of cell wall degrading enzymes such as exo- and endoglucanases, cellulases, xyloglucanases and pectolytic enzymes including polygalacturonase (Harrison, 1999). Entry into the plant root is followed by the growth of infective hyphae which pass the epidermis, enter the cortex

and branch to form intercellular mycelium (Provorov *et al.,* 2002). Intercellular hyphae penetrate the cortical cell walls and differentiate within the cell to form highly branched structures known as arbuscules (Harrison, 1999; Provorov *et al.*, 2002). The arbuscule develops within the plant cell and is apoplastic as a plant plasma membrane surrounds it. This interface is the site of phosphate and possibly carbon transfer. The life span of the arbuscule is only a few days; it then collapses, decays and another one forms in the plant (Harrison, 1999).

Plant genes involved in AM development have been identified by blocking mutations, which stop AM development. In the *Myc-1* mutant the development is stopped after appressoria formation with the infective hyphae being aborted immediately after penetrating the epidermis (Provorov *et al.,* 2002). In the *Myc-2* mutants the formation of arbuscules was blocked (Provorov *et al.,* 2002). It has been determined that there are five stages of AM development; Pid the pre-infection stage, Apf the appressoria stage, Img the intercellular mycelium growth, Ard the arbuscule development stage and Myp the mycobiont persistence (Provorov *et al.,* 2002). The Pid and Apf stages have not been genetically characterised yet.

Although the mycorrhizae association is symbiotic, plant defence-like reactions are induced within the root when infected with AM fungi. The defence reactions include modification of the cell walls, synthesis of phytoalexins and accumulation of callose and induction of pathogen-response proteins (Hirsch and Kapulnik, 1998; Provorov *et al.,* 2002). The plant defence reaction is not strong and has a short time span compared to the response elicited upon pathogen invasion, indicating mycorrhizal fungi either do not trigger strong host defence reactions or are

able to suppress the reaction (Hirsch and Kapulnik, 1998).

1.2.2.2 Bacterial Endophytes

Endophytic bacteria originate from the rhizosphere, seeds or plant material (Hallmann *et al.,* 1997). A subset of rhizobacteria may enter the interior of the root by hydrolysing wall-bound cellulose, through auxin-induced tumours, with water flow, through wounds or through lateral branching sites (Hallmann *et al.,* 1997; Siciliano *et al.,* 1998). There may be chemical signals that are required for the bacteria to enter the root and form a symbiotic relationship with the plant, as is the case for the mycorrhizal fungi and rhizobia. Therefore, a subset of endophytes would originate from the rhizosphere while other bacteria may spend their entire lifespan as an endophyte and be passed to the next generation of the plant via the seed. Endophytic bacteria have been isolated from ovules, seeds and tubers from a variety of plants (Sturz and Nowak, 2000). Seed-borne or rhizosphere-derived endophytes may also be plant specific and make up a host-specific component of the endophyte population, whereas opportunistic endophytes derived from the rhizosphere may make up a non-host specific endophytic population that is common among plants. The close relationship between the rhizosphere and endophytes was demonstrated when it was found that in some cases when complementary crops are grown in rotation they can share 70% of the same endophytic bacterial species (Sturz *et al.,* 1998).

Population densities of bacterial endophytes are low and rarely exceed 10^6 CFU per gram of fresh plant tissue, where as pathogens range from 10^7 - 10^{10} CFU (Hallmann, 2001). Endophytic bacterial populations are larger in the roots and less in

the stems and leaves (Lamb *et al.*, 1996). Once endophytic bacteria have entered the interior of the root they can colonise root between the epidermal cells, below collapsed epidermal cells, within epidermal cells and between intercellular spaces in the root cortex. There have been reports of the endophyte *Acetobacter diazotrophicus* living in the xylem apoplast (James *et al.*, 1994; Reis *et al.*, 1994); however this was discounted by Dong *et al.* (1997) who previously shown *A. diazotrophicus* lives in a sugar solution in the intercellular-space apoplast of the stem (Dong *et al.*, 1994). Hallmann *et al.* (2001) and McCully, (2001) provide good reviews on the associations of bacterial endophytes with their host plants and their niches.

Endophytic bacteria have been isolated from the tissues of healthy tomato (*Solanum lycopersicum* L.) (Nejad and Johnson, 2000), potato (*Solanum tuberosum* L.) (Sturz *et al.*, 1998; Sturz *et al.*, 1999; Garbeva *et al.*, 2001; Sessitsch *et al.*, 2001; Sessitsch *et al.*, 2004), wheat (*Triticum aestivum* L.) (Germida *et al.*, 1998; Coombs and Franco, 2003a), sweet corn (*Zea mays* L.) (McInroy and Kloepper, 1995), cotton (*Gossypium hirsutum* L.) (McInroy and Kloepper, 1995), citrus plants (Araujo *et al.*, 2001; Araujo *et al.*, 2002) and carrot plants (Surette *et al.*, 2003).

The endophytic population differs among plant species and cultivars at different field sites and in response to soil contamination (Siciliano *et al.*, 1998; Dunfield and Germida, 2001; Siciliano *et al.*, 2001). It has been suggested that altering the genetic composition of the plants results in altered root exudates. Two endophytes of rice (*Oryza sativa* L.), *Corynebacterium flavescens* and *Bacillus pumilus*, have been shown to have a higher chemotactic attraction to root exudates than bacteria from the rhizosphere (Bacilio-Jimenez *et al.*, 2003). Therefore, a change

in root exudates could alter the endophytic population.

1.2.2.3 Nitrogen-Fixing Endophytes

Nitrogen-fixing endophytes have been isolated from a number of different plants. The nitrogen-fixing endophyte *Acetobacter diazotrophicus* was isolated from sugarcane and has been associated mainly with sugar-rich plants such as sugarcane, sweet potato and Cameroon grass (Dong *et al.,* 1994; Dong *et al.,* 1995; Baldani *et al.,* 1997). Endophytic isolates from four genera: *Herbaspirillum*, *Ideonella*, *Enterobacter* and *Azospirillium* were isolated from the wild rice species, *Oryza officinallis* (Elbeltagy *et al.,* 2001), while six closely related strains of *Serratia marcescens* were isolated from the roots and stems of four different rice varieties (Gyaneshwar *et al.,* 2001). However, the most extensively studied endophytic nitrogen fixing bacteria are the Rhizobia.

Rhizobia (*Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*) are soil bacteria that can form a symbiosis with leguminous plants that produce nitrogen-fixing nodules. The symbiosis has also been important agronomically as crop rotations with legumes can enhance the productivity on non-leguminous crops by enriching the soil with available nitrogen (Hirsch *et al.,* 2001). Rhizobia symbionts are cultivated easily *ex planta* and provide a good model to study plant functions such as signalling, gene expression, cell differentiation, organogenesis and nitrogen and carbon metabolism. Unlike the AM fungi the legume-rhizobia symbiosis is highly specific as the rhizobia nodulate taxonomically defined plant groups (Provorov *et al.,* 2002).

The symbiotic interaction between the rhizobia and the host plant begins when the bacteria colonise the root surface and induce curling of the root hair tips (Schultze and Kondorosi, 1998). The Rhizobia invade the plant tissue via infection threads and traverse the outer cell layers to reach the nodule primordium. Within the infection thread the bacteria multiply but remain confined by the plant cell wall in a differentiated that exists inside the cells of the host plant (Schultze and Kondorosi, 1998; Spaink, 2000). This differentiated form of the bacteria called bacteroids can fix gaseous nitrogen into ammonia or alanine, which is supplied to the host plant and in turn the plant provides the bacteria with nutrients. Nodulation leads to colonisation of plant cells by the invading rhizobia. Though some rhizobia can enter into a symbiosis with more than one host, only certain combinations will result in the formation of nitrogen-fixing nodules. Incompatible associations lead to empty nodules or nodules containing non-fixing bacteroids (Perret *et al.*, 2000).

Like AM fungi, rhizobia recognise the roots of the appropriate host plant and colonise the surface. Flavonoids secreted from the roots and seed activate the rhizobial nodulation genes (*nod* gene induction). More than 4,000 different flavonoids have been identified in vascular plants, and subsets are involved in mediating the host specificity in legumes (Perret *et al.*, 2000). Isoflavonoids enables the rhizobia to distinguish their hosts from other legumes (Hirsch *et al.*, 2001). Specific flavonoids induce *nod* gene expression and rhizobial chemotaxis (Hirsch *et al.*, 2001). Flavonoids are perceived as aglycones, which induce rhizobial *nod* genes by interacting with the gene product of *nodD*, a LysR-type regulator (Hirsch *et al.*, 2001). Other non-flavanoid molecules such as betaines and aldonic acids are also

inducers of *nod* genes, however, at much higher concentrations (Perret *et al.*, 2000). *Nod* genes induce the synthesis of lipo-chito-oligosaccharide Nod factors which induce the early stages of nodule development (Provorov *et al.*, 2002). *NodD* acts as a sensor of the plant signal and as an activator of transcription of nod loci (Perret *et al.*, 2000). *Nod* genes represent a molecular interface between the bacterium and the plant. The *nod* factors are pivotal to the induction of root nodules and various other responses related to the infection process in the host plant (Spaink, 2000).

1.2.2.4 Endophytic Biocontrol Agents.

As endophytic bacteria are in intimate contact with the plant they are an attractive choice as biological control agents. The use of endophytes as biological control agents would reduce or eliminate the problem of rhizobacteria being unable to compete with indigenous microflora and being adversely affected by other members of the rhizosphere community.

Endophytic bacteria have been shown to promote growth and inhibit plant disease. Endophytes are an important source of biologically active compounds, some of which are active against plant pathogens (Strobel, 2003). Sturz *et al.* (1999) found that 61 out 192 endophytic bacterial isolates from potato stem tissues were effective biocontrol agents against *Clavibacter michiganensis* sp. *sepedonicus*. In oak, endophytic bacteria active against the oak wilt pathogen *Ceratocystis fagacearum* have been isolated (Brooks *et al.*, 1994). Sessitsch *et al.* (2004) isolated seven endophytes from potato that were antagonistic to fungal and bacterial pathogens and were considered as promising biocontrol agents. Nejad and Johnson (2000) isolated

endophytic bacteria from healthy oilseed rape and tomato plants that had the ability to improve seed germination, seedling length and plant growth. When the endophytes were used as a seed treatment they were able to significantly reduce the symptoms caused by the vascular wilt pathogens, *Verticillium dahliae* and *F. oxysporum* f. sp. *lycopersici*, which cause disease on oilseed rape and tomato respectively.

As applying bacterial seed treatments prior to planting does not guarantee the establishment of a beneficial endophyte or yield enhancement (Sturz and Nowak, 2000), molecular mechanisms underlying root colonisation, host specificity, growth promotion and pathogen inhibition need to be understood so that endophytes can be successfully manipulated for use as biological control agents.

1.2.3 Techniques used in the study of Endophytes

Bacterial endophytes have been isolated primarily by cultivation-based methods. Over 192 bacterial species have been isolated from internal plant tissues including *Pseudomonas*, *Bacillus*, *Enterobacter* and *Agrobacterium* (Sturz *et al.*, 1999; Sessitsch *et al.*, 2001). Characterisation of endophytic isolates obtained through microbial cultivation techniques is commonly performed by the carbon source utilisation method (BIOLOG) or FAME method. These approaches require the microorganisms to be culturable. As some microorganisms cannot be cultured, molecular methods need to be adopted to characterise endophytic populations.

Techniques such as ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis

(TGGE), single strand conformation polymorphism (SSCP), ITS-restriction fragment length polymorphism (ITS-RFLP) and random amplified polymorphic DNA (RAPD) can give complex profiles that allow comparisons of communities but no phylogenetic information (Kent and Triplett, 2002). A common method now being used to analyse endophytic communities is one based on analysis of a variable portion of the 16S rRNA gene by polymerase chain reaction (PCR) and further analysis by DGGE, TGGE or Terminal Restriction Fragment Length Polymorphism (T-RFLP).

The rRNA genes are highly conserved because of the fundamental role of the ribosome in protein synthesis. The rRNAs are molecules with universal, constant and highly conserved functions that were established at an early stage in evolution and not affected by changes in the organism's environment (Rossello-Mora and Amann, 2001). There are three rRNAs in bacteria which are classified by their sedimentation rates in ultracentrifugation, 23S, 16S and 5S which are 3300, 1650 and 120 bases, respectively (Rossello-Mora and Amann, 2001). The 16S rRNA is most commonly used for phylogenetic analysis due to its length and large sequence database available.

PCR of a variable portion of the 16S rRNA followed by DGGE or TGGE can provide profiles of endophytic communities. The DGGE or TGGE method separates DNA fragments by sequence dependent helix denaturation and the accompanying change in electrophoretic mobility (Muyzer *et al.*, 1993). DNA fragments of the same length but with one or two base pair differences can be resolved on the polyacrylamide gel. DGGE and TGGE have been using to study microbial

populations in the rhizosphere and endophytic environment (Heuer *et al.,* 1997; Heuer *et al.,* 1999; Smit *et al.,* 1999; Duineveld *et al.,* 2001; Garbeva *et al.,* 2001; Smalla *et al.,* 2001).

T-RFLP requires amplification of the 16S rRNA gene with one of the primers being fluorescently labelled, followed by digestion of the PCR product with restriction endonucleases which normally recognise 4 bp sites. The products are separated by electrophoresis with laser detection of the labelled fragments by an automated analyser. Upon analysis, only the terminal end-labelled restriction fragments are detected. The variable fragment lengths can be correlated with data available in the TAP-T-RFLP database (Marsh *et al.,* 2000). T-RFLP has an advantage over DGGE because it is rapid and has the ability to provide semi-quantitative taxonomic information. The major limitation of this technique is that it relies on 16S rRNA sequence information to be available in the database. T-RFLP cannot determine species identity either, therefore, cloning and sequencing of the amplified 16S rRNA fragments should be performed. T-RFLP has been used to study microbial populations and is the favoured technique over DGGE and TGGE (Dunbar *et al.,* 2000; Lukow *et al.,* 2000; Klamer *et al.,* 2002; Blackwood *et al.,* 2003; Egert and Friedrich, 2003; Sessitsch *et al.,* 2004)

Surface sterilisation of plant material before isolation of a microbe is insufficient evidence to denote the bacteria is endophytic and it is now considered that only direct localisation using microscopy can provide such evidence (Gyaneshwar *et al.,* 2001). The green fluorescent protein (GFP) of *Aqurea victoria* can be used as a reporter for bacterial localisation. The GFP protein is a 238 amino

acid peptide that does not require exogenous substrates or co-factors for fluorescence (Chalfie *et al.,* 1994). GFP can be used to tag an endophyte and study the location and life cycle of the endophyte *in planta*.

Elbeltagy *et al.* (2001) tagged the *Herbaspirillum* sp. strain B501 which was isolated from wild rice, *Oryza officinalis*. After inoculating aseptically grown wild and cultivated rice seeds, sections of the rice were observed by fluorescence stereomicroscopy. This revealed that the GFP-tagged *Herbaspirillium* sp. colonised the intercellcular spaces of the shoots and roots of the wild rice but only weakly colonised the cultivated rice species. An endophytic actinobacteria strain, *Streptomyces* sp. EN27, previously isolated from a healthy wheat root in our laboratory was tagged with the *eGFP* gene. The tagged strain was used to inoculate wheat (*T. aestivum* L. cv. Excalibur) seeds and the endophytic colonisation was observed by laser scanning confocal microscopy. The endophytic actinobacteria colonised very early in the plant development with colonisation of the embryo, endosperm, and emerging radicle (Coombs and Franco, 2003b).

1.3 The Actinobacteria

1.3.1 General Characteristics

Actinobacteria are phylogenetically defined as a number of taxa within the high G+C subdivision of the gram-positive phylum, the Actinomycetales (Stackebrandt *et al.,* 1997). Actinobacteria can be characterised as differentiating prokaryotes which exhibit strain specific types of morphological differentiation

including germination of spores, elongation and branching of vegetative mycelium, formation of aerial mycelium, septation of hyphae and spore maturation (Miyadoh, 1997). This morphological variation was previously used to characterise the actinobacteria. With the advent of molecular biology actinobacteria are now characterised by 16S rRNA sequencing and based on the 16S rRNA gene sequencing the order of Actinomycetales is comprised of 102 genera (Figure 1.6) (Embley and Stackebrandt, 1994; Stackebrandt *et al.,* 1997). Actinobacteria often contain plasmids which vary in size and copy numbers, with the most common being 10-40 kb and a copy number less than 30 (Piret and Demain, 1989). The function of the plasmids includes gene transfer, fertility, genomic rearrangements and antibiotic production (Piret and Demain, 1989).

Streptomyces coelicolor, a soil-dwelling actinobacteria, was the first actinobacterium for which the full genome sequence was obtained. *S. coelicolor* has a single linear chromosome of 8,667,507 base pairs and is the largest bacterial genome to be sequenced (Bentley *et al.,* 2002).

The *S. coelicolor* genome sequence reveals that this actinobacterium is well equipped to deal with the complex, dynamic and competitive soil environment. The organism has a complex life cycle, adapting to a wide range of environmental conditions and exploiting a large number of nutrients and has a huge metabolic potential. The genome codes for a predicted 7,825 genes. Many of the genes are predicted to be involved in secondary metabolism (Bentley *et al.,* 2002). Of the putative proteins there is a strong emphasis on regulation, with 965 proteins thought to have a regulatory function.

Figure 1.6: Phylogenetic relationship of 90 genera of actinomycetes based on 16S rRNA sequences (Miyadoh, 1997).

Actinobacteria were first researched due to their role as human pathogens. The genus *Actinomadura* is responsible for foot infections known as mycetoma or madura foot. Actinobacteria primarily have a saprophytic existence within the soil and the populations are influenced by environmental conditions such as humidity, temperature, pH and vegetation (Crawford *et al.,* 1993; Basilio *et al.,* 2003). The thermophilic actinobacteria from a variety of genera such as *Saccharomomospora*, *Saccharomopolyspora*, *Thermoactinomyces*, *Thermobifida* and *Thermomonospora* are involved in important environmental processes such as the decomposition of organic materials in soil including lignin, starch, chitin and other recalcitrant polymers (Crawford *et al.,* 1993; Heuer *et al.,* 1997; Song *et al.,* 2001). Relatively few actinobacteria are phytopathogenic and actinobacteria have been shown to be quantitatively and qualitatively important in the rhizosphere in improving plant growth and protecting the roots from pathogenic fungi (Crawford *et al.,* 1993)

1.3.2 Secondary Metabolites

The principal reason behind the actinobacteria having such important roles in the soil and in plant relationships comes from the ability of the actinobacteria to produce a large number of secondary metabolites, many of which possess antibacterial activity. Actinobacteria produce approximately two-thirds of the known antibiotics produced by all mircoorganisms. The genus *Streptomyces* produces nearly 80% of the actinobacterial antibiotics, with the genus *Micromonospora* producing one-tenth as many as the *Streptomyces* (Kieser *et al.*, 2000). In addition to the production of antibiotics the actinobacteria produce many secondary metabolites with

a wide range of activities. Activities of the secondary metabolites include antifungal agents that degrade cell walls and inhibit the synthesis of mannan and β-glucan enzymes (Nolan and Cross, 1988), antiparasitic agents (Goetz *et al.*, 1985) and insecticidal agents (Burg *et al.*, 1979). Figure 1.7 shows the range of activities of secondary metabolites of actinobacteria. Actinobacteria produce a number of plant growth regulatory compounds, some of which have been used commercially as herbicides. Not all secondary metabolites are anti-microbial. Others are enzyme inhibitors, immunomodulators and antihypertensives (Franco and Coutinho, 1991). The actinobacteria produce over 60% of secondary metabolites produced by microorganisms, with *Streptomyces* accounting for over 80% (Kieser *et al.*, 2000).

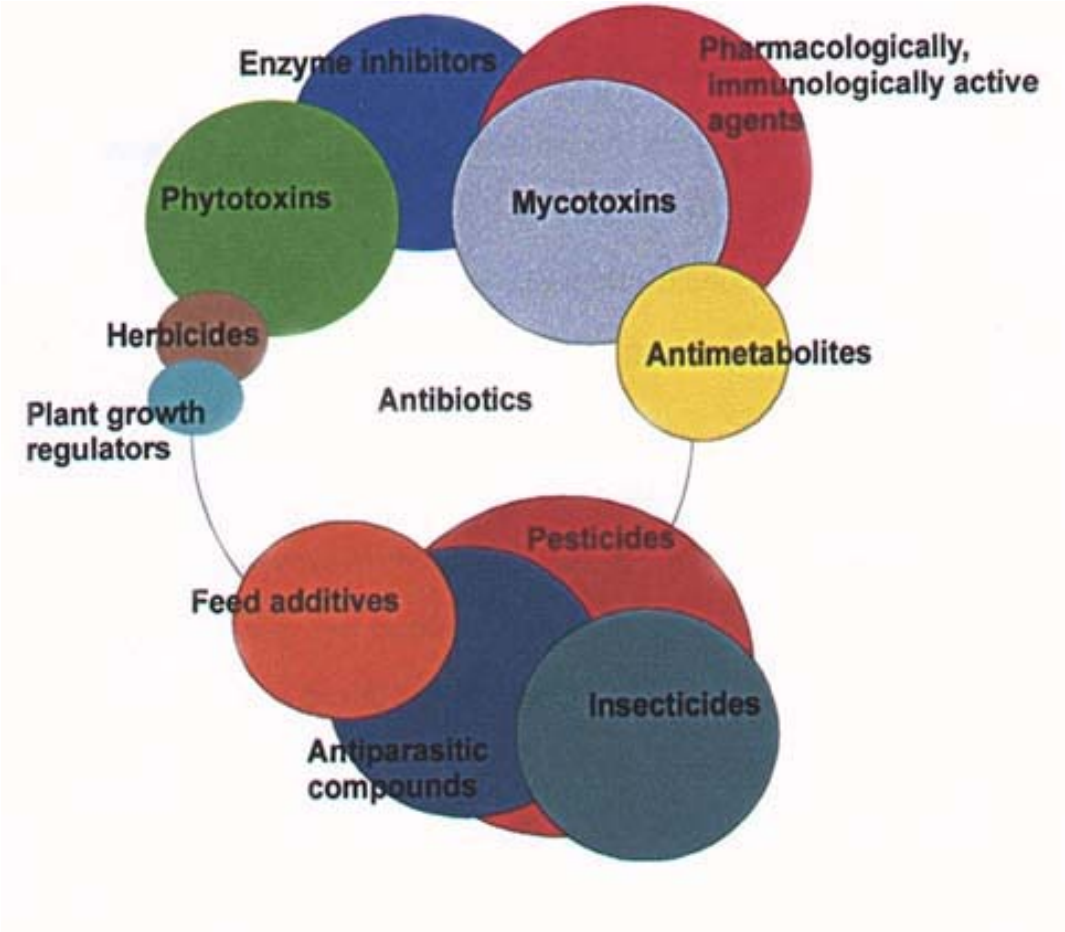


Figure 1.7: Bioactive secondary metabolites produced by actinobacteria (Berdy, 1989).

1.3.3 Plant-Associated Actinobacteria

In some cases Actinobacteria form a pathogenic relationship with plants. *Streptomyces scabies* is a soil-borne actinobacterium that is the principal causal agent of scab diseases, which affect a variety of underground tuberous vegetables such as potato (Schottel *et al.,* 2001). *S. scabies* produces thaxtomin, a family of phytotoxins (Neeno-Eckwall and Schottel, 1999), that induce the development of necrotic lesions in potato (Loria *et al.,* 1995). There is a 100% correlation between pathogenicity and the ability to produce thaxtomin (Neeno-Eckwall and Schottel, 1999). Scab suppressive soils have been identified and it has been found that the lenticels on these tubers are colonised by *Streptomyces* (Schottel *et al.,* 2001). Suppressive strains of *Streptomyces* isolated from a naturally scab suppressive soil produced antibiotics that inhibited *S. scabies in vitro* (Neeno-Eckwall and Schottel, 1999).

Streptomyces species have also been implicated in the biological control of a number of other pathogens. *S. ambofaciens* inhibited *Pythium* damping-off in tomato plants and Fusarium wilt in cotton plants (Yuan and Crawford, 1995). *S. hygroscopius* var. *geldanus* was able to control Rhizoctonia root rot in pea plants and the inhibition was due to the production of the antibiotic geldanamycin (Yuan and Crawford, 1995). *Streptomyces lydicus* WYEC108 inhibited *Pythium ultimum* and *R. solani in vitro* by the production of antifungal metabolites (Yuan and Crawford, 1995). Table 1.2 shows a number of other actinobacteria that are biocontrol agents for phytopathogenic fungi.

Table 1.2: Actinobacteria Biocontrol agents for phytopathogenic fungi*.

Actinobacteria	Pathogen	Host Plant
Streptomyces netropis	*Verticillium* sp.	Cotton
Actinomadura sp.	*Phytophthora cinnamomi*	Snapdragon
Micromonospora carbonacea	*Phytophthora cinnamomi*	Banksia
Micromonospora globosa	*Fusarium udum*	Cotton
Actinoplanes missouriensis	*Phytophthora megasperma*	Soy Bean
Actinoplanes utahensis	*Phytophthora megasperma*	Soy Bean
Amorphosporangium auranticolor	*Phytophthora megasperma*	Soy Bean
	Phytophthora megasperma	Soy Bean
Micromonospora sp.	*Pythium ultimum*	N/A
Actinoplanes sp.	*Pythium aphinidermatum*	N/A

*Table adapted from El-Tarabily *et al.* (1997).

Basal drop disease is caused by either *Sclerotinia minor* or *S. sclerotiorum* and is the most serious and common disease of lettuce (El-Tarabily *et al.*, 2000). El-Tarabily *et al.* (2000) isolated 94 *Streptomyces* and 35 non-*Streptomyces* species from the rhizosphere of lettuce. Of their isolates, *Streptomyces viridodiaticus* and *Micromonospora carbonacea* significantly reduced the growth of *S. minor in vitro*. The actinobacteria produced high levels of chitinase, β-1-3-glucancase and an antifungal compound and caused extensive plasmolysis and cell wall lysis of the pathogen. This is not the first report of an actinobacteria producing chitinolytic enzymes as Singh *et al.* (1999) used a chitinolytic *Streptomyces* sp. to control cucumber wilt caused by *Fusarium oxysporum*.

1.3.4 Endophytic Actinobacteria

The first endophytic actinobacteria to be identified and studied were the *Frankia* species. *Frankia* are nitrogen-fixing actinobacteria that form actinorrhizae with eight families and over 200 species of angiosperms (Lechevalier, 1989; Provorov *et al.,* 2002;). This symbiosis is closest to the legume-rhizobia in terms of evolution, structure and function (Provorov *et al.,* 2002). While it had been well established that *Frankia* formed a symbiotic relationship with angiosperms it was not until the first electron micrograph was made that *Frankia* was identified as an endophyte and an actinobacterium (Lechevalier, 1989).

In vitro Frankia form branching vegetative hyphae which bear characteristic multilocular sporgania filled with non-motile spores and there is no aerial mycelium. *Frankia* enters the root system either through a root hair or directly through the epidermis. Once inside it invades the cortical tissues, passing from cell to cell, sometimes invading the plant cell walls. Vesicles, round or club-shaped structures, are the site of nitrogen fixation. Senescent nodule tissue decays, returning the endophytic propagules to the rhizosphere where they can persist for long periods (Lechevalier, 1989).

Since the isolation of *Frankia* a number of other biologically active endophytes and root-colonising microorganisms belonging to the actinobacterial phylum have been isolated or detected. The most common endophytic actinobacterium isolated from surface-sterilised plant tissue is *Streptomyces* (Sardi *et al.,* 1992; Taechowisan *et al.,* 2003; Coombs and Franco, 2003a). Sardi *et al.* (1992) isolated 49 actinobacteria from a range of plant species with approximately 96% of

isolates belonging to the genus *Streptomyces*. The remainder belonged to *Norcardia*, *Micromonospora* and *Streptosporangium*. Taechowisan *et al.* (2003) isolated 330 actinobacteria from 26 different plant species and the majority were *Streptomyces* spp., with the remainder identified as *Microbispora* spp., *Nocardia* spp. and *Micromonospora* spp.

Streptomyces lydicus WYEC108, a root-colonizing actinobacterium capable of mycoparasitism of fungal root pathogens and excretion of anti-fungal metabolites in the rhizosphere is capable of increasing root nodulation frequency in pea (*Pisum sativum*) (Tokala *et al.*, 2001). *Actinoplanes missouriensis*, isolated from surface sterilised lupin roots, was found to be an antagonist of *Plectosporium tabacinum*, the causal agent of lupin root rot in Egypt (El-Tarabily, 2003). The inhibition of *P. tabacinum* appears to be through the production of chitinase and the ability of *A. missouriensis* to degrade the hyphae of *P. tabacinum in vitro* (El-Tarabily, 2003).

Population-based studies of endophytic actinobacteria using cultivation independent methods were carried out in potato by Sessitsch *et al.* (2001). Characterisation of the endophytic actinobacteria in three potato varieties was performed by analysis of the 16S rRNA PCR using actinobacteria-specific primers followed by denaturing gradient gel electrophoresis (DGGE). The results indicated that several *Streptomyces* species of the *S. scabies* subgroup were present.

In our laboratory, a number of endophytic actinobacteria were isolated by culture-dependent methods, with the major genera being *Streptomyces*, *Microbispora*, *Micromonospora* and *Nocardioides* (Coombs and Franco, 2003a). A number of these isolates were capable of suppressing fungal pathogens of wheat including *Rhizoctonia*

solani, *Pythium* spp. and *Gaeumannomyces graminis* var. *tritici*, *in vitro* and *in planta* indicating their potential use as biocontrol agents (Coombs, 2002; Coombs *et al.*, 2004).

1.4 Plant-Microbe Molecular Interactions

1.4.1 Plant Genetic Factors

The plant genotype affects the type of microbial associations that can be formed. Root exudates from the plant alter according to genotypic differences; therefore the plant genotype can affect the microbial population supported in the rhizosphere. Likewise, the endophytic population is influenced by the plant genotype in terms of colonisation and interaction of microbes with the plant host.

It is well known that some endophytes, such as rhizobia, have a defined host range. Variation in the host specificity of rhizobia has been reviewed by Smith and Goodman (1999) where they discuss host specificity at two levels, quantitative and qualitative. In relation to qualitative host specificity the interaction is either compatible or incompatible. Compatible bacteria infect plants and form nitrogen-fixing nodules and the strength of this interaction can be measured. With incompatible bacteria early infection events may occur, but the nodules are either not formed or are aborted. The rhizobium strain, sym-2, interacts with a specific gene, *nodX*, and the gene product acetylates a lipo-oligosaccharide nodulation factor specifically to bring about a compatible interaction within the host (Smith and

Goodman, 1999).

The effect of host genotype on the strength of compatible interactions in four alfalfa cultivars with *R. meliloti* strains has been investigated by measuring the plant respiration, nitrogenase activity, H_2 evolution, yield, number of nodules per plant and average weight per nodule. The study revealed significant cultivar/strain interactions with some host genotypes having stronger interactions with the rhizobium than others (Smith and Goodman, 1999). Most research has gone into manipulation of rhizobium strains to improve nitrogen fixation, however, as the plant genotype can affect this interaction more research into the role of the plants in the interaction needs to be explored. Similar situations then would be expected of other microbe plant interactions. Like rhizobia, AM fungi, form a beneficial symbiosis with plants. In contrast to rhizobia, AM fungi have a broad host range. AM fungi are chemotactically attracted to the host root exudates and it has been found that development of appressoria will only occur on a host plant. A signal, located in the epidermal cell wall, confers host specificity (Harrison, 1999).

The rhizosphere population is affected by plant genotype. It has been suggested that altering the plant genome can alter root exudate composition and hence the microbial population supported in the rhizosphere. Indeed, it has been discovered that transgenic canola plants have a different rhizosphere and endophytic population compared to the non-engineered plants (Siciliano *et al.*, 1998). Dunfield and Germida (2001) investigated the effect of genetically modified plants on the rhizosphere and endophyte populations, supported the fact that the plant genotype does affect these populations. Reiter *et al.* (2002) investigated the response of

endophytic bacterial communities in potato to infection with *Erwinia carotovora* subsp. *atroseptica*. The results of the study indicated that plant stress, due to infection, increased the diversity in the infected plant compared to the uninfected control plants. It was also found that pathogen stress had a more significant effect on the endophytic community than the plant genotype (Reiter *et al.*, 2002).

1.4.2 Growth Promotion

A number of bacterial endophytes have been shown to promote plant growth. The endophytic actinobacteria isolated in our laboratory were able to promote the growth of wheat (Coombs, 2002). Sturz *et al.* (1995) found that 10% of bacterial endophytes isolated from potato tubers promoted plant growth. Sturz *et al.* (1998) also found that in a crop rotation with clover and potatoes, 21% of the endophytic bacterial isolates were able to promote plant growth. The mechanism of plant growth promotion may be through the production of plant growth hormones, the inhibition of minor or major pathogens or nitrogen fixation in the case of nitrogen-fixing endophytes such as *Acetobacter diazotrophicus* (Sevilla *et al.*, 2001).

The plant hormones auxin and cytokinin have profound effects on growth and differentiation. Auxin (indoleacetic acid) plays an important role in cell division, cell elongation and differentiation, and in processes such as meristem maintenance, root elongation, lateral root development and senescence (Moller and Chua, 1999). Many plant-associated bacteria synthesise auxin. While the production of auxin by phytopathogenic bacteria has been implicated in the induction of plant tumours it has

also been linked to plant growth promotion by beneficial rhizobacteria. Phytopathogenic bacteria such as *Agrobacterium tumefaciens* and *Pseudomonas syringae* synthesise auxin from tryptophan via the intermediate indole-acetamide, whereas beneficial bacteria have been found to synthesis auxin mainly through an alternate pathway via indolepyruvic acid (Patten and Glick, 2002).

Patten and Glick (2002) investigated the link between auxin production by plant growth promoting rhizobacteria and plant growth promotion, specifically root elongation. The plant growth promoting bacterium *Pseudomonas putida* GR12-2 enhances seedling root growth in canola and tomato. *P. putida* GR12-2 overproduces auxin, synthesises siderophores and produces 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which lowers growth inhibiting levels of ethylene. It was found that the production of auxin increases canola root length on average 35% to 50% by comparing *P. putida* GR12-2 inoculated plants to plants inoculated with an auxin-deficient strain of *P. putida* GR12-2. Low levels of auxin stimulate primary root elongation while high levels of auxin secreted by bacteria can stimulate the formation of lateral and adventitious roots. In addition, the production of the enzyme ACC deaminase is also important. ACC deaminase hydrolyses plant ACC, the precursor of ethylene, thereby reducing the levels of ethylene which can inhibit plant growth (Penrose *et al.,* 2001). Therefore it is most likely that auxin and ACC deaminase function together to stimulate root elongation.

The rapid establishment of roots, either by elongation of the primary roots or by proliferation of lateral and adventitious root is advantageous for a number of reasons. Firstly, it increases the ability of the seedlings to anchor in the soil and to

obtain water and nutrients from the environment, enhancing their chances for survival (Patten and Glick, 2002). Secondly, the rapid elongation of roots minimises the opportunity for pathogen infection because the root quickly passes through the pathogen court allowing pathogens, especially fungi, insufficient time to infect the plant.

1.4.3 Disease Resistance

Plants have developed complex mechanisms to recognise and respond to pathogen invasion. Disease resistance occurs at different levels including non-host resistance, parasite- and race-specific resistance, plant age- and organ-specific resistance, systemic acquired resistance and induced systemic resistance (Hammerschmidt, 1999).

Gene-for-gene resistance occurs when the product of a single resistance gene (*R* gene) located in the plant specifically recognises the product of a pathogen avirulence (avr) gene. *R* gene-mediated resistance is often accompanied by an oxidative burst, by rapid production of reactive oxygen species (ROS), and a hypersensitive response (HR) is triggered (Glazebrook, 2005). The HR response is a type of programmed cell death which culminates in rapid cell death around the site of infection to effectively prevent further spread of the pathogen (Scholthof, 2001; Glazebrook, 2005). Gene-for-gene resistance will only provide protection against a specific pathogen.

Upon viral, bacterial or fungal infection the host plant elicits a localised HR

around the infected cells. Other local responses include changes in cell wall composition to inhibit penetration by pathogens and *de novo* synthesis of antimicrobial compounds, such as phytoalexins and pathogenesis-related proteins (Heil and Bostock, 2002). In addition to local responses plants have developed a mechanism to protect uninfected parts through a signal that spreads systemically inducing changes in gene expression. Systemic resistance to a wide range of pathogens can be induced by two different methods, systemic acquired resistance (SAR) and induced systemic resistance (ISR).

1.4.3.1 Systemic Acquired Resistance

Systemic Acquired Resistance (SAR) is induced by prior inoculation with a necrotizing pathogen or the application of chemical agents such as salicylic acid (SA), 2-6-dichloro isonicotinic acid (INA) and benzo (1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Uknes *et al.*, 1992). Once the SAR pathway has been activated resistance can be conferred to a number of pathogens.

SAR has been extensively studied in the dicots, tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana*. SAR was first characterised in tobacco plants that expressed increased resistance systemically after infection by tobacco mosaic virus (Ross, 1961). SAR is characterised by an early increase of endogenously synthesised SA and the enhanced production of pathogenesis-related (PR) proteins. Eleven different PR families are recognised in tomato and tobacco (van Loon and van Stein, 1999). The PR-1 family is the main group of PRs induced by pathogens or SA and is commonly used as a marker for SAR. PR-1, though, is the only family for which no

function or relationship is known (van Loon and van Stein, 1999).

The SAR response is characterised by three stages. The first is the initiation of immunisation stage in which a necrotic pathogen infects the plant causing the formation of localised necrotic lesions and local resistance such as the HR or induced necrosis. Necrotic lesions induce a set of PR proteins and SA increases 10-50 times above background levels (Cameron *et al.,* 1999).

A mobile signal is also produced and thought to move from the site of attack via the phloem to the rest of the plant establishing systemic resistance. Maldonado *et al.* (2002) screened T-DNA-tagged Arabidopsis lines and identified a mutant *dir1-1* (*defective in induced resistance 1-1*) which showed local resistance by PR gene expression but resistance was abolished in un-inoculated distance leaves and failed to develop SAR. *DIR1* encodes a putative apoplastic lipid transfer protein and it was proposed that DIR1 interacts with a lipid-derived molecule to promote long distance signalling (Maldonado *et al.,* 2002).

The second stage, the establishment phase, involves perception of the mobile signal (Cameron *et al.,* 1999). The final stage of SAR requires the plant to be challenged with a second normally virulent pathogen and the plant responds to this pathogen as if it was avirulent (Cameron *et al.,* 1999). In Arabidopsis the SAR pathway confers resistance to *Pseudomonas syringae* (P.v) *maculicola* ES 4326 and *Peronospora parasitica* (Ryals *et al.,* 1996).

In Arabidopsis, the SAR pathway is initiated when a pathogen infects the plant and leads to CPR5 (Constitutive Expression of PR Proteins) activation. The importance of SA has been demonstrated using plants that express the bacterial *NahG* gene. *NahG* encodes salicylate hydroxylase responsible for the degradation of SA to

catechol. In *NahG* plants, *PR* gene expression and resistance to several pathogens is compromised (Gaffney *et al.,* 1993; Delaney *et al.,* 1994). In *cpr5* recessive mutants the plant exhibits spontaneous HR-like lesions and a constant expression of the *PR* genes as well as the *PDF.1.2* gene (Moller and Chua, 1999). After *CPR5* activation, SA accumulates leading to the accumulation of the PR proteins, PR-1, PR-2 and PR-5. It has been reported that production of the PR proteins requires the *NPR1* (non-expression of PR genes) gene product to be functioning correctly. It has been suggested that a SA-dependent but *NPR1*-independent pathway for regulation of *PR-1* gene expression and resistance to bacterial pathogens exists (Bowling *et al.,* 1997; Clarke *et al.,* 1998; Rate *et al.,* 1999; Devadas *et al.,* 2002; Rairdan and Delaney, 2002). Shah *et al.* (1999) characterised the Arabidopsis mutant *ssi1* (*suppressor of SA sensitivity*), which demonstrated that a signal can bypass the requirement of *NPR1* gene, and the expression of *PR* genes and disease resistance occurs. The NPR1 protein has two protein-protein interaction domains, an ankyrin repeat and a BTB/POZ (Broad-Complex, Tramtrack, Bric-a-brac/Poxvirus, Zinc finger) domain, as well as a putative nuclear localization signal and phosphorylation sites (Cao *et al.,* 1997; Ryals *et al.,* 1997). NPR1 activates *PR-1* gene expression by physically interacting with a subclass of basic leucine zipper protein transcription factors that bind to promoter sequences required for SA-inducible *PR* gene expression (Zhang *et al.,* 1999; Zhou *et al.,* 2000). The SAR pathway is currently being dissected through the analysis of Arabidopsis mutants, some of which are shown in Table 1.3.

Table 1.3: Arabidopsis mutants and their function in relation to systemic acquired resistance.

Name	Function	Reference
sid1, sid2 *SAR induction deficient*	SAR is reduced but not totally suppressed. *PR-1* abolished but *PR-2* and *PR-5* expression is the same as wild-type plants. *sid1* is allelic to *eds5*.	(Nawrath and Metraux, 1999)
NahG	Contains an insert of the bacterial *NahG* gene which encodes salicylate hydroxylase that converts SA to catechol. Cannot express *PR-1*.	(Lawton *et al.*, 1995)
cpr5: *constitutive expression of PR proteins*	*CPR5* acts upstream of SA and leads to constitutive expression of the *NPR1*-dependent and *NPR1*-independent pathways. Plants form spontaneous HR lesions.	(Bowling *et al.*, 1997)
npr1: *no PR gene expression* *nim1*: *non inducible immunity*	A point mutation in an IkB-like signal-transduction component acting downstream of SA.	(Cao *et al.*, 1994; Delaney *et al.*, 1994; Cao *et al.*, 1997)
jar1: *jasmonate resistant*	*JAR1* has similarity to the auxin-induced *GH3* gene product from soybean and is insensitive to jasmonates.	(Staswick *et al.*, 2002)
coi1: *coronatine-insensitive*	Coronatine is a chlorosis-inducing toxin produced by pathogens. Confers male sterility, insensitivity to coronatine, and insensitivity to methyl jasmonate (MeJA).	(Feys *et al.*, 1994)
etr1 (formerly *ein1-1*) *ethylene insensitive*	*ETR1* is a single gene dominant mutation. Plants are defective in promotion of seed germination, peroxidase activity, acceleration of senescence of detached leaves, and negative feedback of ethylene biosynthesis.	(Guzman, 1990; Lawton *et al.*, 1994; Ecker, 1995)
ein2, ein3, ein4, ein5, ein6 ein7	*ein2* show increased ethylene production. *ein3*, *ein5*, *ein6* and *ein7* have a less severe phenotype than *etr1*, *ein4*, or *ein2*. Ethylene-regulated genes are induced by ethylene higher than *ein3* than in alleles of *etr1* or *ein2*.	(Ecker, 1995)
lsd: *lesion simulating disease resistance response*	Spontaneous HR lesions and constitutive SAR expression.	(Dietrich *et al.*, 1994)
acd2: *accelerated cell death*	Spontaneously develops lesions similar to wild-type plants undergoing a hypersensitive response when challenged with avirulent bacterial pathogens.	(Greenberg *et al.*, 1994)
ssi1 *suppressor of SA sensitivity*	Constitutively expresses *PDF1.2* and renders expression of ethylene- or JA-responsive defence genes sensitive to SA and vice versa,	(Nandi *et al.*, 2003)
eds: *enhanced disease susceptibility, eds4, eds5-1, eds6-1, eds7-1, eds9-1*	Inactivation of SA biosynthesis enzymes. *eds4*/*eds5* do not express *PR-1* but can be rescued with SA. *eds5-7*/*eds6-1*/*eds7-1*/*eds9-1* do not affect HR response, synthesis of camalexin, and, the expression of 11 defence-related genes.	(Parker *et al.*, 1996; Rogers and Ausubel, 1997)
pad4: *phytoalexin deficient* *eds1*	Encode lipase-like proteins that function in R-gene mediated and basal plant disease resistance. Act upstream of SA promoting SA accumulation and have reduced levels of camalexin	(Glazebrook and Ausubel, 1994; Glazebrook *et al.*, 1996)

In addition to the SAR pathway, the jasmonic acid (JA)/ethylene (ET) pathway operates in a SA-independent manner and confers resistance to a broad range of pathogens. Jasmonates are important plant signalling molecules involved in a variety of critical functions including fruit ripening, senescence, tuber formation, tendril coiling, pollen formation and defence response against pests and pathogens (Creelman and Mullet, 1997). In Arabidopsis, jasmonates inhibit root elongation and are required for pollen development and pathogen defence (Ellis and Turner, 2001). Pathogen defence genes induced by jasmonates include the vegetative storage proteins (VSPs) (Benedetti *et al.,* 1995), a thionin, Thi2.1 (Epple *et al.*, 1995), and the plant defensin, PDF1.2 (Penninckx *et al.,* 1996).

Like jasmonates, ET (C_2H_4) is an important plant signalling molecule involved in many plant functions including seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening (Wang *et al.*, 2002). ET is produced by a two step process that consists of the enzymatic conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) followed by the conversion of ACC to ET which is catalysed by ACC oxidase (Ecker, 1995). Application of ET or the precursor ACC has been shown to trigger a pathway leading to the up-regulation of the defence genes, *PDF1.2* and *Hel*. ET production is increased upon exposure to abiotic and biotic stresses, including extreme temperatures, drought, anaerobic conditions, wounding, herbivory, and infection by viral, bacterial, and fungal pathogens (Geraats *et al.,* 2002).

JA and ET both are implicated in the activation of genes encoding plant defensins and enzymes involved in phytoalexin biosynthesis (Pieterse *et al.,* 1998). The JA/ET pathway also starts with *CPR5* activation. *CPR5,* is situated upstream of

the SAR and JA/ET pathways and acts as a negative regulator before the pathways diverge (Bowling *et al.,* 1997). The JA/ET pathway requires the concomitant activation of JA and ET signalling as was demonstrated in Arabidopsis mutants which were blocked in JA response (*coi-1* and *jar1*) and in ET response (*ein2-1* and *etr1*) (Moller and Chua, 1999).

The JA and ET signalling leads to the induction of the PR proteins, PR-3 a basic chitinase, PR-4 a chitin-binding protein and the plant defensin, PDF1.2. PDF1.2 is a member of the group of plant defensins, which is a family of peptides with anti-microbial activities and includes the thionins, lipid transfer proteins, hevein-type, knottin-type and anti-microbial peptides (van Loon and van Stein, 1999). The NPR1 protein is also thought to be required for JA/ET-mediated resistance and the mechanism underlying the divergence of the two pathways downstream of *NPR1* is not currently known (van Wees *et al.*, 2000). In Arabidopsis, the JA/ET pathway induces resistance against the fungal pathogens *Alternaria brassicola*, *Botyrtis cinerea* and *Fusarium oxysporum* f.sp. *matthiolae* (van Loon and van Stein, 1999).

Systemic resistance has not been studied extensively in monocots. SAR has been reported in rice with *Pseudomonas syringae* pv. *syringae* as the inducing pathogen. Although SAR has not been conclusively demonstrated in wheat, it has been reported that *Erysiphe graminis* infection appears to induce SAR as does treatment with BTH leading to induced resistance against *Septoria* spp., *P. recondita* and *E. graminis* (Gorlach *et al.,* 1996). In contrast to dicots, the orthologous PR-1 proteins in wheat do not correlate to SAR induction (Molina *et al.,* 1999). Pathogen-induced resistance has been correlated with the expression of Wheat Induced

Resistance (WIR) genes and chemically induced resistance with Wheat Chemical Induced (WCI) genes (Kmcel *et al.,* 1995). The behaviour of the WIR and WCI genes has been postulated to be different from genes in the Arabdiopsis SAR pathway (Schaffrath *et al.,* 1997).

1.4.3.2 Induced Systemic Resistance

Induced Systemic Resistance (ISR) is phenotypically similar to SAR; however, the resistance is induced by non-pathogenic biotic agents. It is believed that ISR is distinct from the SAR pathway, but mediated by a JA/ET pathway and it has been reported that there is no up-regulation of PR proteins (Hammerschmidt, 1999; van Wees *et al.,* 2000; Pieterse, 2002). There are some conflicting reports on this matter. Park and Kloepper (2000) investigated the effect of ten PGPR strains on the induction of the *PR-1a* gene promoter in regards to systemic resistance in tobacco against *Pseudomonas syringae* pv. *tabaci*. The results of this study indicated that *PR-1a* promoter activity and PGPR-mediated induced systemic resistance are linked events but this finding contradicts the model for PGPR mediated ISR proposed by Pieterse *et al.* (1998). However, the architecture of the SAR and ISR pathways may vary among different plant species.

Pieterse *et al.* (1998) investigated ISR in Arabidopsis, using the non-pathogenic, root-colonising *Pseudomonas fluorescens* WCS417r as the inducing agent. *P. fluorescens* WCS417r triggers ISR in carnation (van Peer *et al.,* 1991), radish (Leeman *et al.,* 1995), tomato (Duijff, 1996) and Arabidopsis (Pieterse *et al.,* 1996). *P. fluorescens* WCS417r induced systemic resistance independent of SA

accumulation and *PR* gene activation in Arabidopsis. Using the Arabidopsis mutants *jar1*, *etr1* and *npr1*, ISR was blocked indicating the ISR pathway triggered by *P. fluorescens* requires JA and ET perception and *NPR1* function. Pieterse *et al.* (1998) found that there was no induction of *PDF1.2*, *PR-1*, and *Hel* genes, suggesting the final ISR defensive compounds are different to the compounds up-regulated in the SAR and JA/ET pathways. The defence compounds induced by *P. fluorescens* WCS417r confer resistance to *Fusarium oxysporum* f.sp. *raphani*, the oomycetous leaf pathogen *Peronospora parasitica*, and the bacterial leaf pathogens *Xanthomonas campetris* pv. *campetris* and *Pseudomonas syringae* pv. *tomato* indicating ISR is effective against different types of pathogens (van Wees *et al.*, 2000). Figure 1.8 outlines the proposed SAR and ISR signalling pathways in Arabidopsis. Conrath *et al.* (2002) believe that the non-pathogenic bacteria prime the plant for accelerated and enhanced response when the plant is challenged by a second stress stimulus such as a pathogen.

Verhagen *et al.* (2004) used the microarray technique to identify ISR-related genes in Arabidopsis. Over 8000 genes were surveyed and it was found, when using the ISR-inducing bacterium *P. fluorescens* WCS417r, there was a substantial change in the expression of 97 genes in the roots but no changes in expression could be detected in the leaves. However, after subsequent challenge with *Pseudomonas syringae* pv. *tomato* DC3000 there was a change in the expression of 81 genes in the leaves. This indicates the role of rhizobacteria in priming the plant for ISR.

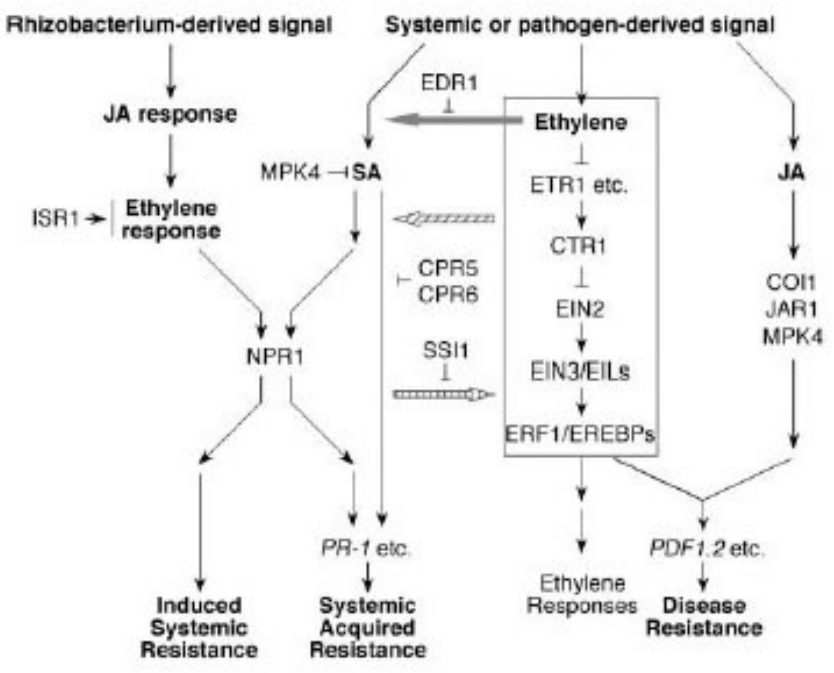


Figure 1.8: Proposed model of the rhizobacteria-mediated ISR signalling pathway and the systemic or pathogen-derived signal pathway (Wang *et al.*, 2002).

There is cross-talk between the SA-dependent and JA-dependent pathway. Schenk *et al.* (2000) investigated the changes in the expression patterns on 2,375 genes in Arabidopsis after inoculation with the fungal pathogen *Alternaria brassicola* and application of SA and MeJA. Treatment with *A. brassicola* resulted in 168 mRNA sequences increasing by 2.5 fold whereas 39 mRNA sequences showed a reduction. The abundance of 193, 221, and 55 mRNA sequences were increased 2.5 fold in the SA, MeJA and ET treatments, respectively. 169 mRNA sequences were regulated by multiple treatments. The results indicated that there is a substantial network of regulatory interactions and coordination during plant defence signalling, discounting reports that the JA/ET pathway acts in an antagonistic fashion to the SA

pathway. *NPR1* is required for this cross-talk as shown by Spoel *et al.* (2003) and *ssi2*-mediated signalling modulates the cross-talk between the SA and JA/ET pathways (Kachroo *et al.*, 2003).

1.5 Broad Research Objectives

- To characterise the indigenous endophytic actinobacterial and eubacterial population of wheat plants grown in various field soils by T-RFLP.
- To characterise the indigenous endophytic actinobacterial population in wheat in response to inoculation by actinobacteria endophyte coated seeds or in soil containing a mixed microbial inoculant by T-RFLP.
- To characterise the fungal endophyte population in wheat by molecular methods.
- To determine if previously isolated actinobacteria endophytes induce systemic resistance in *Arabidopsis thaliana*.

Chapter Two: Analysis of the Endophytic Bacterial, and specifically the Actinobacterial, Population of Wheat (*Triticum aestivum* L.) by Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Partial 16S rRNA Gene Sequencing.

2.1 Introduction

A number of the biologically active endophytes isolated belong to the actinobacteria phylum and specifically the genus *Streptomyces* (Shimizu *et al.*, 2000; Sessitsch *et al.*, 2001; Smalla *et al.*, 2001; Tokala *et al.*, 2001; Coombs and Franco, 2003a). Cultivation based methods were used in our laboratory to isolate a number of endophytic actinobacteria from wheat (Coombs and Franco, 2003a). The major genera identifed were *Streptomyces*, *Microbispora*, *Micromonospora* and *Nocardioides*. A number of these isolates were capable of suppressing fungal pathogens of wheat *in vitro* including *Rhizoctonia solani*, *Pythium* sp. and *Gaeumannomyces graminis* var. *tritici*.

Endophytic populations have been isolated and characterised primarily by cultivation-based methods. However, as not all microorganisms can be grown *in vitro* and there is a bias population overview due to the use of selective media, molecular methods of community analysis were used. The most common molecular technique to study endophytic communities involves analysis of a variable portion of the 16S rRNA gene sequence by PCR followed by DGGE, TGGE or T-RFLP.

The T-RFLP technique was chosen to analyse the microbial community as it provides high resolution and can be performed rapidly. The DGGE or TGGE separation of 16S rRNA sequences can provide a community fingerprint but the information cannot be directly translated into taxonomic information (Osborn *et al.*, 2000). In contrast, T-RFLP allows detection of bacteria to the genus level and the abundance each member of the community present in the sample can be determined.

T-RFLP requires PCR amplification of the 16S rRNA gene with one of the primers being fluorescently labelled, followed by restriction endonuclease digestion. The digestion products are separated by electrophoresis with laser detection of the terminal-labelled fragments using an automated analyser. The variable fragment lengths can be correlated with data available in an online database (Marsh *et al.,* 2000) and translated into semi-quantitative taxonomic information. The T-RFLP technique previously has been used to study the changes in the bacterial communities in soil, marine sediments and internal potato tissue (Dunbar *et al.*, 2000; Lukow *et al.,* 2000; Sessitsch *et al.,* 2001). From these studies it was concluded that the T-RFLP technique is a sensitive tool appropriate for analysing changes in microbial communities.

Variations in the indigenous populations of endophytes in a variety of plants have been reported (Germida *et al.,* 1998; Araujo *et al.,* 2001; Garbeva *et al.*, 2001; Sessitsch *et al.*, 2001; Adams and Kloepper, 2002; Araujo *et al.,* 2002; Zinniel *et al.,* 2002). These variations were attributed to plant cultivar, plant age, tissue type, time of sampling and environment (Zinniel *et al.,* 2002). Culture-dependent studies have investigated changes in bacterial endophyte populations in cotton, pea, canola and wheat (McInroy and Kloepper, 1995; Hallmann *et al.,* 1999). It is well known that soil type, plant type, cropping practices, growth stage and other environmental factors are capable of affecting the microbial population present in the rhizosphere (Siciliano *et al.,* 1998; Smalla *et al.,* 2001). However the effect of soil type on the indigenous endophytic populations in wheat has not been investigated. This is the first study to investigate the response of the endophytic actinobacteria population in wheat by the culture-independent method T-RFLP.

2.2 Materials and Methods

2.2.1 Actinobacterial Cultures

A number of pure actinobacteria cultures were used in this study for validation of methods (Table 2.1). Cultures numbers starting with EN, PM, SE or AB were isolated from the roots of healthy wheat plants previously in our laboratory. Cultures were maintained on oatmeal agar, YME, MS or ½ PDA media (Appendix 1). For the remainder of chapter two, cultures will be referred to by the culture number eg. EN2.

Table 2.1: Actinobacteria Cultures used in this Study

Culture No.	GenBank Ac. No.	Actinobacteria Culture Name
AB6	N/A	*Kitasatospora cochleate*
AB11	N/A	*Streptomyces bottropensis*
EN2	AY148073	*Microbispora* sp.
EN16	AY148072	*Streptomyces* sp.
EN27	AY148075	*Streptomyces* sp.
EN31	N/A	*Streptomyces* sp. (Scab Isolate)
EN41	N/A	*Micromonospora yulongensis*
EN45	N/A	*Streptomyces galilaeus*
EN46	AY148081	*Nocardioides albus*
PM23	N/A	*Streptomyces* sp.
PM124	N/A	*Tsukamurella* sp.
SE2	AY148089	*Streptomyces* sp.
*S. caviscabies (*ATCC 51928)	AF112160	Type Strain *Streptomyces caviscabies*
S. scabies (ATCC 49173)	AF091226	Type Strain *Streptomyces scabies*
S. setonii (ATTC 25497)	N/A	Type Strain *Streptomyces setonii*

2.2.2 Cultivation of Wheat Plants

2.2.2.1 Growth of Actinobacteria and Harvesting of Spores

Actinobacteria cultures EN27 and EN46 were grown on MS agar and ½ PDA media respectively (Appendix 1), while EN2 was grown on oatmeal agar (Appendix 1). Plates were incubated at 27°C for 3 to 10 days until luxuriant sporulation had occurred. Spores were harvested by scraping them off the plate with a sterile loop and suspending them in 2 ml sterile H_2O.

2.2.2.2 Coating of Wheat Seeds with Actinobacteria Inoculum

Approximately 40 wheat seeds (cultivar Krichauff) were placed in a sterile petri dish and treated separately with 2 ml of the actinobacteria spore suspensions or water as a control. The actinobacteria (EN2, EN27 and EN46) spores were harvested as in 2.2.2.1. After mixing the seeds well in the spore suspensions the petri dish was left on an angle in the laminar flow cabinet overnight to evaporate the water and coat the spores onto the seeds.

2.2.2.3 Wheat Plant Cultivation

Wheat plants (cv. Krichauff) were grown in pots (100 mm height x 50 mm diameter) with approximately 110-120 g of field soil per pot and seeds were sown at a depth of 1 cm. Plants were grown for 6 weeks in a glasshouse with watering as required.

2.2.2.3.1 Field Soil Experiment

Wheat seeds were grown in different field soils to assess the effect of soil type on the diversity of endophytic actinobacterial populations. Four different field soils were obtained from the South-East of South Australia (Figure 2.1). The two Swedes Flat soils differed in that one had the addition of a commercial microbial inoculant, Nutri-life 4/20™ (NutriTech, Australia) to the soil. Nutri-life 4/20™ contained three strains of *Bacillus subtilis*, two strains of *Bacillus megaterium*, two strains of *Azotobacter vinelandii*, two strains of *Pseudomonas fluorescens*, two strains of *Pseudomonas putida*, two strains of *Pseudomonas stutzeri*, two strains of *Rhizobium japonicum*, two strains of *Rhizobium leguminosarum*, *Streptomyces albidoflavus*, *Streptomyces cellulosae*, *Saccharomyces cerevisiae*, two strains of *Chaetomium globosum*, *Trichoderma lignorum* and *Trichoderma harzianum* (personal communication, NutriTech). For each field soil, three seeds were sown per pot and three replicate pots were used.

2.2.2.3.2 Soil Microbial Count

One gram of soil was diluted in 25 ml of phosphate buffer and kept at RT for 5 mins with 125 rpm shaking. Soil suspensions were then sonicated for one minute in a sonicator bath (SoniClean, Adelaide, Australia). The sonicated soil suspension was used to make a 10^2 dilution using sterile saline (0.9% NaCl). Further serial dilutions up to 10^4 were made with sterile H_2O. From each dilution 50 µl and 20 µl was spread onto ¼ strength nutrient agar (Appendix 1) and incubated at 27°C for 7 days. The colony forming units (CFU) per gram of soil was then calculated.

Figure 2.1: Four field soils were collected from the south-east region of South Australia. Three soils were collected from the area Swedes Flat (Blue Box), a sandy loam from a pasture (referred to as Red Loam) and two dark loams from a wheat crop (referred to as Swedes Flat). One Swedes Flat soil had been treated with NutriLife 4/20™ (NutriTech, Australia). The fourth soil was obtained from Western Flat (Red Box). Western Flat had water repellent sand from virgin scrub.

2.2.2.3.3 Endophyte Coated Seed Experiment

Wheat seeds were coated with an actinobacteria inoculum and sown in Haslam field soil (Figure 2.2) and grown as per six weeks. For each endophyte (EN2, EN27 and EN46) plus a non-coated seed control, three seeds were sown per pot as per 2.2.2.3 and three replicate pots were used.

2.2.1.4 Harvesting Wheat Plants

Wheat plants were harvested after 6 weeks of growth. The pots were removed and the soil around the roots gently loosened before washing the plants with tap water. The roots were cut from the shoots and stored in separate plastic bags at –20°C before the endophytic eubacterial DNA extraction was performed.

2.2.3 Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis of the Endophytic Bacteria and Actinobacteria in Wheat Roots

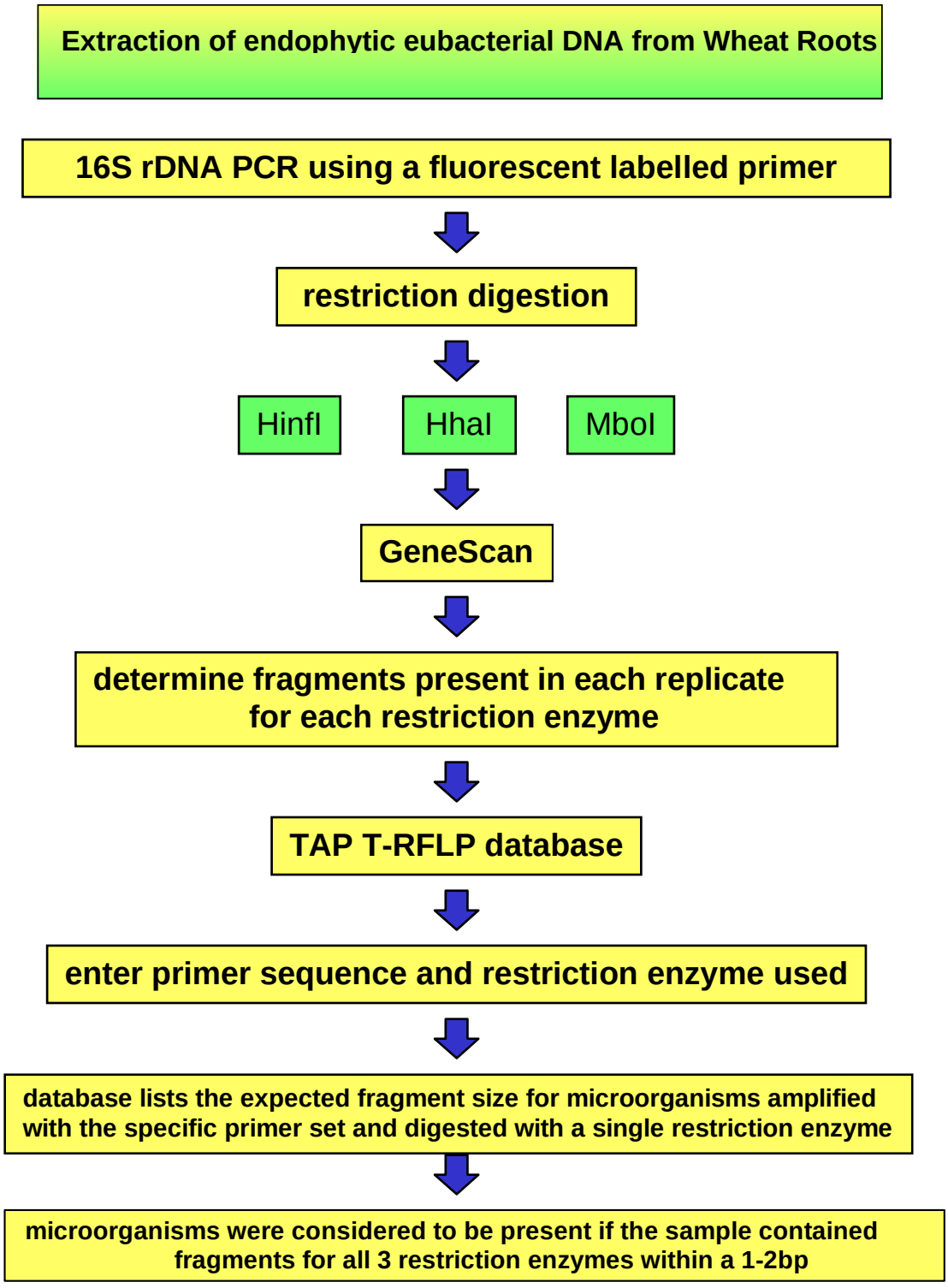
The endophytic actinobacterial population inhabiting the wheat root were assessed by the T-RFLP method using the strategy outlined in Figure 2.3.

2.2.3.1 Growth of Actinobacteria Cultures

Actinobacteria cultures listed in Table 2.1 were grown on either oatmeal agar, YME or MS agar (Appendix 1) and incubated at 27°C for 3 to 10 days until sporulation had occurred. The actinobacteria cultures were used as controls for T-RFLP analysis.

Figure 2.2: The field soil (Haslam) was obtained from a wheat field in Haslam located on the Eyre Peninsula, South Australia.

Figure 2.3: Strategy for determining endophytic microbial populations using the T-RFLP technique.



2.2.3.2 DNA Extraction from Actinobacteria Cells

Total genomic DNA was extracted using a modified CTAB/NaCl protocol (Kieser *et al.,* 2000). For each isolate, two loopfuls of mycelium and spores were scraped from colonies grown on agar media and resuspended in 500 µl of Tris-EDTA (10 mM Tris; 10 mM EDTA, pH 8.0) by vortexing. This suspension was lysed with 10 µl of lysozyme (10 $mg.ml^{-1}$) for 1 hr at 37°C. Subsequently, 10 µl of 1% (w/v) proteinase K and 33.5 µl of 10% SDS were added, followed by incubation for 1 hr at 55°C. Added to this were 100 µl of 5 M NaCl and 65 µl of CTAB/NaCl (700 mM NaCl, 275 mM CTAB) and incubated for a further 10 min. The lysates were centrifuged (12,000*g*, 15 min at RT) to precipitate the cell debris, before the supernatant was extracted with 500 µl of chloroform/isoamyl alcohol (24:1) at RT for 30 min with intermittent shaking. After centrifugation (12,000*g*, 15 min), DNA was precipitated from the supernatant with 3 volumes of absolute ethanol and 0.1 volume 3 M sodium acetate. Precipitated DNA was washed with 70% ethanol, resuspended in 75 µl sterile H_2O and stored at –20°C. The DNA was semi-quantified on a 2% agarose gel in 0.5X Tris-borate-EDTA (Appendix 1) stained with ethidium bromide (10 $\mu g.ml^{-1}$) and visualised under UV.

2.2.3.3 DNA Extraction from *Pseudomonas fluorescens*

Two loopfuls of *Pseudomonas fluorescens* (E7 and E5a, isolated in our laboratory) from glycerol stocks were used to inoculate King's B medium (Appendix 1) and incubated at 27°C for 4 days. DNA was extracted from these cultures using a method modified from Ausubel *et al.* (1990). Two loopfuls of the bacteria were resuspended in 500 µl of Tris-EDTA and mixed by vortexing. To

this 30 µl of 10% SDS and 50 µl of 1% (w/v) proteinase K were added and the contents incubated at 37°C for 1 hr. After this 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl (700 mM NaCl, 275 mM CTAB) was added. After vortexing the samples were incubated at 65°C for 10 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added and the samples were kept at RT for 1h with intermittent shaking. Samples were centrifuged at 16,000*g* at RT and the aqueous, viscous supernatant transferred to a sterile 1.5 ml eppendorf. Two volumes of absolute ethanol and 0.5 times the volume of 3 M sodium acetate were added to precipitate the DNA. Precipitation was performed overnight at -20°C. Samples were centrifuged at 16,000*g* for 5 min at RT. The supernatant was discarded and the pellet washed three times with 70% ethanol. The pellet was dried at 55°C in a heat block with the eppendorf lids open and redissolved in 50 µl of sterile H_2O. Samples were stored at -20°C.

2.2.3.4 Extraction of Eubacterial DNA from Wheat Roots and Shoots

A protocol for the extraction of endophytic eubacterial DNA from wheat roots was developed as there was no established protocol. Endophytic eubacterial DNA was extracted from one gram of the wheat root or shoot material obtained in 2.2.1.4. Surface sterilisation of the plant material was first performed to ensure that eubacterial DNA obtained was from endophytic eubacteria. This was done by immersing the plant material in absolute ethanol for 1 min followed by 6 min in 3% sodium hypochlorite and 30 seconds in absolute ethanol. The plant material was rinsed in sterile H_2O, dipped in absolute ethanol and flamed. Surface sterilisation of the plant material was checked by rolling the sterilised material on

YME and MS agar plates and incubating the plates for seven days at 27°C. Only the roots that were confirmed to be surface sterilised were chosen for further analysis. Extraction of the endophytic eubacterial DNA was performed by cutting the sterilised plant material into 0.1 mm to 0.5 mm sections. The sectioned material was transferred to a sterile 1.5 ml eppendorf tube and 1 ml of NAP buffer (124 mM $Na_2HPO_4.H_2O$) added. Sonication was performed in a sonicater bath (SoniClean, Adelaide, Australia) for 1 min to facilitate further cleaning of the plant material. Samples were centrifuged at 12,000*g* for 10 min. The supernatant was discarded and the plant material transferred to a 1.5 ml screw top tube with 1 g of 0.1 mm silica beads (BioSpec Products, Oklahoma, U.S.A) and 1 ml Tris-EDTA (10 mM Tris; 10 mM EDTA, pH 8.0). Samples were homogenised in a mini bead beater (Daintree Scientific, Queensland, Australia) at 4800 rpm for 3 min. Further treatment of the eubacterial cell homogenate to extract DNA was performed as described in 2.2.3.2, from the lysozyme treatment step onwards.

2.2.3.4.1 DNA Purification

The endophytic eubacterial DNA extracted in 2.2.3.4 was purified twice using the Prep-A-Gene® DNA Purification kit (Bio-Rad) as per the manufacturer's instructions to remove PCR inhibitors. To further remove any inhibitors the DNA was re-precipitated with two volumes of absolute ethanol and 0.5 volumes of 3 M sodium acetate. After overnight precipitation at -20°C the DNA was pelleted by centrifugation at 12,000*g* for 10 min. The pellet was washed three times in 70% ethanol and dried in a heating block at 55°C before resuspending in 30 µl of sterile H_2O.

2.2.3.5 16S rRNA T-RFLP

The T-RFLP method measures the size polymorphism of the terminal restriction fragments from a PCR amplified marker. The University of Michigan, U.S.A, developed a database (http://rdp.cme.msu.edu/html/TAP.trflp.html) that allows one to correlate the 16S rRNA gene fragment sizes obtained from restriction digestion (normally with 4 bp cutters) with data from known bacterial species. Analysis of the endophytic actinobacterial population of wheat was performed by amplifying a portion of the 16S rRNA gene using the 243f (*Escherichia coli* numbering) primer developed by Heuer *et al.* (1997) and the 1492r 5' fluorescent 6-carboxy-2',4,7,7'-tetrachlorofluorescein (TET)-labelled primer (Weisburg *et al.,* 1991). This combination of 16S rRNA primers amplifies the majority of actinobacteria genera and only a limited number of non-actinobacteria genera. Analysis of the endophytic eubacterial population of wheat was performed using the universal bacterial primers developed by Weisburg *et al.* (1991) to amplify the most eubacterial 16S rRNA gene sequences. Primers fD1 and rP2 from Weisburg *et al.* (1991) are the same as 8f and Bacr primers used in this study, respectively. The Bacr reverse primer was labelled with hexachlorofluorescein phosphoramidite (HEX). For each plant group (four field soils, three endophyte coated seeds and an uncoated control) the 16S rRNA PCR was performed in duplicate giving a total of six PCR reactions per plant treatment group.

2.2.3.5.1 Actinobacteria 16S rRNA T-RFLP PCR

Actinobacteria Primer	Sequence
243f:	5’ GGA TGA GCC CGC CGC CTA 3’
1492r:	5’ *TA CGG GTA CCT TGT TAC GAC TT 3’

*TET fluorescent label

The 243f and 1492r primers were used in a 50 µl PCR reaction with the following contents: Primers (20 ng.µl^{-1}) 2 µl each, 5X Taq buffer (inc. dNTPs) 10 µl, H_2O 33 µl, Taq polymerase (Biotech, Australia) (2 U.µl^{-1}) 1 µl, template DNA 2 µl. The following thermal profile was followed: 94°C - 5 min, (94°C - 1 min, 58°C - 1 min, 72°C - 1 min) × 40 cycles, 72°C - 10 min.

2.2.3.5.2 Eubacterial 16S rRNA T-RFLP PCR

Eubacterial Primer	Sequence
8f	5’ *AG AGT TTG ATC CTG GCT CAG 3’
1492r (Bacr)	5’ ACG GCT ACC TTG TTA CGA CTT 3’

*HEX fluorescent label

The 8f and 1492r (Bacr) primers were used in a 50 µl PCR reaction with the following contents: Primers (20 ng.µl^{-1}) 2 µl each, 5X Taq buffer (inc. dNTPs) 10 µl, H_2O 33µl, Taq polymerase (Biotech, Australia) (2 U.µl^{-1}) 1 µl, template DNA 2 µl. The following thermal profile was followed: 94°C - 5 min, (94°C - 1 min, 56 °C - 1 min, 72°C - 1 min) × 40 cycles, 72°C - 10 min.

2.2.3.6 Restriction Digestion

Single restriction digestions of PCR products were performed with the following enzymes obtained from Promega:

16S rRNA PCR Product	Enzyme	Recognition Sequence 5' to 3'
Actinobacteria (243f-1492r)	*Hinf*I	G↓ANT
Eubacteria (8f-1492r)	*Hinf*I	G↓ANT
Actinobacteria (243f-1492r)	*Hha*I	GCG↓C
Eubacteria (8f-1492r)	*Hha*I	GCG↓C
Actinobacteria (243f-1492r)	*Mbo*I	↓GATC
Eubacteria (8f-1492r)	*Msp*I	C↓CGG

The 16S rRNA PCR products (10 μl) were digested with 3-5 units of restriction enzyme in bovine serum albumin and 1X buffer according to the manufacturer's (Promega) instructions. Restriction digestions were performed at 37°C for 16 to 18 hrs to achieve complete digestion. Digested products were analysed by agarose gel electrophoresis.

2.2.3.7 Agarose Gel Electrophoresis

DNA and PCR samples were analysed using agarose gel electrophoresis. In all cases the gel was composed of 1.5% to 2.0% agarose in 0.5X TBE with 0.5X TBE as the running buffer. The samples were stained with ethidium bromide (10 $mg.ml^{-1}$) in the agarose gel and visualised under UV. The gels were run at 120V for 50 to 55 min. The DNA or PCR products were prepared for electrophoresis by mixing the sample (5 μl to 10 μl) with 2 μl of 6X loading buffer (Appendix 1).

2.2.3.8 Genescan

The size of the terminal 16S rRNA gene fragments present in the restriction digestions were determined on an automated sequencer (Applied Biosystems, Australia) 373 DNA sequencer, Stretch, using 1 µl of the restriction digestion. Data was analysed using GeneScan Analysis program V.3.1.2 (Applied Biosystems, Australia).

2.2.3.9 Statistical and Data Analysis

For each soil treatment, replicate T-RFLP profiles were obtained. These were from two to three wheat root samples subjected to duplicate PCR. For each soil sample the terminal restriction fragments (TRFs) obtained from each of the restriction enzymes for each of the replicates were aligned and the average length for each representative TRF was determined. Only TRFs above 35 bp and present in a minimum of two of the replicates were considered for further analysis.

The relative abundance of each bacterial genus present in the sample was determined by first calculating the abundance percentage (Ap) for each fragment and for each replicate separately using the formula Ap = $(n_i / N) \times 100$: where n_i is the peak area of one distinct fragment and N is the sum of all peak areas in a given T-RFLP profile (Lukow *et al.,* 2000). The minimum and maximum Ap value for each TRF across the replicates was recorded.

The TRFs to be included for further analysis were then compared with data from bacterial genera/species in the Ribosomal Database Project (Maidak *et al.,* 1999) using the TAP T-RFLP software available online (http://rdp.cme.msu.edu/html/TAP.trflp.html) (Marsh *et al.,* 2000). A bacterial

genus/species was considered present in a sample only if all three corresponding TRFs (from the 3 separate restriction enzyme digests) within a 1-2 bp range were present in the sample. In those instances where the TRF for the third restriction enzyme digest (usually *Mbo*1) was not present in the TAP T-RFLP database, a decision to include the genus was made on a case-by-case basis after rechecking the T-RFLP electropherograms. A TRF was considered to be 'validated' if it correlated with TRFs from the other 2 enzyme digests that matched a bacterial genus/species. These three TRFs were called a 'triple TRF-genus match'.

A triple TRF-genus match often corresponded to more than one bacterial species/genus. When this was the case, the bacterial genera were listed under the same set of three TRFs. A table of the TRFs and Ap values for all three restriction enzymes that had a corresponding bacterial genus was prepared. The minimum and maximum Ap values for each of the validated TRFs were corrected for the number of times the TRF was part of a combination in the prepared table. This was done for each of the TRFs to provide the theoretical minimum and maximum Ap values for the bacterial genus/species that was represented by the triple TRF-genus match. As actinobacterial genera can have more than one set of triple TRF-genus matches due to presence of more than one species, the minimum and maximum corrected Ap values for each genus were combined to give the final percentage for that genus.

2.2.4 Partial Sequencing of the Endophytic Actinobacterial 16S rRNA from Wheat Roots

2.2.4.1 Actinobacteria Partial 16S rRNA PCR

The actinobacteria 16S rRNA gene from 243 bp to 1492 bp (*E. coli* numbering) was amplified as described in 2.2.3.5.1 using the endophytic eubacterial DNA extracted from wheat samples grown in the different field soils.

2.2.4.2 PCR Product Purification

Actinobacteria 16S rRNA PCR products were purified using the MoBio (California, U.S.A) UltraClean PCR Product Purification Kit to remove fluorescent labels as well as residual primers and dNTPs.

2.2.4.3 Cloning of Actinobacteria Partial 16S rRNA Gene Sequence

Purified actinobacteria 16S rRNA PCR products were ligated to the Bluescript pGEM T-vector obtained from Promega. Ligations were performed as per the manufacturer's instructions using 3 μl of the purified PCR product in a 10 μl volume, incubated overnight at 4°C. Competent cells, JM109, were obtained from Promega and used for transformations. For transformations, 2 μl of the ligation product were placed in a 1.5 ml eppendorf tube. The JM109 cells, stored at -80°C, were thawed on ice and mixed gently by flicking the tube; 50 μl of the cells were placed into the eppendorf tube containing the ligation product. This ligation/cell mix was left on ice for 20 min before heating at 42°C for 45 to 50

seconds. The tubes were returned to ice for 2 min before adding 950 μl of SOC media (Appendix 1). The samples were incubated at 37°C for 1.5 hrs with 150 rpm shaking on a rotary shaker. For each transformation 100 μl, 300 μl and 600 μl were plated on LB plates containing ampicillin, IPTG and X-gal (Appendix 1), incubated overnight at 37°C.

2.2.4.4 Plasmid Preparation

For each field soil group 100 white colonies were picked from the transformation plates with sterile toothpicks. Each toothpick was placed in a 5 ml McCartney bottle containing 2 ml LB medium with 100 $\mu g.ml^{-1}$ ampicillin. The cultures were incubated overnight at 37°C with 120 rpm shaking. Plasmid isolation was performed by the alkali lysis method (Sambrook *et al.,* 1989). The plasmid pellet was resuspended in 25 μl of H_2O. The resuspended plasmid was treated with 2 μl of 10 $mg.ml^{-1}$ RNase A (Boehringer Mannheim, Germany) and incubated at 37°C for 30 min to remove RNA.

2.2.4.5 Selection of Candidates for Sequencing by Restriction Analysis

For each of the field soils, the 100 plasmids containing the actinobacteria 16S rRNA gene sequences were isolated and digested with 2 restriction enzymes *Hha*I and *Hinf*I. For each field soil 2 μl of each of the 100 plasmids were digested with 0.3 μl of *Hha*I according to the manufacturer's instructions (Promega). Digestions were performed for 16 to 18 hrs at 37°C so that digestion was complete. For each digestion, 5 μl was run on an agarose gel. From the restriction

patterns generated with *Hha*I, 30 plasmids that appeared to have different restriction fragment patterns were further digested with the restriction enzyme *Hinf*I (Promega, Australia) to identify 20 plasmids with dissimilar 16S rRNA RFLPs.

2.2.4.6 Sequencing

Sequencing was performed on an automated sequencer (Applied Biosystems, Australia, 373 DNA sequencer, Stretch). Sequencing was performed from the pGEM T-vector with the SP6 forward primer (5' TAT TTA GGT GAC ACT ATA 3') and dynamic ET terminator sequencing chemistry (Amersham, Australia). Plasmids were diluted by half with sterile H_2O and 3-10 μl used per sequence reaction. Sequences were analysed using the Sequencing Analysis V. 3.4.1 program (Applied Biosystems, Australia). Sequences were compared to online databases using the BLAST program located at the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). The standard blastn (nucleotide-nucleotide) algorithm was used with the default settings (Altschul *et al.*, 1997). The three highest match coefficients by the bit score were recorded in the table and this data was compared to the species identified by T-RFLP.

2.2.5 Electron Microscopy

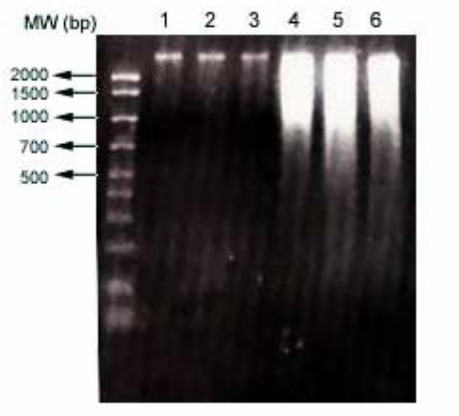
Electron microscopy of the pure actinobacteria endophyte cultures was performed by Kerry Gascoigne, Electron Microscope Suite, Department of Anatomy and Histology, School of Medicine, Flinders University. Electron

microscopy of the actinobacteria treated wheat roots was performed by Dr. Margaret McCully and Dr. Cheng Huang at CSIRO Plant Industry, Canberra using a cryo scanning cryoelectron microscope, JSM-4600. Samples were prepared by Cheng Huang and transferred to the cryostage of the scanning electron microscope. Samples were etched lightly by slowly warming to 183°K, which was done under observation at 1 kV until faint cell outlines were detected. Samples were recooled to 153°K and coated with 50nm of evaporated high-purity aluminum then observed at 7 to 15 kV. The images were recorded as video prints and on Tmax 100 film (McCully *et al.*, 2000).

2.3 Results

2.3.1 Extraction of Eubacterial DNA from Wheat Roots and Shoots

The endophytic eubacterial DNA extracted from wheat roots was of a moderate yield and integrity (Figure 2.4 lanes 1-3). In comparison, the eubacterial DNA obtained from wheat shoots was of a higher yield but appeared to be severely sheared (Figure 2.4 lanes 4-6). The sheared DNA most likely originated from plant chloroplast DNA.



Lane
1: EN2 1 roots
2: EN2 2 roots
3: EN2 3 roots
4: EN2 1 shoots
5: EN2 2 shoots
6: EN2 3 shoots

Figure 2.4: Eubacterial DNA (10 μl) extracted from the roots and shoots of six week old wheat grown from seeds coated with the endophyte EN2 (3 replicates) and run on a 2% agarose gel.

2.3.2 Soil Microbial Count

Total eubacteria were enumerated for each soil type and are shown in Table 2.2. The Swedes Flat soil with the added inoculant NutriLife 4/20™ supported the highest number of microorganisms and this decreased in Swedes Flat without NutriLife 4/20™. The Red Loam and Western Flat soil both

supported a lower number of microflora compared to the Swedes Flat soil with and without NurtriLife 4/20™.

Table 2.2: Eubacteria CFU estimated per gram of soil for each field soil type.

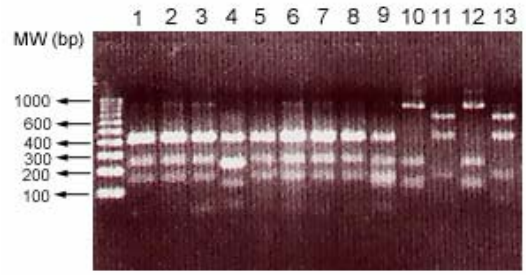
Soil Type	CFU.g^{-1} of Soil
Swedes Flat with NutriLife 4/20™	1.75×10^7
Swedes Flat without NutriLife 4/20™	1.0×10^6
Red Loam	6.25×10^5
Western Flat	5.0×10^5

2.3.3 Actinobacteria and Eubacteria 16S rRNA PCR

The 16S rRNA PCR using pure culture actinobacterial DNA amplified a product of approximately 1250 bp for each actinobacteria tested. Amplification of the actinobacteria 16S rRNA gene from endophytic eubacterial DNA extracts could only be achieved after the DNA had been purified three times and only from wheat roots. The endophytic DNA extraction method extracts many plant phenolics and polysaccharides which can inhibit the PCR reaction. Once removed, the PCR amplification of actinobacteria (243f - 1492r) and eubacteria (8f - 1492r) 16S rRNA gene sequences from wheat roots was achievable and was reproducible. No actinobacteria 16S rRNA sequences could be amplified from the wheat shoots but the universal bacterial primers were able to amplify 16S rRNA sequences from shoot material.

2.3.4 Endophytic Actinobacterial Population of Wheat Grown in Four Different Field Soils

The T-RFLP technique was first validated using pure actinobacteria cultures for which the 16S rRNA sequence was known. Figure 2.5 shows the restriction fragment pattern for the pure actinobacteria cultures.



Lane

1: EN31 – *Hha*I
2: EN27 – *Hha*I
3: EN45 – *Hha*I
4: EN46 – *Hha*I
5: *S. bottropensis* - *Hha*I
6: *S. scabies* - *Hha*I
7: SE2 - HhaI
8: PM124 - *Hha*I
9: *S. setonii* - *Hha*I
10: PM124 - *Hinf*I
11: PM124 - *Mbo*I
12: *S. setonii* - *Hinf*I
13: *S. setonii* - *Mbo*I

Figure 2.5: Restriction digestion (10 μl) of actinobacteria 16S rRNA PCR products amplified from pure cultures.

The digested actinobacterial 16S rRNA gene sequences were run on an agarose gel to confirm that complete digestion had been achieved. The restriction digestions yielded three fragments but the terminal fragment could not be distinguished on the agarose gel. Therefore the digested products were run on an automated sequencer which will detect only the 5' terminal fragment as it contains a fluorescent label. The size of each terminal restriction fragment (TRF) can then be determined using the GeneScan program.

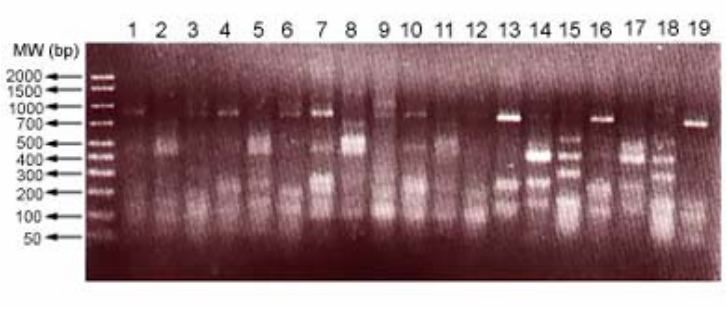
In order to validate the system, the TRFs of the pure actinobacteria strains were determined for the separate restriction enzymes, *Hinf*I, *Hha*I and *Mbo*I. These restriction enzymes were chosen on the basis of their ability to target the polymorphic sites of the actinobacteria 16S rRNA sequence. Table 2.3 shows the

fragmentation patterns of the actinobacteria species. In some cases the actinobacterial species were present in the TAP-T-RFLP database and the expected fragment sizes were within four base pairs of the TRF sizes determined in this study which validated the approach.

An example of the TRF pattern obtained for the actinobacterial 16S rRNA gene sequences amplified from the endophytic eubacterial DNA extracted from the roots of wheat grown in different field soils is shown in Figure 2.6. There appeared to be number of actinobacterial species present in the wheat roots grown in different field soils as a large number of bands were present on the gel. The TRF patterns of these actinobacterial 16S rRNA gene sequences was analysed by GeneScan. Examples of TRF profiles are shown in Figure 2.7 where the three different TRF patterns obtained from the roots of six week old wheat grown in Swedes Flat soil are shown and each peak represents the TRF for a specific actinobacterial species. The three TRF profiles demonstrate that different TRF patterns are obtained with different restriction enzymes and each member of the community will have a triple TRF. The TAP-T-RFLP database was then used to correlate triple TRFs to the actinobacterial genera present in the samples.

Table 2.3: Terminal restriction fragment sizes of pure actinobacterial species.

Name	**Species**	**Enzyme**	**Expected Fragment Size (bp)**	**Obtained Fragment Size (bp)**
EN 45	*Streptomyces galilaeus*	*Hinf*I	N/A	240.6
		*Hha*I	N/A	419.2
		*Mbo*I	N/A	158.1
AB6	*Streptomyces bottropensis*	*Hinf*I	236	238.7
		*Hha*I	419	418.5
		*Mbo*I	158	158.1
PM 23	*Streptomyces* sp.	*Hinf*I	N/A	180.0
		*Hha*I	N/A	N/A
		*Mbo*I	N/A	158.1 162.4
S. scabies	*Streptomyces scabies*	*Hinf*I	236	239.7
	(ATCC 49173)	*Hha*I	418	415.7
		*Mbo*I	157	157.3
SE2	*Streptomyces* sp.	*Hinf*I	N/A	238.5
		*Hha*I	N/A	419.0
		*Mbo*I	N/A	158.0
PM 124	*Tsukamurella* sp. IM-7430	*Hinf*I	N/A	239.8
		*Hha*I	N/A	419.4
		*Mbo*I	N/A	162.3
S. setonii	*Streptomyces setonii*	*Hinf*I	236	239.9
	(ATCC 25497)	*Hha*I	419	-
		*Mbo*I	158	158.3



Lane

1: EN46 1.1 –H	11: EN46 2.2 - Hh
2: EN46 1.1 – Hh	12: EN46 2.2 -M
3: EN46 1.1 – M	13: EN46 3.1 – H
4: EN46 1.2 - H	14: EN46 3.1 - Hh
5: EN46 1.2 - Hh	15: EN46 3.1 -M
6: EN46 1.2 - M	16: EN46 3.2 - HI
7: EN46 2.1 - H	17: EN46 3.2 - Hh
8: EN46 2.1 - Hh	18: EN46 3.2 - M
9: EN46 2.1 - M	
10: EN46 2.2 - H	

Figure 2.6: Restriction digestion (H - *Hinf*I, *Hh* - *Hha*I, M - *Mbo*I) of actinobacteria 16S rRNA PCR products amplified from endophytic eubacterial DNA extracted from the roots of 6 week old wheat inoculated with EN46. The first number after EN46 refers to the plant replicate number and the second number the PCR replicate.

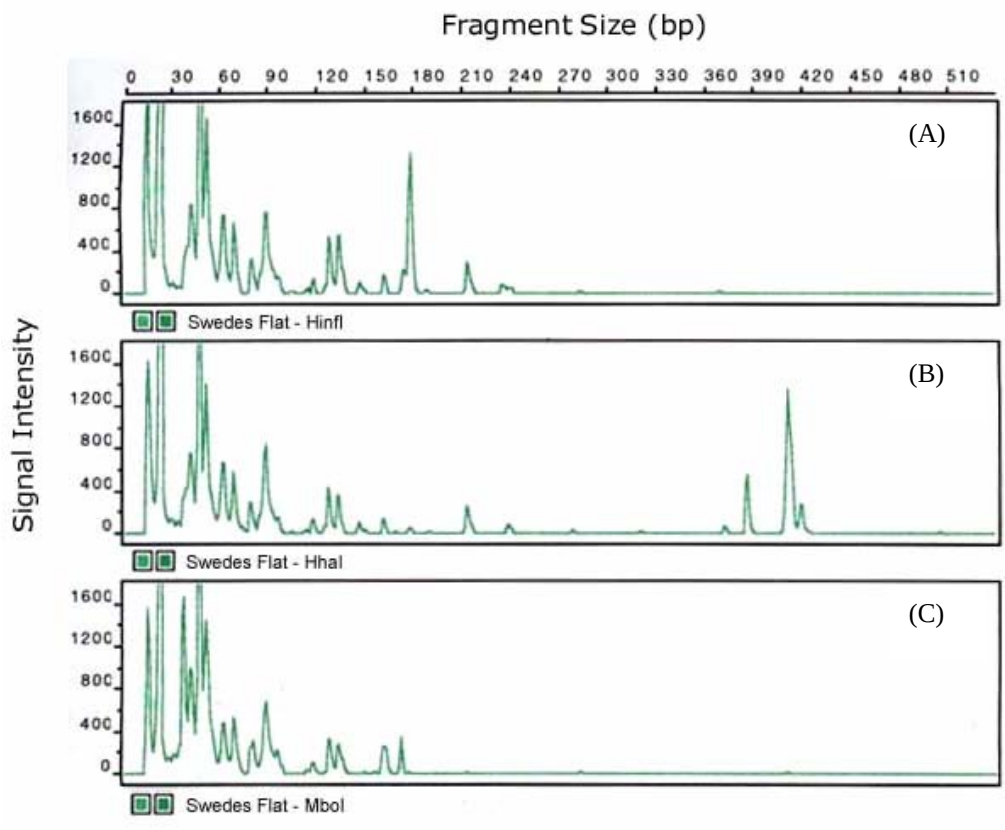


Figure 2.7: T-RFLP profile for the roots of six week old wheat grown in the field soil obtained from Swedes Flat (without NutriLife 4/20™) and digested with (A) *Hinf*I, (B) *Hha*I and (C) *Mbo*I.

Figure 2.8 shows the restriction pattern obtained with the *Hinf*I enzyme for the roots of six week old wheat grown in the field soils from Swedes Flat, Western Flat and Red Loam. The *Hinf*I TRF pattern was different for each of the roots grown in different field soils indicating a different actinobacterial composition.

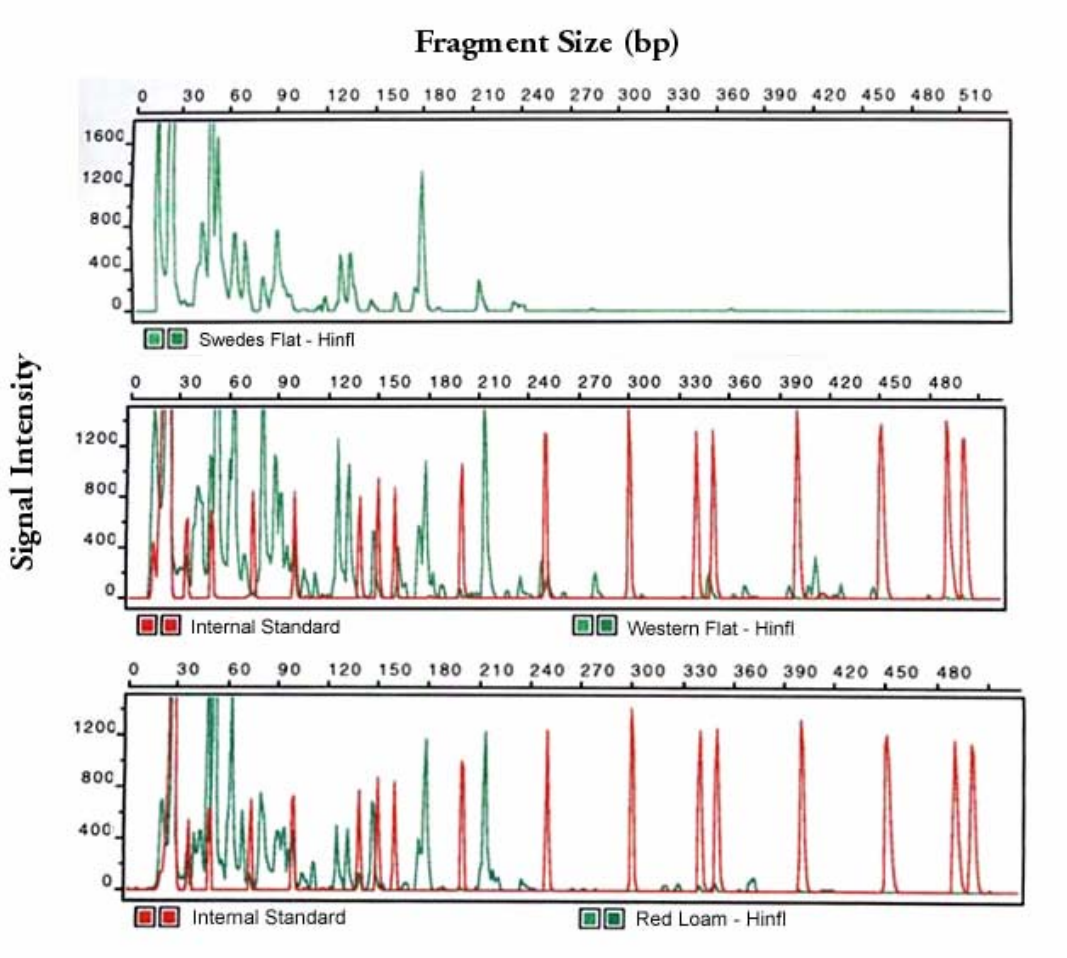


Figure 2.8: T-RFLP profile for the roots of six week old wheat grown in the field soils obtained from Swedes Flat (without NutriLife 4/20™), Western Flat and Red Loam and digested with the restriction enzyme *Hinf*I. The red peaks represent the internal standards and the green peaks the detected TRFs.

There was very little difference in the size of the TRFs obtained in the six repliciates. The TRFs were highly reproducible with a standard deviation from 0.04 bp to 4.52 bp; but, for the majority there was less than a 1 bp difference in size in the six replicates for all fragments. An example of the T-RFLP raw data for wheat grown in Red Loam, Swedes Flat (minus NutriLife 4/20™) and Western Flat is available as an online appendix that is supplementary data to a paper that was published from this work

http://som.flinders.edu.au/FUSA/Biotech/Acrobatfiles/VConnFranco/appendices.htm. The tables are quite lengthy and to keep the thesis concise the data was kept as an online appendix.

A number of TRFs did not have a match in the database, or there was a match for one enzyme but the corresponding TRFs from the other two enzyme digests were not present. Table 2.4 shows the percentage of peak areas for each restriction enzyme that matched to bacterial species that had the other two corresponding matches.

Table 2.4: Percentage of peak areas for restriction enzymes *Hinf*I, *Hha*I and *Mbo*I, that had all three matches in the TAP-T-RFLP database.

Enzyme	Red Loam	Western Flat	Swedes Flat minus NutriLife 4/20™	Swedes Flat plus NutriLife 4/20™
*Hinf*I	51.4%	30.7%	65.0%	70.2%
*Hha*I	43.9%	18.5%	61.5%	50.4%
*Mbo*I	12.8%	4.3%	45.2%	19.9%

The triple TRFs were then corresponded to a genus using the TAP-T-RFLP software. Table 2.5 and Figure 2.9 summarise the results and shows the maximum abundance percentenage each genus was detected by T-RFLP in the roots of wheat grown for six weeks in the field soils obtained from Red Loam, Western Flat and Swedes Flat.

Figure 2.10 shows the differences in peaks obtained with the *Hinf*I enzyme for wheat roots grown for six weeks in soil obtained from Swedes Flat with and without NutriLife 4/20™. The raw data for Swedes flat with NutriLife 4/20™ is available in Appendix 2, Tables A.2.1 and A.2.2. Table 2.6 and Figure 2.11 show the maximum abundance percentage for the genera identified and the difference in percentage for Swedes Flat with NutriLife 4/20™ compared to without. In all tables and figures, genera with less than one percent abundance in all soil types were omitted.

The highest diversity and level of endophytic colonisation by actinobacteria was found in wheat grown in Swedes Flat soil. This soil supported the highest number of indigenous microbes per gram of soil which is shown in Table 2.2. Swedes Flat with the NutriLife 4/20™ inoculum did have a higher $CFU.g^{-1}$ of soil, however, the number was inflated due to the addition of the non-indigenous microbes.

The dominant genera as determined by T-RFLP for the Swedes Flat soil were *Mycobacterium*, *Bifidobacterium*, *Rhodococcus*, *Streptomyces*, *Nocardia* and *Geodermatophilus*. The other two soils showed a concomitant decrease in endophytic diversity and colonisation with the bacterial load in the soil. Red Loam had the second highest diversity as determined by T-RFLP and the second highest indigenous microbial level. The major genera were identified as

Streptomyces, *Arthrobacter*, *Kineococcus*-like bacterium, *Amycolatopsis* and *Nocardia*. Wheat plants grown in Western Flat soil, a water-repellent sandy soil which supported the lowest number of microorganisms, also had the least diversity and level of endophytic colonisation. The major genera identified were *Kitasatospora*, followed by *Mycobacterium*.

The T-RFLP method detected 41 actinobacteria genera present in the roots of wheat grown in Swedes Flat soil without the added microbial inoculant. When NutriLife 4/20™ was added both the endophytic actinobacteria population and the level of colonisation reduced by approximately half (21 genera) for the majority of genera but between a range of 14% to 86%. Three genera increased in the soil with the addition of NutriLife 4/20™. *Kribella* increased from 1.06% to 15.69%, *Thermonospora* 5.47% to 6.39% and *Nocardioides* 1.06% to 2.82%. These actinobacteria genera were not a component of the inoculant (see 2.2.1.3 for inoculant details). The inoculant did contain *Streptomyces albidoflavus* and *Streptomyces cellulosae*, however, the endophytic *Streptomyces* decreased from 18.53% to 9.15%. *S. cellulosae* and *S. albidoflavus* though have not been reported to be endophytes and not all soil microbes are expected to have the capability of endophytic colonisation.

Table 2.5: Maximum abundance percentage of each genus identified by T-RFLP in the roots of wheat grown for six weeks in field soils obtained from Red Loam, Western Flat and Swedes Flat (without NutriLife 4/20™).

Genus	Red Loam	Swedes Flat	Western Flat
Mycobacterium	1.30	21.02	2.45
Bifidobacterium	5.78	20.78	1.24
Rhodococcus	1.95	20.64	1.24
Streptomyces	14.35	18.53	0.00
Nocardia	6.22	16.75	1.18
Geodermatophilus	1.30	13.76	1.24
Saccharomonospora	1.30	9.45	0.62
Arthrobacter	11.49	7.52	0.62
Microbacterium	0.65	7.41	0.62
Frankia	1.30	6.51	0.62
Gordonia	0.65	6.88	1.26
Saccharothrix	0.00	6.44	0.59
Brevibacterium	4.50	6.05	1.24
Thermonospora	2.62	5.47	0.00
Kitasatospora	1.95	5.52	15.02
Pimelobacter	0.65	4.99	0.62
Lentzea	1.30	4.99	0.62
Agromyces	0.00	4.84	0.00
Actinomyces	0.00	4.72	0.00
Williamsia	0.65	4.46	0.00
Thermocrispum	2.62	4.46	0.00
Sanguibacter	0.65	4.46	0.00
Rubrobacter	4.93	4.46	0.00
Promicromonospora	0.65	4.46	0.00
Corynebacterium	1.30	1.06	1.24
Micrococcus	0.65	2.95	0.62
Micromonospora	0.00	2.51	0.59
Leifsonia	0.00	2.42	0.00
Dietzia	0.00	2.42	0.00
Curtobacterium	0.00	2.42	0.00
Kineococcus-like bacterium	11.49	1.54	0.62
Actinosynnema	2.46	1.98	1.18
Actinoplanes	4.92	2.00	0.59
Catellatospora	0.65	1.06	0.62
Sarraceniospora	0.65	1.06	0.00
Nocardioides	3.27	0.53	0.00
Lechevalieria	0.65	1.06	0.62
Kribella	0.00	1.06	0.00
Streptoalloteichus	2.46	1.01	0.00
Spirilliplanes	2.46	1.01	0.00
Amycolatopsis	7.73	0.00	0.00

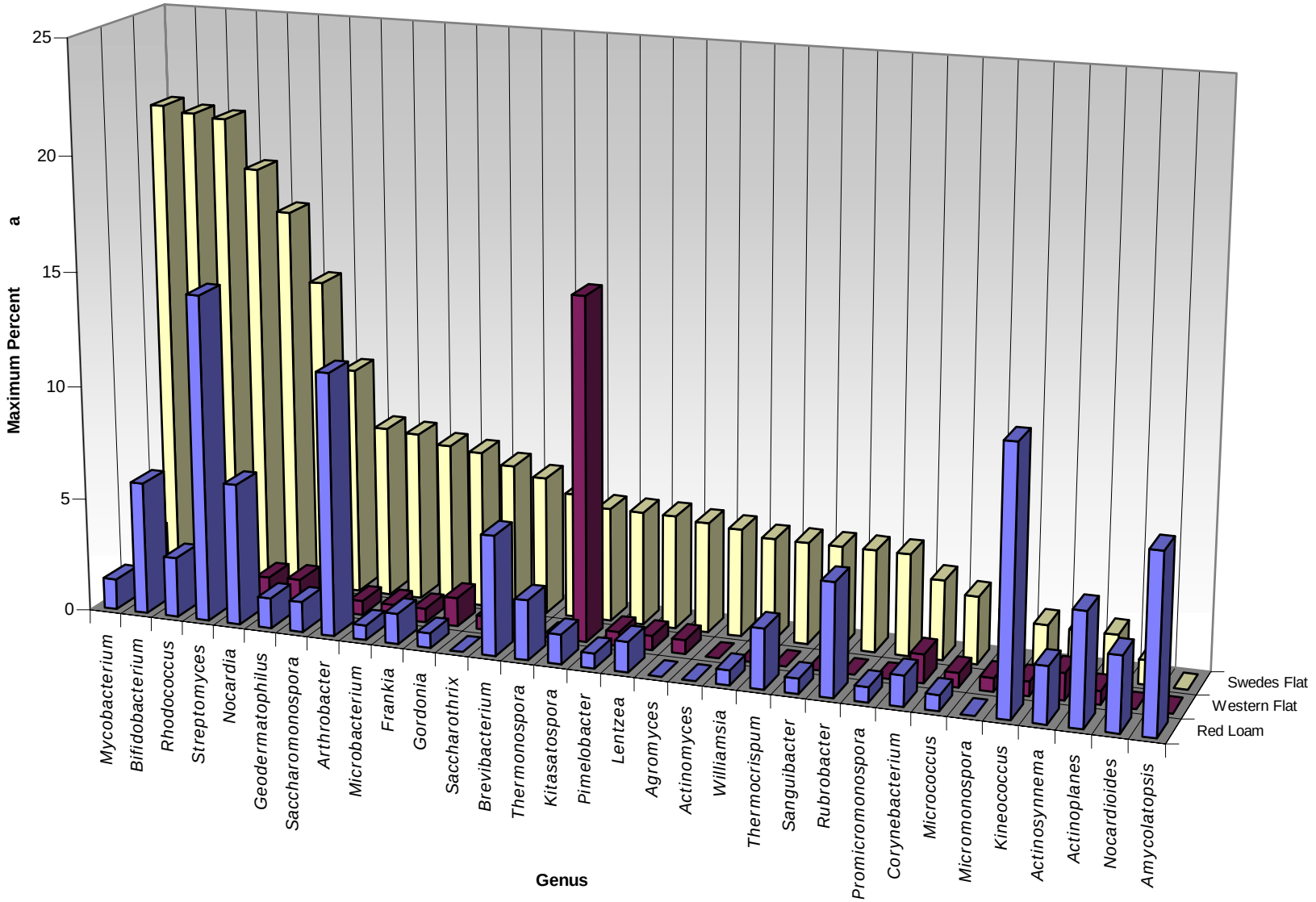


Figure 2.9: Endophytic actinobacteria genera indentified in the roots of wheat grown in three different field soils.

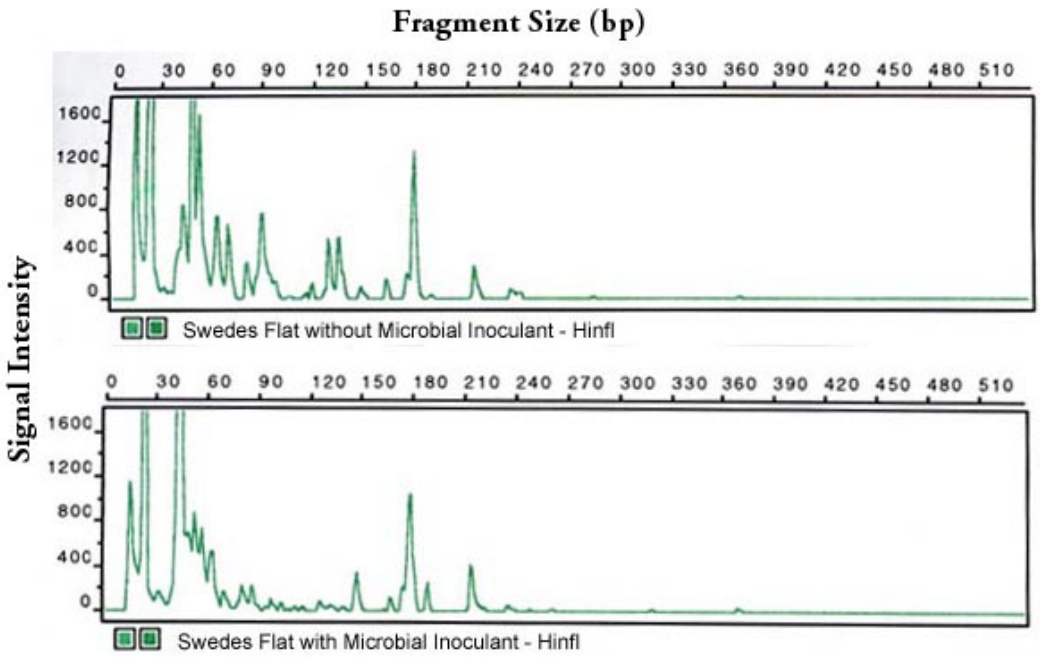


Figure 2.10: T-RFLP profile for the roots of wheat grown for six weeks in the field soil Swedes Flat with and without NutriLife 4/20™ and digested with *Hinf*I.

Table 2.6: Maximum abundance percentage of each genus identified by T-RFLP in the roots of wheat grown for six weeks in soil obtained from Swedes Flat with and without NutriLife 4/20™.

Genus	With NutriLife 4/20™	Without NutriLife 4/20™	Decrease (%) of Genera in soil with NutriLife 4/20™
Mycobacterium	9.75	21.02	53.62
Bifidobacterium	2.82	20.78	86.43
Rhodococcus	9.49	20.64	54.02
Streptomyces	9.15	18.53	50.62
Nocardia	3.25	16.75	80.60
Geodermatophilus	6.24	13.76	54.65
Saccharomonospora	0.00	9.98	100.00
Arthrobacter	0.00	7.52	100.00
Microbacterium	6.24	7.41	15.79
Frankia	0.00	7.04	100.00
Gordonia	4.16	6.88	39.53
Saccharothrix	0.00	6.44	100.00
Brevibacterium	0.00	6.05	100.00
Thermonospora	6.39	5.47	-16.82
Kitasatospora	0.00	4.99	100.00
Pimelobacter	2.08	4.99	58.32
Lentzea	0.00	4.99	100.00
Agromyces	2.08	4.84	57.02
Actinomyces	0.00	4.72	100.00
Williamsia	0.00	4.46	100.00
Thermocrispum	0.00	4.46	100.00
Sanguibacter	0.00	4.46	100.00
Rubrobacter	2.08	4.46	53.36
Corynebacterium	2.08	3.48	40.23
Micrococcus	2.08	2.95	29.49
Micromonospora	0.00	2.51	100.00
Leifsonia	1.17	2.42	51.65
Dietzia	2.08	2.42	14.05
Curtobacterium	1.17	2.42	51.65
Brachybacterium	1.17	2.42	51.65
Kineococcus-like bacterium	0.00	2.07	100.00
Actinosynnema	0.00	2.00	100.00
Actinoplanes	0.00	2.00	100.00
Catellatospora	0.00	1.59	100.00
Sarraceniospora	0.00	1.06	100.00
Nocardioides	2.82	1.06	-166.04
Lechevalieria	0.00	1.06	100.00
Kribella	15.69	1.06	-1380.19
Streptoalloteichus	0.00	1.01	100.00
Spirilliplanes	0.00	1.01	100.00
Planomonospora	2.82	0.00	-100.00

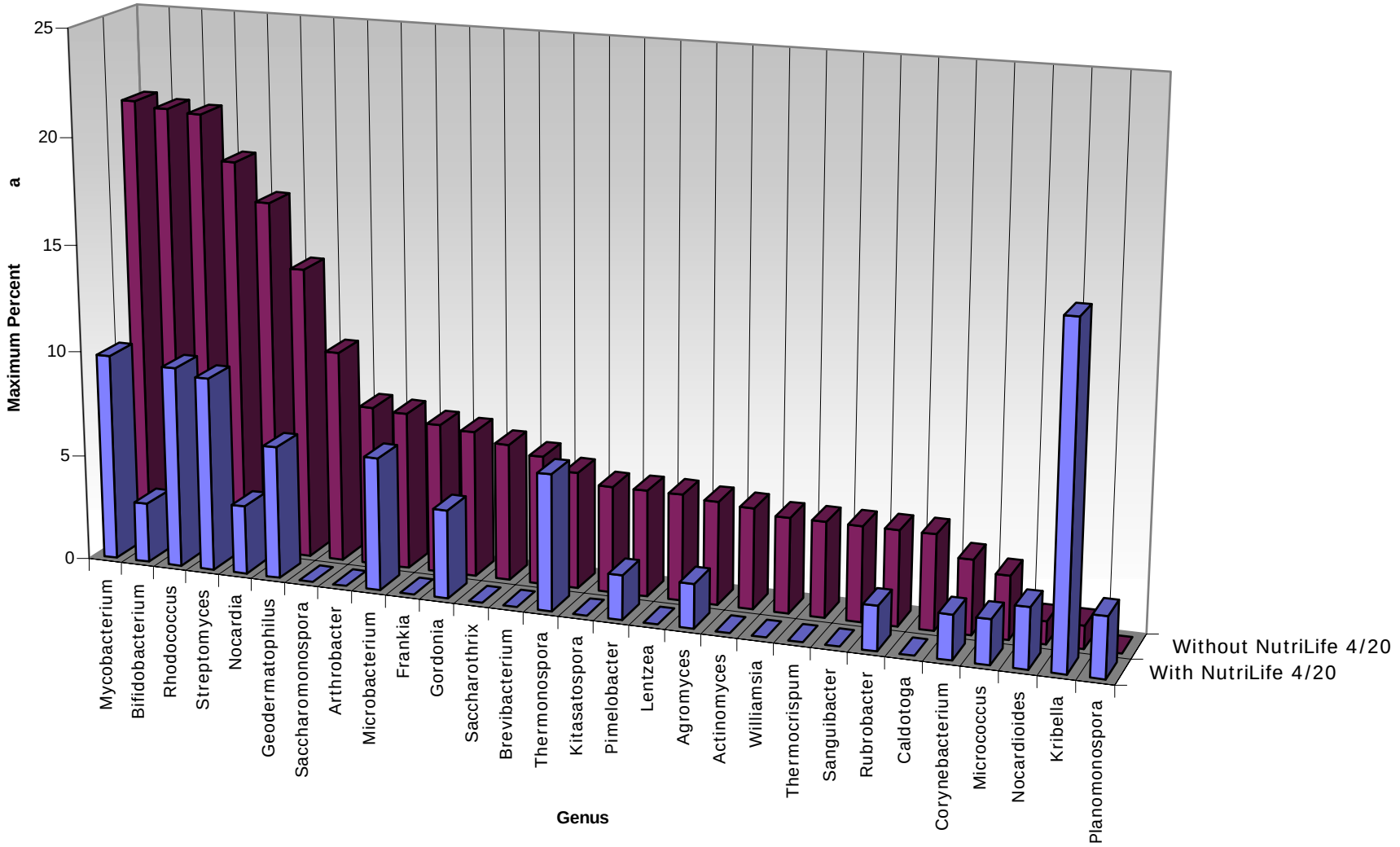


Figure 2.11: Comparison of actinobacteria genera in wheat roots grown in soil from Swedes Flat with and without NutriLife 4/20™.

2.3.4.2 Partial 16S rRNA Gene Sequencing

The T-RFLP technique can determine the possible species that are present within a sample. However, the fragments often match a number of different species and even different genera based on a set of three fragment matches. To conclusively identify endophytic actinobacteria species the 16S rRNA gene sequences were cloned and sequenced. The partial 16S rRNA gene sequences were compared to sequences available in the NCBI database ((http://www.ncbi.nlm.nih.gov) and the results are shown in Tables 2.7 and Table 2.8. Table 2.9 lists the closest actinobacteria sequence match identified by sequencing with the corresponding reference when deposited in the GenBank database. Appendix 3 shows the three highest matches to sequences in the Genbank database available through NCBI with the corresponding bit score and percentage identity.

Eight different genera were identified by sequencing and all genera were detected by T-RFLP. The predominant genus identified was *Mycobacterium* (62%) with 16 different species identified followed by *Streptomyces* (12%) with 4 different species sequenced. The remainder were identified as *Rhodococcus* (6%), *Amycolatopsis* (3%), *Micromonospora* (3%), *Gordonia* (1.5%), Nocardia (1.5%) and *Nocardioides* (1.5%). A number of uncultured bacteria (6%) were also identified.

Table 2.7: Highest sequence match of partial 16S rRNA actinobacteria sequences isolated from the roots of wheat grown for six weeks in the field soils from Red Loam, Western Flat and Swedes Flat with or without NutriLife 4/20™.

Highest Seq. Match	Red Loam 17 Clones	Western Flat 12 Clones	Swede Flat - NutriLife 4/20™ 16 Clones	Swede Flat + NutriLife 4/20™ 20 Clones
Amycolatopsis sp. GY152	-	-	2	-
Gordonia polyisoprenivorans	-	-	1	-
Micromonospora endolithica	1	-	-	-
Micromonospora peucetica	1	-	-	-
Mycobacterium aichiense	-	-	1	3
Mycobacterium austroafricanum	-	-	-	1
Mycobacterium bohemicum	-	2	-	-
Mycobacterium chubuense	-	-	-	1
Mycobacterium cookii	1	-	1	1
Mycobacterium flavescens	-	-	1	-
Mycobacterium heidelbergense	1	-	-	-
Mycobacterium interjectum	-	-	-	1
Mycobacterium IWGMT 90174	-	-	-	2
Mycobacterium lacus	-	-	-	1
Mycobacterium palustre	-	1	-	-
Mycobacterium scrofulaceum	-	-	-	1
Mycobacterium sp.	1	-	-	1
Mycobacterium sp. 2333	1	2	2	-
Mycobacterium sp. IMVS B76676	5	4	5	1
Mycobacterium sp. 'MCRO 33'	-	-	1	-
Nocardia pseudobrasiliensis	1	-	-	-
Nocardioides sp. 4P1-A	-	-	-	1
Rhodococcus coprophilus	1	-	-	3
Streptomyces griseochromogenes	-	-	-	1
Streptomyces sp. EF-91	-	1	-	-
Streptomyces sp. SE2	1	-	1	1
Streptomyces thermolineatus	3	-	-	-
Uncultivated soil bacterium clone C019	-	-	-	1
Uncultured actinobacterium clone SMW4.128WL	-	-	1	-
Uncultured eubacterium WD294	-	1	-	-
Uncultured maize root bacterium Zmrc174	-	1	-	-

Table 2.8: Relative percentage of genera identified from the sequencing of actinobacteria 16S rRNA partial gene sequences isolated from the roots of wheat grown in four different field soils.

Genera	Percent of Clones
Amycolatopsis	3.08%
Gordonia	1.54%
Micromonospora	3.08%
Mycobacterium	64.62%
Norcardia	1.54%
Nocardioides	1.54%
Rhodococcus	6.15%
Streptomyces	12.31%
Uncultured bacteria	6.15%

Table 2.9: Actinobacteria species identified as the closest match by partial 16S rRNA gene sequencing.

Actinobacteria Species	GenBank Ac. No.	Reference	Comments
Amycolatopsis sp. GY152	AY222828	Tan *et al.* Unpublished	No comments.
Gordonia polyisoprenivorans	AF416719	Arenskotter *et al.* (2001)	Taxonomic characterization of two species of *Gordonia polyisoprenivorans* that were capable of degrading isosprene rubber.
Micromonospora endolithica	AJ56035	Hirsch *et al.* (2004)	Cryptoendolithic actinomycetes were isolated from Antarctic sandstone rock.
Micromonospora peucetica	X92603	Koch *et al.* (1996)	No comments
Mycobacterium achiense JS618	AF498656	Coleman *et al.* (2002)	Isolated from a industrial soil site in Germany and capable of degrading vinyl chloride.
Mycobacterium austroafricanum	AF544626	Leys *et al.* Unpublished. Springael *et al.* Unpublished	Isolated from an environmental site and is a fast-growing polycyclic aromatic hydrocarbon (PAH) degrading species.
Mycobacterium bohemicum	AJ277283	Torkko *et al.* (2001)	Characterization of *Mycobacterium* isolates from human and veterinary samples and stream water.
Mycobacterium chubuense	AF480597	Turenne *et al.* (2001)	Characterization of 16S rRNA sequences from nontuberculous mycobacteria using laboratory and type strains for comparisons against databases entries.
Mycobacterium cookii	AF480598	Turenne *et al.* (2001)	As above.
Mycobacterium flavescens	AF480579	Turenne *et al.* (2001)	As above.
Mycobacterium heidelbergense	AJ000684	Haas *et al.* (1993)	Isolated as the causative agent of cervical lymphadenitis in children.
Mycobacterium interjectum	AF014936	Lumb *et al.* (1997)	Characterization of three *Mycobacterium interjectum* clinical isolates. Two isolated from lymph nodes of children with cervical lymphadenitis and one from sputum of an elderly male with chronic lung disease.
Mycobacterium IWGMT 90174	X88908	Wayne *et al.* (1996)	Semantide and chemotaxonomy based analyses of some problematic phenotypic clusters of slowly growing mycobacteria.
Mycobacterium lacus	AF406783	Turenne *et al.* (2002)	Isolated from the synovial tissue from a female with bursitis of her right elbow and proposed as a pathogen.
Mycobacterium palustre	AJ308603	Torkko *et al.* (2002)	Isolated from a lymph-node biopsy from a child with cervical lymphadenitis and should be listed as a potential inducer of paediatric lymphadenitis.
Mycobacterium scrofulaceum	AF480604	Turenne *et al.* 2001.	As above.
Mycobacterium sp.	AB010912	Colquhoun *et al.* Unpublished.	Taxonomy and biotransformation activities of some deep-sea actinomycetes.
Mycobacterium sp. 2333	AY065649	Englund *et al.* (2002)	A strain very closely related to *M. cooki* and *Mycobacterium* sp. strain IMVS B76676.

Table 2.9 cont.

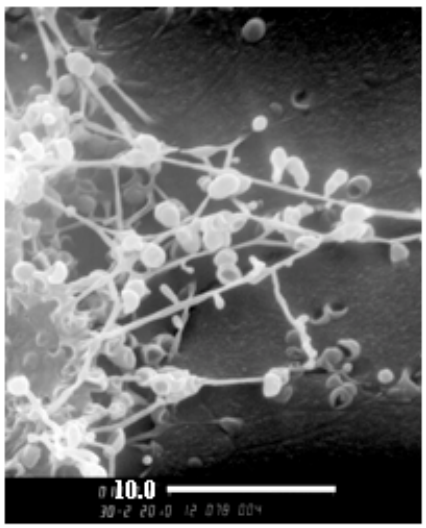
Actinobacteria Species	GenBank Ac. No.	Reference	Comments
Mycobacterium sp. IMVS B76676	AF016407	Goodwin *et al.* (1998)	Isolated from the cervical lymph node of an adolescent male and 16S rRNA sequencing and HPLC suggest isolate belongs to an unrecognised pathogenic species.
Mycobacterium sp. 'MCRO 33'	AF152559	Roth *et al.* (2000)	A clinical isolate very closely related to *Mycobacterium scrofulaceum.*
Nocardia pseudobrasiliensis	AB086861	Poonwan *et al.* Unpublished.	No comment.
Nocardioides sp. 4P1-A	AY027587	Behrend and Heesche-Wagner (1999)	Actinobacteria containing highly conserved genes encoding ring hydrogenation of picric acid and 2,4-Dinitrophenol.
Rhodococcus coprophilus	X80626	Rainey *et al.* (1995)	No comment.
Streptomyces griseochromogenes	AJ310923	Rashidian *et al.* Unpublished	No comment.
Streptomyces sp. EF-91	AF112174	Doumbou *et al.* (2001)	Isolated from potato and does not cause common scab disease indicating and endophytic relationship. Closely related to *Streptomyces albidoflavus.*
Streptomyces thermolineatus	Z68097	Kim *et al.* unpublished	No comment.
Streptomyces sp. SE2	AY148089	Coombs and Franco (2003a)	Isolated from the roots of healthy wheat plants grown in South Australia.
Uncultivated soil bacterium clone C019	AF013522	Kuske *et al.* (1997)	Isolated from soil in the arid Southwestern United States.
Uncultured actinobacterium clone SMW4.128WL	AY043872	Axelrood *et al.* (2002)	Isolated from surface organic matter and mineral soil samples from a British Columbia Ministry of Forests Long-Term Soil Productivity installation.
Uncultured eubacterium WD294	AJ292686	Nogales *et al.* (2001)	Isolated from polychlorinated biphenyl (PCB)-polluted soil.
Uncultured maize root bacterium Zmrc174	AF226216	Chelius and Triplett (2001)	Isolated from a maize root.

2.3.5 Analysing the Impact of the Introduction of Endophytes in Wheat by T-RFLP

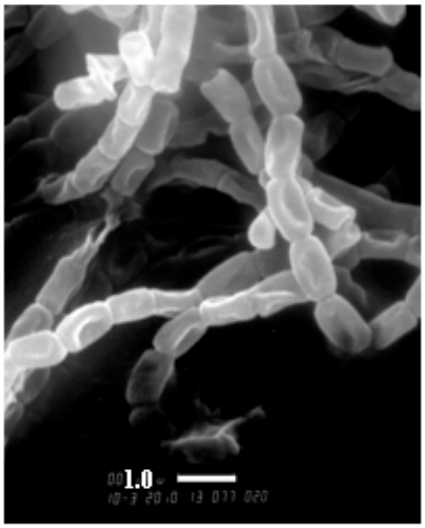
Wheat seeds were coated with the actinobacteria spores before being sown. Coating seeds with the inoculum has previously been found to be a suitable way of introducing the actinobacteria strains into the wheat roots (Coombs and Franco, 2003a). The level of colonisation can be determined by culture methods; however, in this study the T-RFLP technique was used to investigate two issues: (1) can the technique detect and quantify the introduced endophytes? (2) does introducing an endophyte affect the indigenous microbial population?

2.3.5.1 Detection and Quantification of Introduced Endophytes in Wheat Roots by T-RFLP

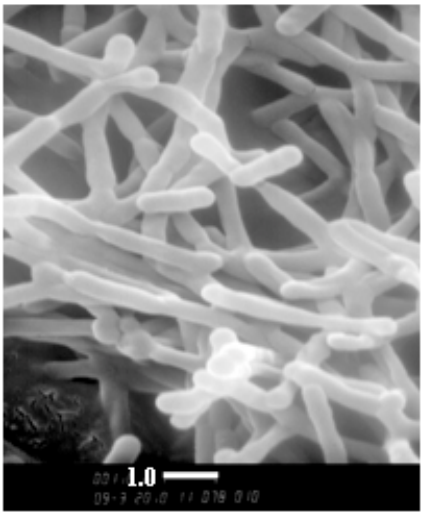
The three actinobacteria endophytes, EN2 (*Microbispora* sp.), EN27 (*Streptomyces* sp.) and EN46 (*Nocardioides albus*) investigated in the study were coated onto wheat seeds (cultivar Krichauff) as spores and the coated seed grown in soil obtained from Haslam in South Australia. Pure cultures of each endophyte were analysed by the T-RFLP technique to determine if the unique fragments obtained with the restriction enzymes (*Hinf*I, *Hha*I and *Mbo*I). Table 2.10 shows the fragment size obtained for each endophyte. Figure 2.12 shows electron micrographs of the actinobacteria endophyte pure cultures. Visual confirmation of wheat root colonisation was also performed by electron microscopy. Figure 2.13 shows the *Streptomyces* sp. EN27 present on the surface of a wheat root, within a dead root cell and in the intercellular space confirming endophytic colonisation.



(A) - *Microbispora* **sp. strain EN2**



(B) - *Streptomyces* **sp. strain EN27**



(C) - *Norcardioides albus* **EN46**

Figure 2.12: Electron micrographs of the spores of *Microbispora* sp. EN2, *Streptomyces* sp. EN27 and *Nocardioides albus* EN46. The white bar indicates distance in microns.

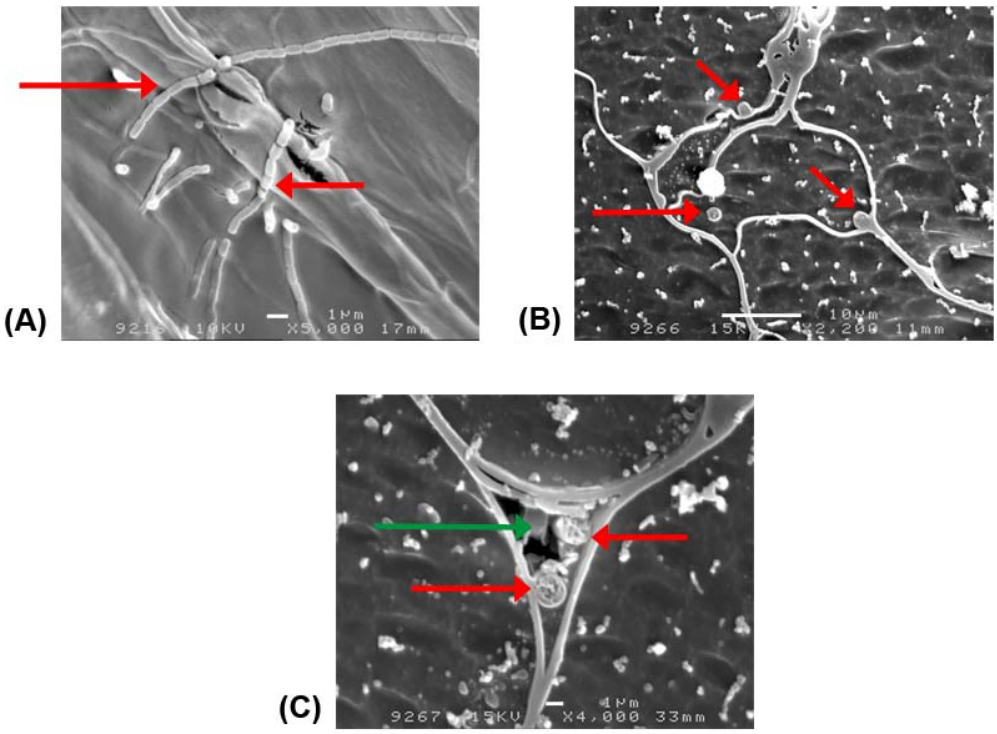


Figure 2.13: Endophytic colonisation of the wheat root by *Streptomyces* sp. EN27. Red arrows indicate EN27 spores. (A) *Streptomyces* sp. EN27 spores present on the surface of a wheat root. (B) Cross section of a wheat root showing the *Streptomyces* sp. EN27 spores or hyphae present within a dead cell. (C) Cross section of wheat root showing *Streptomyces* sp. EN27 present within the intercellular space which is indicated by the green arrow.

Table 2.10: Termina fragment sizes of EN2, EN27 and EN46 16S rRNA digested with *Hinf*I, *Hha*I and *Mbo*I.

Name	**Species**	Enzyme	Fragment of Interest (bp)
EN46	*Nocardioides albus*	*Hinf*I	178.46
		*Hha*I	410.54
		*Mbo*I	162.68
EN27	*Streptomyces* sp.	*Hinf*I	240.61
		*Hha*I	419.71
		*Mbo*I	163.30
EN2	*Microbispora* sp.	*Hinf*I	175.32
		*Hha*I	418.66
		*Mbo*I	162.37

The 16S rRNA gene was amplified from DNA extracted from the roots of the endophyte-inoculated wheat and from an uninoculated plant at six weeks of growth. The T-RFLP profile obtained with *Hinf*I for each of these plants in shown in Figure 2.14, the annotated peaks indicate the fragment corresponding to the introduced endophyte. Only the *Hinf*I T-RFLP profile has been shown as the fragments obtained are of different sizes for each endophyte. Table 2.11 shows the three specific fragments belong to the inoculated endophytes and the minimum and maximum percentages of the peak areas (not corrected).

When analysing the minimum and maximum abundance percentages (not corrected) of the specific fragments for the EN2-inoculated plant the *Hinf*I and *Hha*I fragments increased by approximately two-fold and three-fold, respectively, whereas the *Mbo*I fragment increased by 1.1 fold in relation to the maximum percentage.

For the EN27-inoculated plant the specific 241 bp *Hinf*I fragment was not present in the uninoculated plant and the *Hha*I specific fragment increased by two-fold when the maximum percentages were analysed whereas the *Mbo*I 163 bp fragment decreased by 0.4-fold.

The specific *Hha*I fragment for EN46 decreased by approximately 0.4-fold in the EN46-treated plant compared to the un-inoculated plant. The *Hinf*I also descreased but only very slightly, from 5.78% in the un-inoculated plant to 5.41% in the EN46-treated plant. However, the *Mbo*I specific fragment increased with by 2.2 fold, but only in one of the three replicate plants.

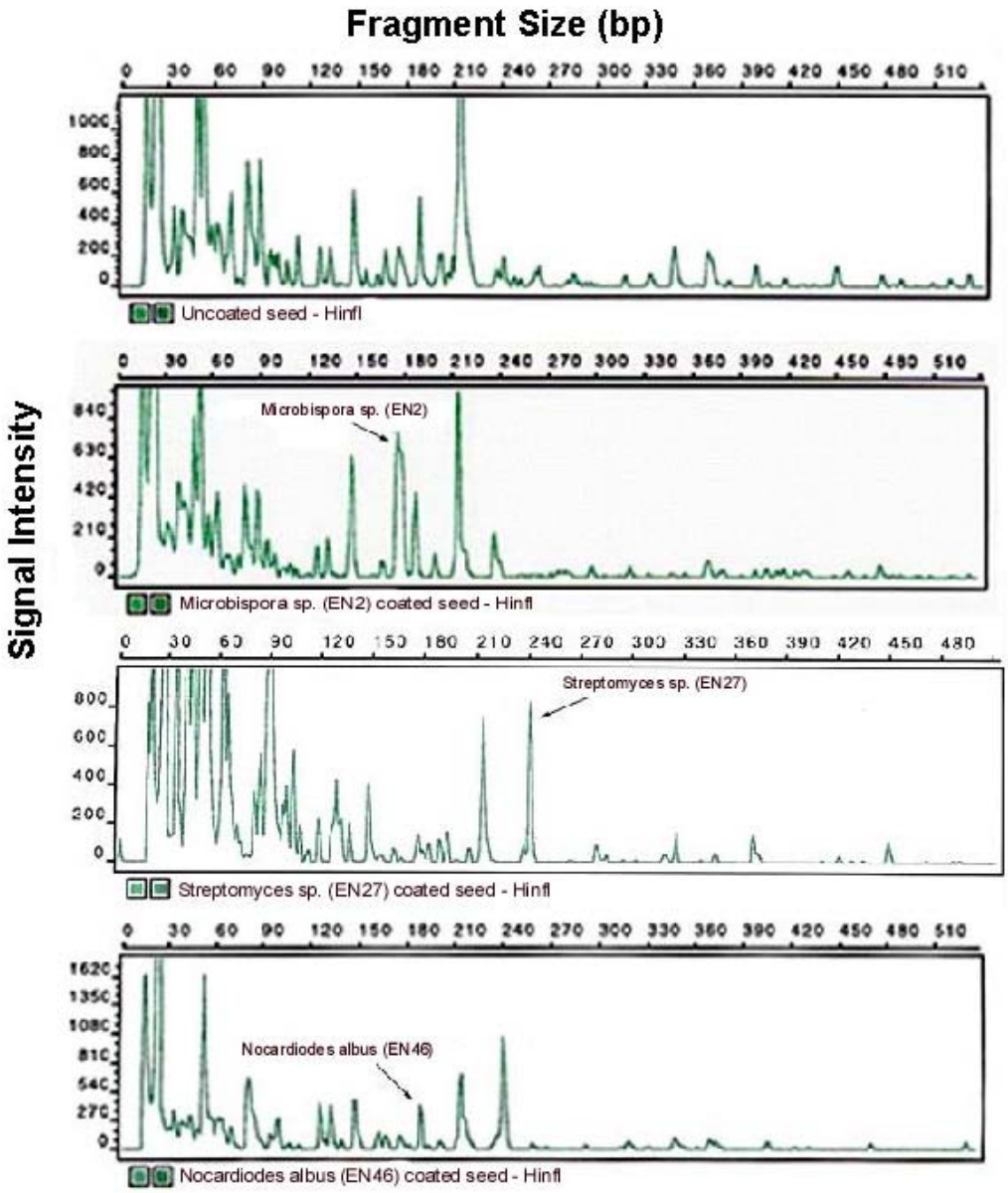


Figure 2.14: T-RFLP *Hinf*I profiles for the roots of wheat grown from endophyte inoculated and uninoculated seed. The highlighted peaks correspond to the specific fragment of the actinobacteria endophyte inoculated onto the seed.

Table 2.11: Comparison of the minimum and maximum fragment abundance percentages that correlate to the actinobacteria inoculants obtained from T-RFLP profiles in the roots of wheat inoculated with an endophyte to a non-inoculated plant.

Restriction Enzyme	**Fragment of Interest**	**EN2 Inoculated Wheat Roots**		**Uninoculated Wheat Roots**	
		Min %	Max %	Min %	Max %
*Hinf*I	176	0.96	6.17	0.69	3.33
*Hha*I	420	2.98	10.94	1.69	3.33
*Mbo*I	162	1.58	11.08	3.62	10.13
Restriction Enzyme	**Fragment of Interest**	**EN27 Inoculated Wheat Roots**		**Uninoculated Wheat Roots**	
		Min %	Max %	Min %	Max %
*Hinf*I	241	1.26	3.55	n/a	n/a
*Hha*I	420	2.77	5.80	1.69	2.96
*Mbo*I	163	4.19	7.06	3.62	10.13
Restriction Enzyme	**Fragment of Interest**	**EN46 Inoculated Wheat Roots**		**Uninoculated Wheat Roots**	
		Min %	Max %	Min %	Max %
*Hinf*I	179	0.58	5.41	0.38	5.78
*Hha*I	411	1.02	9.45	4.61	14.57
*Mbo*I	162	1.87	23.19	3.62	10.13

2.3.5.2 The Effect of Introducing Actinobacteria Endophytes on the Indigenous Endophytic Population of Wheat Roots

The fragments obtained from the T-RFLP profiles for each restriction enzyme and plant treatment are shown in Appendix 2, Tables A.2.3, A.2.4 and A.2.5. From this data it can be seen that there are some differences in the roots of the wheat inoculated with the endophytes. The endophytic species present in the roots of all the inoculated and uninoculated plants are presented in Appendix 2 Tables A.2.6, A.2.7, A.2.8, A.2.9 and A.2.10 showing the additional species present within the particular plant treatment. Among the four different plant treatments a total of 58 genera were identified. Table 2.12 and Figure 2.15 show the maximum abundance percentage for each genus in the inoculated and

uninoculated plants. Only 28 genera were included in this table as genera with a maximum abundance percent below one percent in all sample groups were omitted.

The T-RFLP technique detected 58 actinobacterial genera with 49 genera with a maximum abundance percent over 0.6%, with approximately 92% of the genera detected present in at least three of the treatments. The predominant genera in all treatments were *Kribbella*, *Streptomyces*, *Bifidobacterium*, *Arthrobacter*, *Nocardia* and *Rhodococcus*. *Kribbella* was detected in the un-inoculated control at 13.72% and at 13.21% and 13.68% in the *Microbispora* sp. EN2 and *Streptomyces* sp. EN27 inoculated plants, respectively. However, in the *Nocardioides albus* EN46-treated plant *Kribbella* species decreased to 7.19%. The *Streptomyces* species increased from 12.15% (un-inoculated) to 20.12% in the *Streptomyces* sp. EN27 inoculated plants. However, the *Streptomyces* species also increased in the *Microbispora* sp. EN2- (18.37%) and *Nocardioides albus* EN46-treated (30.66%) roots.

Table 2.12: Maximum abundance percentage of actinobacteria genera present in the roots of wheat grown from endophyte inoculated and uninoculated seed for six weeks in soil obtained from Haslam.

Genus	Un-Inoculated	EN2-Inoculated	EN27-Inoculated	EN46-Inoculated
Kribbella	13.72	13.21	13.68	7.19
Streptomyces	12.15	19.75	20.12	32.24
Bifidobacterium	6.05	11.92	3.43	15.96
Arthrobacter	5.26	5.79	2.73	3.08
Nocardia	4.00	3.34	0.39	0.87
Actinoplanes	2.92	0.60	0.00	0.00
Rhodococcus	2.81	7.03	1.52	2.84
Kineococcus-like bacterium	2.54	3.04	0.28	0.86
Nocardioides	2.53	2.91	1.04	4.18
Rubrobacter	2.46	2.08	0.33	3.70
Mycobacterium	2.27	3.45	1.11	2.20
Geodermatophilus	2.27	4.29	1.13	2.20
Microbispora.	2.26	2.34	1.18	1.66
Brevibacterium	2.16	4.38	0.56	1.61
Thermomonospora	2.10	4.42	1.68	3.40
Saccharomonospora	1.73	4.01	0.66	1.07
Corynebacterium	1.62	3.02	0.53	1.41
Frankia	1.62	3.28	0.56	1.61
Microbacterium	1.62	1.64	0.64	1.53
Lechevalieria	1.08	1.64	0.28	0.86
Lentzea	1.08	2.74	0.28	0.75
Sarraceniospora	1.08	1.64	0.28	0.86
Kitasatospora	1.08	2.74	0.28	3.16
Micrococcus	1.08	1.64	0.39	0.98
Pimelobacter	1.08	1.64	0.39	0.98
Gordonia	1.08	1.65	0.91	1.10
Actinomyces	0.83	2.54	0.76	0.64
Thermocrispum	0.65	2.23	0.38	6.07

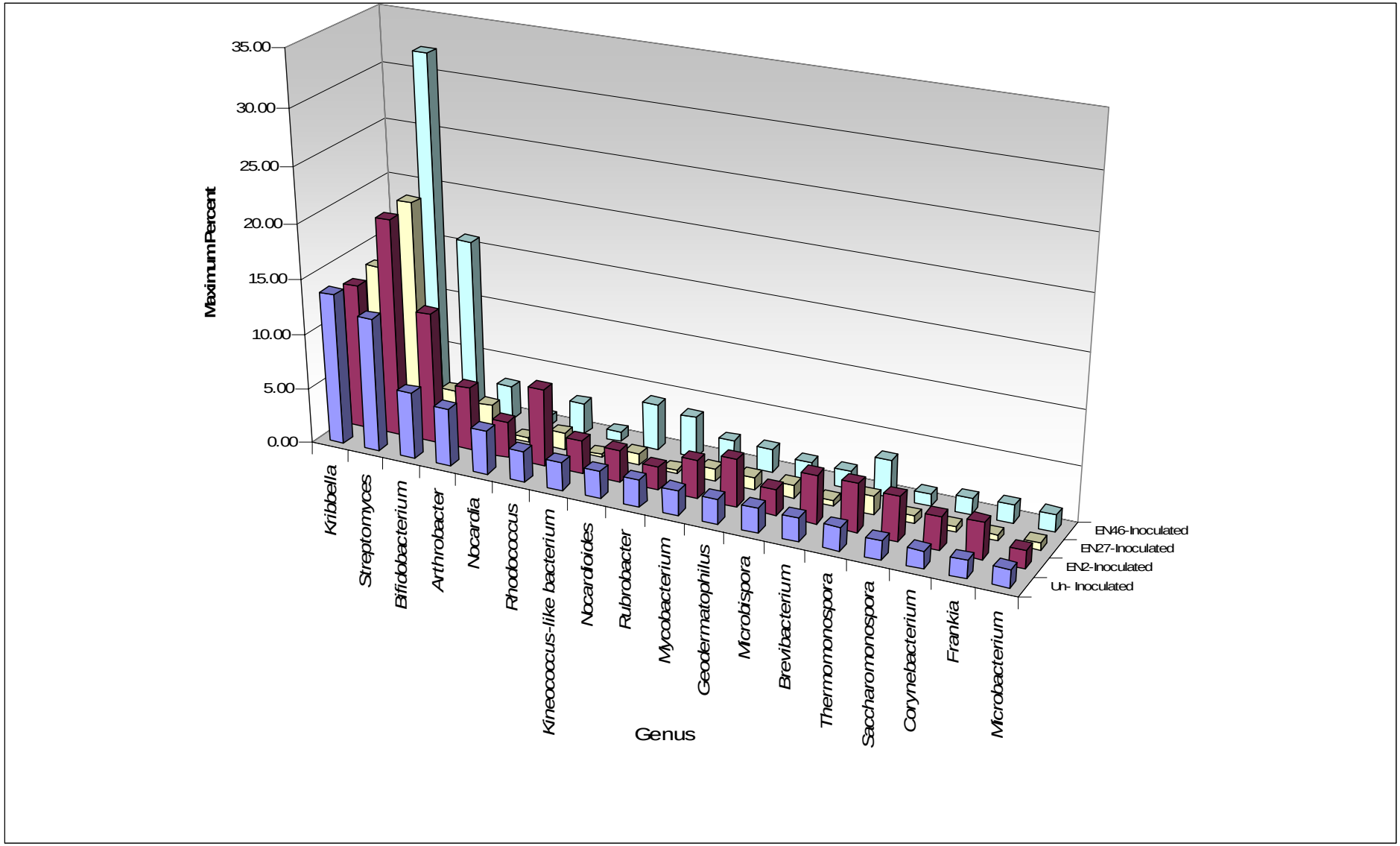


Figure 2.15: Endophytic actinobacteria genera present in inoculated and un-inoculated wheat roots

2.3.6 Analysis of the Endophytic Eubacteria Population of Wheat Roots by T-RFLP

Analysis of the general endophytic bacterial population of wheat roots and shoots was performed by the T-RFLP method. The 16S rRNA PCR was able to be performed from endophytic eubacterial DNA obtained from the wheat root and shoot using the 8f and 1492r (Bacr) primer set. The T-RFLP strategy was as per Figure 2.3, except the restriction enzyme *Msp*I was used instead of *Mbo*I. The T-RFLP technique using the eubacterial primer set was first validated using actinobacteria species EN46, EN2, EN27, a type strain of *Streptomyces scabies* and the non-actinobacteria species *Pseudomonas fluorescens.* The fragment sizes obtained by T-RFLP are shown in Table 2.13.

The *Hinf*I, *Hha*I and *Msp*I digested 16S rRNA gene from the different plant treatments was analysed by GeneScan. The GeneScan files obtained showed that for nearly all of the plant groups there were fewer peaks than for the actinobacteria groups and when peaks were obtained, the peak heights were small. The uninoculated wheat seed grown in soil obtained from Haslam showed the largest number of peaks with reasonable intensity. Therefore only this plant treatment was used for analysis. The *Msp*I and *Hinf*I restriction enzymes generated a number of peaks, whereas *Hha*I did not and was excluded from the analysis. Figure 2.14 and 2.15 show the GeneScan file for the *Msp*I and *Hinf*I digested 16S rRNA gene from the roots and shoots of an un-inoculated wheat seed in Haslam soil. The T-RFLP profiles obtained for the wheat roots had a larger number of peaks than obtained in the shoots indicating a larger bacterial population.

Table 2.14 shows the fragments present in the roots and shoots of this plant and it was noted that there were fewer fragments obtained from the shoots. Table 2.15 shows the matches of the *Msp*I and *Hinf*I fragments to bacterial genera present in the TAP-T-RFLP database. Many of the fragments generated by the restriction digestions did not match to any bacterial species in the database.

Table 2.13: Fragment sizes of the actinobacteria 16S rRNA gene digested with *Hinf*I, *Hha*I and *Msp*I.

Culture Name	Species	Enzyme	Fragment Size (bp)
EF1	*Pseudomonas fluorescens*	*Hinf*I	119.40
		*Hha*I	207.58
		*Msp*I	496.15
EF2	*Pseudomonas fluorescens*	*Hinf*I	119.46
		*Hha*I	207.57
		*Msp*I	496.04
EN2	*Microbispora* sp.	*Hinf*I	128.01
		*Hha*I	360.00
		*Msp*I	163.04
EN27	*Streptomyces* sp.	*Hinf*I	326.78
		*Hha*I	473.64
		*Msp*I	165.16
EN46	*Nocardioides albus*	*Hinf*I	325.32
		*Hha*I	447.63
		*Msp*I	143.22
S. scabies	*Streptomyces scabies*	*Hinf*I	325.21
		*Hha*I	468.50
		*Msp*I	163.18

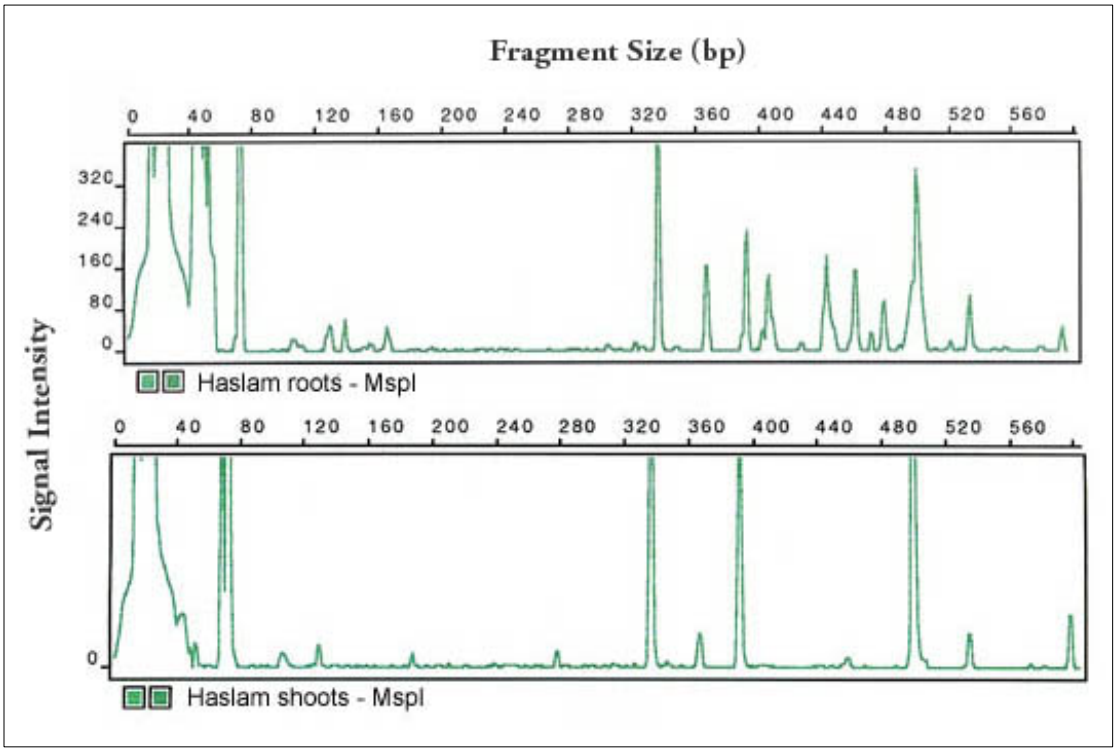


Figure 2.14: GeneScan image of the *Msp*I-digested 16S rRNA gene sequences amplified from the roots and shoots of an uninoculated wheat seed grown for six weeks in soil obtained from Haslam.

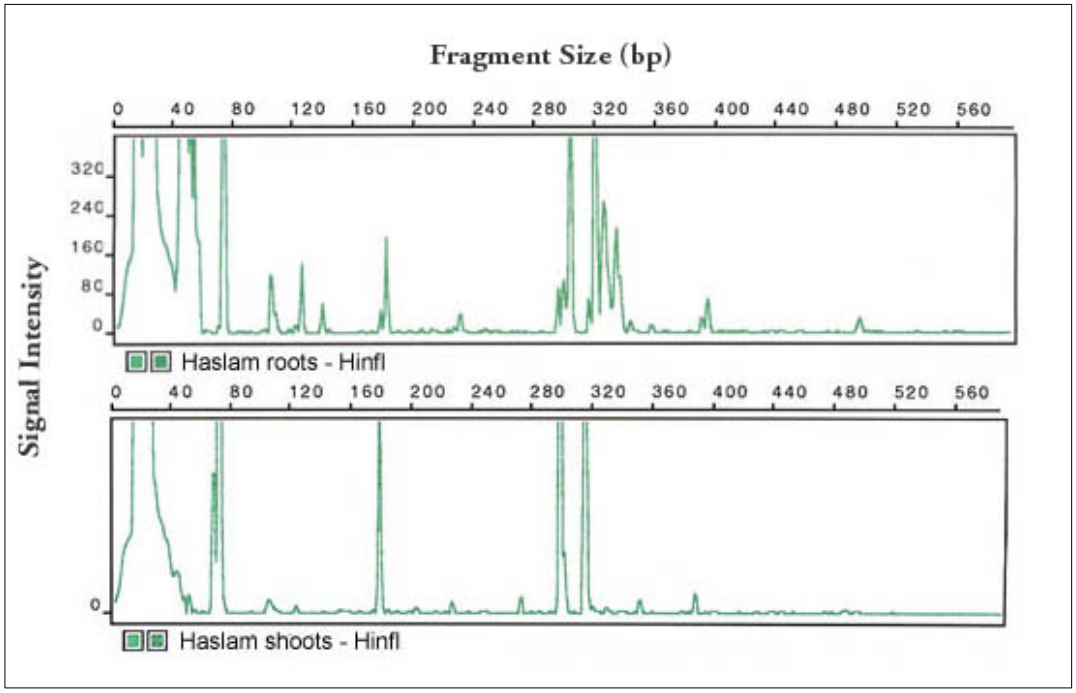


Figure 2.15: GeneScan image of the *Hinf*I-digested 16S rRNA gene sequences amplified from the roots and shoots of an uninoculated wheat seed grown for six weeks in soil obtained form Haslam.

Table 2.14: 16S rRNA gene fragment sizes obtained with *Msp*I and *Hinf*I restriction digestions from the roots and shoots of an uninoculated wheat seed grown for six weeks in soil obtained from Haslam.

Fragment Size	*Msp*I (roots)	*Msp*I (shoots)	Fragment Size	*Hinf*I (roots)	*Hinf*I (shoots)
45	+	+	42	+	-
48	-	+	45	+	+
50	+	+	47	-	+
54	+	-	49	+	+
57	+	-	52	+	+
68	+	+	69	-	+
73	+	+	72	+	+
129	+	-	105	+	-
130	+	-	125	+	-
139	+	-	128	+	-
154	+	-	182	+	+
337	+	+	299	+	-
368	+	+	302	+	-
392	+	+	305	+	+
406	+	-	321	+	+
408	+	-	327	+	-
443	+	-	330	+	-
445	+	-	335	+	-
460	+	-	337	+	-
478	+	-	358	-	+
497	+	-	396	+	-
499	+	+			

Table 2.15: Endophytic bacterial genera present in the roots and shoots (highlighted in yellow) of wheat grown from an uninoculated seed for six weeks in soil obtained from Haslam.

MspI	HinfI	Phylum	Family	Genus
67	128	*Actinobacteria*	*Micrococcaceae*	*Arthrobacter*
67	325	*Actinobacteria*		*Streptomyces*
71	321	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*
72	327	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*
72	321	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*
72	323	*Actinobacteria*	*Coriobacteriaceae*	*Collinsella*
73	323	*Actinobacteria*	*Coriobacteriaceae*	*Collinsella*
127	321	*Fibrobacteres*	*Fibrobacter*	*Fibrobacter*
		Actinobacteria		*Streptomyces*
128	124	*Actinobacteria*	*Nocardiopsaceae*	*Streptomonospora*
128	101	*Proteobacteria*	*Phyllobacteriaceae*	*Mesorhizobium*
		Proteobacteria	*Rhizobiaceae*	*Rhizobium*
129	323	*Actinobacteria*	*Actinosynnemataceae*	*Saccharothrix*
130	298	*Proteobacteria*	*Rhodobacteraceae*	*Rhodobacter*
		Proteobacteria	*Rhodobacteraceae*	*Paracoccus*
130	324	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*
405	104	*Proteobacteria*	*Bartonellaceae*	*Bartonella*
441	300	*Proteobacteria*	*Rhodospirillaceae*	*Rhodovibrio*
442	106	*Proteobacteria*	*Rhodospirillaceae*	*Rhodovibrio*
443	105	*Proteobacteria*	*Acetobacteraceae*	*Acidiphilium*
443	326	*Proteobacteria*		
444	338	*Proteobacteria*	*Cystobacteraceae*	*Stigmatella*
445	304	*Firmicutes*	*Clostridiaceae*	*Clostridium*
446	305	*Viridiplantae*	*Chlamydomonadaceae*	*Chlamydomonas*
461	324	*Proteobacteria*	*Nitrosomonadaceae*	*Nitrosospira*
495	329	*Proteobacteria*	*Vibrionaceae*	*Vibrio*
496	330	*Proteobacteria*		
		Proteobacteria	*Alteromonadaceae*	*Shewanella*
		Proteobacteria	*Neisseriaceae*	*Neisseria*
		Proteobacteria	*Neisseriaceae*	*Kingella*
		Proteobacteria	*Legionellaceae*	*Legionella*
		Proteobacteria	*Piscirickettsiaceae*	*Piscirickettsia*
		Proteobacteria	*Enterobacteriaceae*	*Erwinia*
		Proteobacteria	*Enterobacteriaceae*	*Citrobacter*
		Proteobacteria	*Neisseriaceae*	*Vitreoscilla*
		Proteobacteria	*Enterobacteriaceae*	*Salmonella*
		Proteobacteria	*Enterobacteriaceae*	*Pantoea*
497	330	*Proteobacteria*	*Enterobacteriaceae*	*Salmonella*
		Proteobacteria	*Legionellaceae*	*Legionella*
498	123	*Proteobacteria*	*Xanthomonadaceae*	*Xanthomonas*
498	105		*Cyanophoraceae*	*Cyanophora*
498	332	*Proteobacteria*	*Vibrionaceae*	*Photobacterium*
499	332	*Proteobacteria*	*Alcaligenaceae*	*Taylorella*
499	123	*Proteobacteria*	*Xanthomonadaceae*	*Xanthomonas*

2.4 Discussion

2.4.1 Advantages and Limitations of the T-RFLP Technique

The T-RFLP technique can be a powerful culture-independent technique sensitive enough for microbial community analysis. This technique is gaining popularity as it is rapid and has high resolution. As the technique is molecular based there can be limitations that may give rise to a distorted profile. The preferential extraction of genomic DNA from environmental sources and subsequent amplification bias during PCR are known limitations for all molecular techniques that rely on amplification of community DNA and PCR analysis. Reviews on such limitations have been presented by Wintzingerode *et al.* (1997) and Zhou *et al.* (1996). To minimise problems at the PCR level, care was taken in primer design and the reactions were performed in duplicate. The first set of primers used in this study were designed to be biased for actinobacteria sequences. The results indicate that the primers were in fact relatively specific for actinobacteria as the TAP-T-RFLP database identified the majority of fragments correspond to actinobacteria sequences. The second set of primers were designed to amplify the majority of bacterial genera. The TAP-T-RFLP database can theoretically determine the species that can to be amplified by the labelled primer. However, the universal bacterial primers theoretically amplify fewer species (2335) than the actinobacteria-biased primers (3527). There is considerable overlap between the primers, though, the universal bacterial primers do not amplify a number of actinobacteria species but they do amplify a number of bacterial genera not picked up by the actinobacteria-biased primers.

The T-RFLP technique can be used to determine if microbial populations are significantly different to one another by determining the presence or absence of peaks. However, to exploit the full potential of the technique the TAP-T-RFLP database can be used to correlate these fragments to bacterial species. Further limitations arise at this point, and the most apparent is that the database is not complete. For all of the samples analysed by T-RFLP a number of the fragments obtained with the restriction enzymes did not have matches in the database. In fact only a maximum of 70% of the peak areas obtained from the GeneScan data matched bacterial species in the database. This indicates that there were bacterial species present in the samples for which there was no corresponding entry in the database. However, the number of bacterial species for which fragments were obtained but were not present in the database cannot be speculated on. The use of three restriction enzymes is necessary to eliminate false positives and pseudo-TRFs (Egert and Friedrich, 2003) which can occur when using only one restriction enzyme. The restriction digests should also be performed for the maximum length of time to ensure complete digestion of PCR products.

To overcome the problem of sequences absent from the database the expected fragment sizes can be determined manually. For example, *Microbispora* has been identified as an endophyte of wheat but the 16S rRNA gene sequence was not present in the TAP T-RFLP database. Therefore, the *Microbispora* 16S rRNA gene sequences were downloaded from the GenBank database located on the NCBI website (http://www.ncbi.nlm.nih.gov) and the restriction analysis program Webcutter 2.0 (http://www.firstmarket.com/cutter/cut2.html) was used to determine

the restriction sites when digested with *Hinf*I, *Hha*I and *Mbo*I. The theoretical fragments were then used for analysis.

It should be noted that the T-RFLP technique can only identify the species that are likely to be within the sample. To conclusively identify species, sequencing of the 16S rRNA gene should be performed. Alternatively, isolation can be performed using media selective for bacterial species detected by T-RFLP.

2.4.2 Diversity of the Endophytic Actinobacteria Population in Wheat Roots grown in Different Field Soils

This is the first study to investigate the endophytic actinobacteria population of wheat roots grown in different field soils by the molecular based T-RFLP technique. The microbial community associated with the plant rhizosphere is known to change according to the soil type, host plant, growth stage, disease state, cropping practices and other environmental factors (Miller *et al.*, 1989; Germida *et al.*, 1998; Siciliano *et al.,* 1998; Dunfield and Germida, 2001; McSpadden Gardener and Weller, 2001; Smit *et al.,* 2001). Culture-dependent studies have investigated changes in bacterial endophyte populations in cotton, pea, canola and wheat (McInroy and Kloepper, 1995; Germida *et al.,* 1998; Hallmann, 1999; Zinniel *et al.,* 2002). However, this study used the molecular based T-RFLP technique to gain a broader insight into the population diversity of the actinobacteria endophytes and the changes that occur due to soil type.

T-RFLP was used to assess the effect of different field soils on the endophytic

actinobacteria population in the roots of wheat. The endophytic actinobacteria population could not be determined in the wheat shoots, as there appeared to be a very low colonisation of actinobacteria in the shoots. Even though the DNA obtained from the shoots was sheared, 16S rRNA gene could be amplified from shoot DNA using the universal bacterial primers intended to amplify a wide range of bacterial genera, but not using the actinobacteria-biased primers. This corresponds to information reported by Lamb *et al.* (1996) who found that endophytes colonise the roots of plants at a higher concentration than in the shoots. Larran *et al.* (2002) also isolated only three bacterial endophytes identified as *Bacillus* sp. from 450 wheat leaves. The natural concentration of all bacterial endophytes can vary between 2.0 and 6.0 $\log_{10}$ CFU per gram for alfalfa, sweet corn, sugar beet, squash, cotton and potato, compared to pathogens which can range from 7.0 to 10 $\log_{10}$ CFU per gram of tissue (Zinniel *et al.*, 2002).

It has been suggested that the endophyte population is a subset of the rhizosphere microbes. Germida *et al.* (1998) investigated the diversity of bacteria in the rhizosphere and roots of canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.) and found while the endophytic population was less diverse, they appeared to originate from the rhizosphere. Therefore, plants grown in soils that support a higher number of indigenous microbial species could be colonised by a larger and more diverse endophytes, which is supported by the results of this study. The endophytic actinobacterial population of the wheat roots was significantly different for each field soils (Figure 2.9). As the same seed batch of the cultivar was sown the results indicate the soil microbial population influences the endophyte population. Soils that

support a higher number of indigenous microbes, such as in Swedes Flat without NutriLife 4/20™, the level of endophytic diversity and colonisation is much higher than in soils with a lower number of microbes.

The diversity of actinobacteria detected by T-RFLP was higher than that found by culture-dependent methods from wheat roots performed in our laboratory. Coombs and Franco (2003a) identified five different genera (*Streptomyces*, *Microbispora*, *Nocardioides*, *Micromonospora*, *Tsukamurella*) from wheat plants sampled from field sites in South Australia over a two year period. Nine actinobacterial genera with a maximum Ap value over 1% (*Kitasatospora*, *Mycobacterium*, *Bifidobacterium*, *Rhodococcus*, *Geodermatophilus*, *Brevibacterium*, *Corynebacterium*, *Nocardia*, *Actinosynnema*) were identified in all of the field soils, and a total of 40 different actinobacterial genera were identified as possible endophytes among the three different field soils. The diversity of the endophytes may be influenced by the difference in field sites but also by the dection method. Actinobacteria are common soil microorganisms and some species have been identified as endophytes. For example *Arthrobacter* has been indentified in the soil (Holt *et al.*, 1986; Smit *et al.*, 2001) but also as an endophyte of wheat and canola (Germida *et al.*, 1998) and pea (van Vuurde and Elivra-Recuenco, 2000).

The endophytic nature of the actinobacteria genera cannot be confirmed by T-RFLP alone therefore sequencing of 16S rRNA clones was performed. With the limited number of clones sequenced, eight different genera identified among the three soils, were also identified by the T-RFLP analysis. The predominant actinobacterial genus identified by sequencing was *Mycobacterium*, with 64% of clones belonging to

this genus. This is the first time *Mycobacterium* spp. have been identified as endophytes of wheat, but they have been shown to be present in the soil (Covert *et al.*, 1999; Cheung and Kinkle, 2001)

A large number of *Mycobacterium* species have been identified as human pathogens such as *M. tuberculosis* (Holt *et al.*, 1986). A number of the clones had close identity to *Mycobacterium* species previously identified as clinical isolates (see Table 2.9). It is possible that some *Mycobacteria* were introduced via the tap water used for watering the plants, as *M. kansasii*, *M. fortuitum*, *M. chelonae*, *M. avium* and *M. xenopi* have been indentified in tap water (Le Dantec *et al.*, 2002). The role of these species within healthy plant tissue is unknown; isolation and characterisation of the *Mycobacterium* species needs to be performed to gain more insight. Four 16S rRNA clones obtained from the roots of wheat grown in Swedes Flat were related to *M. achiense* sp. JS618 (92-97%) which was isolated from an industrial soil site (Coleman *et al.*, 2002). Coleman (2002) isolated number of *Mycobacterium* species from groundwater, soil and activated sludge that were capable of degrading vinyl-chloride. One clone with 93% identity to *M. austroafricanum* was isolated from an environmental site and is capable of degrading polycyclic aromatic hydrocarbon (PAH). The Swedes Flat soil may have been contaminated with PAH leading to the proliferation of *Mycobacterium* species capable of utilising the contaminant as a carbon source; however, the role of *Mycobacterium* as an endophyte is still undetermined. Interestingly, from the same soil a clone had a high identity to *Gordonia polyisoprenivorans* which had been isolated as a rubber degrading species. According to the literature *Gordonia* has not been previously identified as an

endophyte but it is prevalent in the soil (Maldonado *et al.*, 2003).

The high percentage of the 16S rRNA clones related to *Mycobacterium* sequences was not reflected in the T-RFLP analysis where the maximum abundance percentage of *Mycobacterium* detected was 1.3% in Red Loam, 2.5% in Western Flat and 21% in Swedes Flat. This high frequency of detection is possibly a result of the RFLP screening used to select clones for sequencing, with the mycobacterial clones possessing a higher degree of detectable intra-genus polymorphism than the inter-genera polymorphisms between the actinobacterial genera present. The RFLP patterns of the 16S rRNA sequences of the *Mycobacterium* spp. identified generated with *Hinf*I and *Hha*I were significantly different to one another. Random sequencing of clones may have resulted in lower detection, though it would still not give information on abundance of genera.

The second highest genus identified by sequencing was *Streptomyces*. A number of endophytic *Streptomyces* strains have been isolated from various plant including *Ficus*, *Dieffenbachia*, *Allium porrum*, *Brassica oleracera* and *Quercus* sp. (Sardi *et al.*, 1992; Leifert *et al.*, 1994). Four different *Streptomyces* were identified by sequencing, *S. thermolineatus*, *S. griseochromogenes*, *Streptomyces* sp. SE2 and *Streptomyces* sp. EF-91. *Streptomyces* SE2 has been identified as a wheat endophyte previously by Coombs and Franco (2003a) in this laboratory. Likewise *Streptomyces* sp. EF-91 is a known endophyte of potato, indicating a broad host range. This is the first time that *S. thermolineatus* and *S. griseochromogenes* have been reported as endophytes.

Nocardioides and *Micromonospora* were isolated from healthy wheat roots by

Coombs and Franco (2003a) in this laboratory; however, this is the first time the species *Micromonospora endolithica, M. peucetica* and *Nocardioides* sp. 4P1-A have been detected as endophytes. *Nocardia* endophytes have been isolated from citrus plants (Araujo *et al.*, 2002). The 16S rRNA gene sequence identified in this study was closely related to *Nocardia pseudobrassilensis* (95%), a human pathogen. Transmission of nocardiopsis occurs through soil-contaminated wounds (CDC, 2003). However, isolation of these species needs to be performed for further confirmation and characterisation of their endophytic nature, as with the species *Amycolatopsis* sp. GY152 and *Rhodococcus coprophilus* which have not been previously identified as endophytes.

2.4.3 The Effect of a Soil Microbial Inoculant on the Actinobacteria Endophyte Population of Wheat

There is an emerging trend to replace chemical herbicides, fungicides and fertilisers with microbial inoculants due to environmental and health concerns. It is well known that the addition of non-indigenous microbes to soil can affect the indigenous population. Microbial interactions and their biocontrol in the rhizosphere has been reviewed by Whipps (2001). However, the effect of microbial inoculants on the endophyte population is unknown. Therefore, this study used the T-RFLP technique to assess the effect of a microbial inoculant on the endophytic actinobacteria population present in wheat roots.

Two field soils were collected from Swedes Flat in South Australia, in which

one soil had the addition of NutriLife 4/20™ (NutriTech, Australia) a microbial inoculant consisting of 20 bacterial strains and four predatory fungi. It was found that in the soil with the addition of the commercial inoculant the actinobacteria diversity reduced from 40 genera to 21 genera and the colonisation reduced by approximately half for the majority of genera. These results suggest the microbes present in the NutriLife 4/20™ inoculum are able to proliferate and dominate in the soil and consequently are out-competing the indigenous actinobacteria microflora, with the exception of *Kribella*, *Nocardioides* and *Thermonospora.* This appears to be preventing a number of actinobacteria genera from access to the plant and ultimately endophytic colonisation of the wheat roots. Many biocontrol strains are capable of improving plant growth and disease resistance in controlled pot trials but have failed when taken out into the field. This has been attributed mainly to the inability of the introduced strain to compete in the complex rhizosphere population. In this case it is possible that the addition of biocontrol strains to the soil are causing disruption of the natural actinobacteria endophyte population, which in turn may have a negative effect on plant growth and disease resistance. Of the endophytic actinobacteria isolated from the wheat roots by Coombs and Franco (2003a) a number were capable of causing significant disease resistance and growth promotion, indicating that indigenous actinobacteria endophytes are necessary for maintaining plant health.

2.4.4 Use of the T-RFLP Method As a Semi-Quantitative Tool to Detect Introduced Endophytes

The T-RFLP technique was used as a semi-quantitative tool to detect the level

of colonisation by specific actinobacteria endophytes that were introduced into wheat roots by seed application. The specific TRF peaks correlating to actinobacteria (EN2, EN27, EN46) digested with *Hinf*I, *Hha*I and *Mbo*I were used to monitor colonisation levels in wheat roots. Analysis of the *Hinf*I profiles demonstrated that the EN2 and EN27 actinobacteria endophytes colonised the plants when used as an inoculant as the specific fragments belonging to inoculants increased significantly compared to the uninoculated plant. On the other hand the specific 179 bp *Hinf*I fragment belonging to EN46 did not appear to have increased in the EN46-inoculated plant.

When analysing the minimum and maximum abundance percentages (not corrected) of the specific fragments for the EN2-inoculated plant, the inoculant appeared to increase by approximately two to three-fold. For the EN27-inoculated plant the specific 241 bp *Hinf*I fragment was not present in the uninoculated plant which was a good indicator that EN27 had definetly colonised the plant. The EN27 *Hha*I specific fragment also increased by two-fold. The EN27 *Mbo*I 163 bp fragment decreased by 1.5-fold but this does not necessarily imply that the endophyte did not colonise as the 163 bp fragment is found with many other species, so if they did not colonise the plant the peak may decrease.

The EN46 endophyte though appears not to have colonised the plant roots as the two specific fragments obtained with *Hha*I and *Hinf*I decreased. The *Mbo*I specific fragment increased by 2.2 fold, but only in one of the three replicate plants and this suggests that the EN46 endophyte only colonised one of the plants.

The EN2, EN27 and EN46 were all previously isolated from wheat roots by Coombs and Franco (2003a). These isolates were all capable of significant

suppression of disease symptoms caused by *Gaeumannomyces graminis* var. *tritici* at 4 weeks of growth. EN27 also showed significant growth regulation of wheat at 4 weeks. The T-RFLP results have shown that at six weeks the introduced endophytes increase in colonisation up to three fold, suggesting introduced endophytes do not need to colonise at high levels to exhibit a positive effect on the wheat plant. This is of importance in relation to the use of endophytes as biological control agents.

2.4.5 The Effect of Introduced Actinobacteria Endophytes on the Indigenous Population

As the T-RFLP technique was able to detect the introduced endophytes, the technique was used again to determine whether the indigenous endophytic actinobacterial population changes in response to the introduction of a single actinobacterial endophyte. The results indicated that the introduction of an endophyte to wheat roots did not disrupt the indigenous endophytic actinobacterial microflora as there was very little change in the actinobacterial diversity and level of colonisation.

The natural endophytic actinobacteria population was relatively stable despite the inoculation of an endophyte; these results are substantially different to results obtained when NutriLife 4/20™ was added to the soil. This may be due to the fact the soil inoculum contained over 20 bacterial species, whereas in our experiments only a single competent actinobacteria endophyte was inoculated onto the seed. The results show that the inoculation of an additional endophyte to the wheat plant does not significantly alter the indigenous population which is important if the other

endophytes are integral to maintain plant health.

The results from these studies have been published:

Conn, V.M., and Franco, C.M.M. (2004). Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by Terminal Restriction Fragment Length Polymorphism and sequencing of 16S rRNA clones. Applied and Environmental Microbiology **70,** 1787-1794.

Conn, V.M., and Franco, C.M.M. (2004). Effect of microbial inoculants on the indigenous actinobacterial endophyte population in the roots of wheat as determined by terminal restriction fragment length polymorphism. Applied and Environmental Microbiology **70,** 6407-6413.

2.4.6 Analysis of the Eubacterial Endophyte Population of Wheat Grown in a Field Soil

Bacterial endophytes have been isolated from a variety of plants including pea, rice, cotton, canola, corn, citrus plants, oilseed rape, prairie and agronomic plants and a large amount of research has been conducted in potatoes (McInroy and Kloepper, 1995; Germida *et al.,* 1998; Sturz *et al.,* 1999; Nejad and Johnson, 2000; van Vuurde and Elivra-Recuenco, 2000; Elbeltagy *et al.,* 2001; Araujo *et al.,* 2002; Zinniel *et al.,* 2002). A number of different genera have been isolated from a variety of potato cultivars including α-, β-, γ- *Proteobacteria,* high G+C gram positive microbes (actinobacteria), microbes belonging to the *Flexibacter*/*Cytophaga*/*Bacteriodes* group, *Planctomycetales*, *Pseudomonas*,

Agrobacterium, *Stenotrophomonoas*, *Flavobacterium*, *Cellulomonas*, *Clavibacter*, *Curtobacterium*, *Micrococcus*, *Pantoea*, *Xanthomonas* and more (Sturz *et al.*, 1999; Garbeva *et al.*, 2001; Sessitsch *et al.*, 2001; Reiter *et al.*, 2002). Few studies have isolated bacterial endophytes from wheat. Zinniel *et al.* (2002) isolated 28 bacterial endophytes from 48 wheat plants; however, species identification was not performed. Sardi *et al.* (1992) and Coombs and Franco (2003a) isolated endophytes from wheat roots; however, the focus was on the actinobacteria. In this study the overall bacterial species present in wheat was determined by the culture-independent T-RFLP method.

The results of the study identified 34 different bacterial genera present in the roots and one genus (*Mycobacterium*) in the shoots (Table 2.15). A variety of actinobacteria species were identified in the roots including *Arthrobacter*, *Streptomyces*, *Mycobacterium*, *Cellulomonas*, *Collinsella*, *Streptomonospora* and *Saccharothrix*. All of these actinobacteria were identified, except *Collinsella*, using the actinobacteria biased primers. The universal bacterial 16S rRNA primers were designed by Weisburg *et al.* (1991) to amplify a wide range of bacteria genera and were used by McSpadden Gardener and Weller (2001) to asses the bacterial population in the rhizosphere of wheat with take-all disease. The reverse primer was one base pair different to the actinobacteria bias primers which reduced the range of actinobacteria genera amplified by this primer set.

Of the 27 other bacterial genera that were detected in this study, a number have been previously identified as endophytes including *Xanthomonas*, *Erwinia*, *Pantoea*, *Rhizobium*, *Mezorhibium*, *Kingella*, *Salmonella* and *Nitrospira* (Sturz *et al.*, 1999; Briones *et al.*, 2002; Reiter *et al.*, 2002; Zinniel *et al.*, 2002; Dong *et al.*, 2003).

The endophytic association of the remaining genera cannot be concluded until isolation or sequencing has been performed.

Pseudomonas fluorescens was not identified by T-RFLP but has been isolated as an endophyte from wheat roots previously in our laboratory. The bacterial 16S PCR primers were able to amplify the *P. fluorescens* 16S rRNA gene using DNA isolated from pure cultures. This indicates that while *Pseudomonas* species can be isolated as endophytes using culture-dependent methods, but the population of *Pseudomonas* is not at a level that is significant enough to be detected by the T-RFLP technique. Germida *et al.* (1998) also were not able to identify *P. fluorescens* as an endophyte of canola roots, even when it was found to be present in the rhizosphere at 1.6% of the total microbial population.

From the T-RFLP profiles (Figure 2.14 and 2.15) it was clear that a wider range of bacterial species were present in the roots than in shoots. This corresponds to information reported by Larran *et al.* (2002) where only three *Bacillus* sp. were identified in wheat leaves. While a number of genera were identified in the roots, only one was identified in the shoots by the TAP-T-RFLP database. However, 9 distinct fragments were detected with *Msp*I and *Hinf*I (Table 2.14). As discussed in 2.4.1 the TAP T-RFLP database does not contain all bacterial 16S rRNA sequences limiting the bacterial genera that can be identified using this technique. Further work, including sequencing of 16S rRNA gene sequences and isolation, needs to be performed to gain a better overview of the endophytic eubacterial population present in the roots and shoots of wheat.

Chapter Three: Analysis of the Endophytic Fungal Populations in the Roots of Wheat by Partial 18S rRNA Gene Sequencing.

3.1 Introduction

All plants tested to date have been found to harbour endophytic fungi including woody plants, shrubs, trees, citrus plants and grasses (Arnold *et al.,* 2003). Endophytic fungi represent a wide range of taxa mainly belonging to the phylum *Ascomycota* (Ernst *et al.,* 2003). Fungal endophytes are a common and widespread phenomenon that is thought to improve the fitness of the plant.

As discussed in chapter one, one of the most well studied fungal endophyte plant associations is betwen the arbuscular mycorrhizal (AM) and the roots of higher plants. The colonisation of plants with AM fungi increases the fitness of the plant by improving growth and development by the acquisition of phosphate and other minerals from the soil (Garcia-Garrido *et al.,* 2000; Harrier, 2001). AM fungal colonisation also enhances plant resistance to biotic and abiotic stresses.

Fungal endophytes of cultivated turf and grasses have been shown to significantly affect the growth and reproduction of not only the host plant but of pathogens, herbivores of the grasses and natural enemies of the herbivores (Saikkonen *et al.,* 1998). The best studied endophytes of grasses are the intercellular symbionts in the ascomycota family, Clavicipitaceae. These endophytes colonise the above-ground tissues of cool-season grasses in the temperate zone and are vertically transmitted via the seed (Clement *et al.,* 2001; Arnold *et al.,* 2003; Schardl *et al.,* 2004). Grasses found with the asexual *Neotyphodium* and the sexual *Epichloë* endophytes have improved plant fitness, including drought resistance, competitive abilities, seed dispersal, germination success; and the plants are more resistant to herbivore and abiotic stresses than non-infected grasses (Clay *et al.,* 1993; Miles *et*

al., 1998; Saikkonen *et al., 1*998; Clement *et al.*, 2001; Schardl *et al.*, 2004).

In contrast to the AM fungi and Clavicipitaceae associations, little is known about the fungal endophytes from woody angiosperms, even though endophytes have been isolated from the aerial tissues of all trees and shrubs sampled to date (Arnold *et al.*, 2003). The presence of endophytic fungi has also been verified in healthy wheat crops by Larran *et al.* (2002) and Sieber *et al.* (1988). Larran *et al.* (2002) isolated 130 fungal endophytes from wheat leaf segments. Previous work, presented in chapter two of this thesis, showed the presence of actinobacterial endophytes in the roots of healthy wheat and how this altered in accordance with the soil dynamics and microbial inoculants. However, little is known about the endophytic fungal population within the healthy wheat root. The aim of this work was to gain an insight into the fungal endophyte population in the roots of healthy wheat plants by 18S rRNA gene sequencing. Molecular methods were used as is it known many fungal endophytes, like the AM fungi, cannot be cultured ex-planta.

3.2 Materials and Methods

3.2.1 Wheat Cultivation

Wheat plants were cultivated in four different field soils obtained from the South-East of South Australia as described in 2.2.2.3 and 2.2.2.3.1. The wheat plants were harvested at six weeks as per 2.2.1.4.

3.2.2 Endophytic Fungal DNA Extraction

Endophytic fungal DNA was extracted from the roots of wheat grown in four different field soils. The endophytic fungal DNA was extracted as per the method described in 2.2.3.4 and purified as per 2.2.3.4.1.

3.2.3 Partial 18S rRNA PCR

A 550 bp segment of the fungal 18S rRNA gene sequence was amplified from the endophytic DNA samples using primers EF4 and Fung5 developed by Smit *et al.* (1999).

Fungal Primer Label	Sequence
EF4:	5’ GGA AGG G[G/A]T GTA TTT ATT AG 3’
Fung5:	5’ GTA AAA GTC CTG GTT CCC C 3’

The EF4 and Fung5 primers were used in a 50 μl PCR reaction with the following contents: Primers (20 ng.μl^{-1}) 2 μl each, 5X Taq buffer (inc. dNTP’s) 10 μl, H_2O 31 μl, Taq polymerase (Biotech, Australia) (2 U.μl^{-1}) 1 μl, template DNA 4 μl. The

following thermal profile was followed: 94°C - 5 min, (94°C - 1 min, 51°C - 1 min, 72°C - 1 min) × 40 cycles, 72°C - 10 min. For each sample 5 μl to 10 μl of PCR product was run on an agarose gel as per 2.2.3.7.

3.2.4 Cloning and Sequencing of Partial 18S rRNA PCR Products

The partial 18S rRNA gene sequences were purified using the UltraClean PCR Product Purification Kit (MoBio, California, U.S.A). PCR products were ligated into the bluescript pGEM T-vector (Promega) overnight at 4°C. The ligated PCR product/vector was used to transform the JM109 cells (Promega) as per 2.2.4.3. Plasmids were prepared for sequencing as per 2.2.4.4 and the selection of candidates for sequencing as per 2.2.4.5. Sequencing of clones was performed as per 2.2.4.6. Sequences were compared to online databases using the BLAST program on the NCBI website as per 2.2.4.6. Chimeric sequences were detected using the Ribosomal Databases Project II program (http://35.8.164.52/cgis/chimera.cgi?su=SSU). Chimeric sequences were excluded from further analysis.

3.2.5 18S rRNA Phylogeny

Selected 18S rRNA sequences were aligned with the ClustalW program with the value of 1000 Bootstraps using the BioEdit software. A distance matrix was created and the Neighbour-Joining method used to create a phylogenetic tree.

3.3 Results

A 550 bp segment of the fungal 18S rRNA gene was successfully amplified from the endophytic DNA extracted from the roots of wheat grown in four different field soils. Table 3.1 shows the highest sequence match of the 18S rRNA clones derived from the endophytic fungal DNA extracted from the healthy wheat roots. Appendix 6 (Tables A.6.1 to A.6.4) shows the three highest BLAST matches for each of the fungal clones identified in the four field soils. Figures 3.1 shows the phylogenetic relationship of selected fungal 18S rRNA gene sequences identified in the roots of healthy wheat to fungal gene sequences retrieved from Genbank. From the clones sequenced, 29 different fungal species were identified. Table 3.2 lists the species that were detected and together with a summary of information on these known fungi. Table 3.3 lists the Genbank accession numbers and the relevant publications as listed on the NCBI website (http://www.ncbi.nlm.nih.gov). No fungal endophyte was common to all wheat samples tested, 79% of the endophytes were identified in only one field soil, 17% in two field soils and one species, *Trematosphaeria hydrela,* was detected in three field soils. Some species were detected more than once, 20 out of 87 clones were identified as *Trematosphaeria hydrela,* which is not surprising as it was detected in the roots of wheat from three field soils. *Clathrospora diplospora* was identified in 9 of the 87 clones; however, 8 of these clones were from one wheat sample. Since only a limited number of clones were sequenced (Swedes Flat with and without NutriLife 4/20™ and Red Loam n=21 and Western Flat n=24) no inferences can be drawn on the relationship of fungal endophyte species to different field soils.

Table 3.1: Highest sequence match of partial 18S rRNA gene sequences which were amplified from the endophytic DNA extracted from wheat roots grown in four different field soils.

Fungal Species with the Highest Similarity	Accession Number	Similarity %	Clone Numbers	Soil Type
Aleuria aurantia	U53371.1	93-95	143, 148*	SF -
Cephaliophora muscicola	AB001108	97	39	SF +
Cladophialophora boppii nuclear	AJ232946	99	607	Western Flat
Clathrospora diplospora	U43464	97	3, 15, 17, 19, 42*, 45*, 64*, 73	SF +
Clathrospora diplospora	U43464	99	442*	Red Loam
Dothideomycete sp. G9-S53	AY190273	89-98	584, 632	Western Flat
Endogone pisiformis	X58724	94-95	117, 118, 122, 137, 166	SF -
Engyodontium album NRRL 28022	AF049147	99	585*	Western Flat
Fungal endophyte MUT 585	AF503563	93	431*	Red Loam
Lasallia rossica	AF088238	97	115	SF -
Leotiomycete sp. G2-5-S70	AY190266	98	592*	Western Flat
Massaria platani	AF164363	98	185	SF -
Nectria lugdunensis	AY231639	99	178	SF -
Nectria lugdunensis	AY231639	99	561	Western Flat
Phoma herbarum strain ATCC 22167	AY293777	89-92	423, 450	Red Loam
Phoma sp.201	AY293774	99-100	572, 576, 579,	Western Flat
Pleospora rudis	U00975	98-99	552, 553, 577, 578	Western Flat
Pseudoplectania nigrella	AF104345	88-96	587, 590*, 605	Western Flat
Pyrenophora tritici-repentis	U42486.1	96-97	4, 46, 52	SF +
Pyronema domesticum	U53385	96-97	127*, 194*	SF -
Raciborskiomyces longisetosum	AY016351	93-95	566, 567, 589, 601	Western Flat
Setosphaeria monoceras	AY016352	95-98	616, 432	Red Loam
Sordariomycete sp. pgp-hsf	AF292054	98	444	Red Loam
Talaromyces flavus	M83262	96	63	SF +
Termitomyces sp. O1 gene	AB051893	97	443	Red Loam
Trematosphaeria hydrela	AF164376	97-98	27, 30, 56, 85	SF +
Trematosphaeria hydrela	AF164376	98	101, 121, 181, 116, 133*, 158, 132, 142	SF -
Trematosphaeria hydrela	AF164376	98	404, 411, 417, 422, 435, 410, 433, 439	Red Loam
Ulocladium botrytis strain UPSC 3539	AF548106	99	429	Red Loam
Uncultured ascomycete clone AT2-4	AF530541	98	559	Western Flat
Uncultured ascomycete clone AT2-4	AF530541	99	440	Red Loam
Uncultured soil fungus clone Pent 3.5	AY163417	96-97	581, 588, 639	Western Flat
Westerdykella cylindrical	AY016355	85-99	428, 436, 438	Red Loam
Westerdykella dispersa	U42488	96 – 99	13, 55, 67	SF +
Westerdykella dispersa	U42488	91	176*	SF -

SF = Swedes Flat without NutriLife 4/20 (n=21), SF+ = Swedes Flat with NutriLife 4/20 (n=21), Western Flat (n=24), Red Loam (n=21). * = possible chimeric

Table 3.2: Fungal species detected by partial 18S rRNA gene sequencing and a summary of information known for these species.

Species	Comments
Aleuria aurantia	Known as 'the Orange Peel Fungus' it is initially cup shaped but develops into a contorted bowl. Most frequently found on disturbed soil beside woodland paths.
Cephaliophora muscicola	Found in forest debris and moss.
Cladophialophora boppii	*Cladophialophora* is a mitosporic dematiaceous (pigmented) mould. Its natural habitats are soil and rotten plant material. *Cladophialophora boppii* and *Cladophialophora carrioinii* are isolated from patients with chromoblastomycosis, *Cladophialophora boppii* may also cause skin lesions.
Clathrospora diplospora	*Clathrospora* and *Leptosphaeria* are telemorphs of the genus *Alternaria. Alternaria mould* is a cosmopolitan dematiaceous (phaeoid) fungus commonly isolated from plants, soil, food, and indoor air environment. The production of melanin-like pigment is one of its major characteristics. *Clathrospora pentamera* (P. Karst.) Berl. can cause Platyspora leaf spot in wheat (www.apsnet.org).
Dothideomycete sp. G9-S53	Blackleg caused by the dothideomycete, *Leptosphaeria maculans* is the major disease of canola-oilseed rape (*Brassica napus*) worldwide.
Endogone pisiformis	Habitats mosses, leaf and twig litter, does not form ectomycorrhizae but similar structures to vesicles and arbuscules of VAM fungi.
Engyodontium album NRRL 28022	*Engyodontium* sp. are related to *Beauveria* and *Tritirachium*. Health effects of *Engyodontium album* include reports of keratitis, brain abscess, eczema vesiculosum, and native valve endocarditis. Commonly isolated from paper, jute, textiles, and painted walls.
Fungal endophyte MUT 585	No comment
Lasallia rossica	The "cladoniiform" lichens are currently distributed among a diverse group of ascomycete families
Leotiomycete sp. G2-5-S70	No comment
Massaria platani	*Massaria* causes diseases of sycamore: Massaria canker - dieback *Splanchonema platan* (Ces.) Barr = *Massaria platan* Ces. (anamorph: *Macrodiplodiopsis dasmazieresii* (Mont.) Petr (www.apsnet.org)
Phoma herbarum	*Phoma* is a filamentous fungus that inhabits the soil and plant material. *Phoma* are common plant pathogens.
Phoma sp.201	Has been known to causes leaf spot in corn.
Pleospora rudis	This genus is a teleomorph of *Phoma*. *Pleospora* and *Lewia* are two separate ascomycetous sexual genera. In the genus *Pleospora* (sexual), none of the species have asexual forms that are classified in the asexual genus *Alternaria*.
Pseudoplectania nigrella	No comment
Pyronema domesticum	Often found in burnt soil and cotton gauze.
Raciborskiomyces longisetosum	Has been found in the rhizosphere of maize.
Setosphaeria monoceras	Distributed widely in Japan. Causes leaf blight of the genus *Echinochloa*, such as barnyardgrass, and utilised as a biocontrol agent for the weeds. Disperses by scattering conidia. Teleomorph has never been observed in nature.
Sordariomycete sp. *pgp-hsf*	Sordariomycete, a classification of ascomycetes that includes many phytopathogenic fungi, including the genera *Ophiostoma* (containing the species responsible for Dutch elm disease) and *Magnaporthe*.

Table 3.2 cont.

Species	Comments
Termitomyces sp. *Ol*	Symbiotic relationship with termites.
Talaromyces flavus	Reported to control the plant pathogen *V. dahliae in vitro*. Anamorph is *Penicillium vermiculatum.*
Trematosphaeria hydrela	No comment
Ulocladium botrytis strain 3539	*Ulocladium* is a dematiaceous filamentous fungus that inhabits the soil and decaying herbaceous plants. It is widely distributed in nature and can be isolated from paper, textiles, and wood. *Botrytis* is a filamentous fungus isolated from decaying plants.
Uncultured ascomycete clone AT2-4	No comment
Nectria lugdunensis	Found in decaying leaves in streams.
Pyrenophora tritici-repentis	Causes tan spot in wheat.
Uncultured soil fungus clone Pent 3.5	No comment
Westerdykella cylindrica	No comment
Westerdykella dispersa	No comment

Table 3.3: Accession numbers of the fungal species identified by partial 18S rRNA sequencing and the publications cited on the NCBI website.

Species	Ac. No.	Publications
Aleuria aurantia	U53371.1	Landvik ,S., Egger, K.N. and Schumacher, T. (1997) Towards a subordinal classification of the Pezizales (Ascomycota): phylogenetic analyses of SSU rRNA sequences. Nord. J. Bot. 17, 403-418
Cephaliophora muscicola	AB001108	Tanabe, Y., Nagahama, T., Saikawa, M. and Sugiyama, J. (1999) Phylogenetic relationship of Cephaliophora to nematophagous hyphomycetes including taxonomic and nomenclatural emendations of the genus Lecophagus. Mycologia 91, 830-835
Cladophialophora boppii nuclear	AJ232946	Haase, G., Melzer-Krick, B. and Sonntag-Werkes, L. Phylogeny of black yeasts and allied Herpotrichiellaceae. Unpublished
Clathrospora diplospora	U43464	No publications
Dothideomycete sp. G9-S53	AY190273	Kauhanen, M., Vainio, E.J., Niemela, P. and Hantula, J. Microfungal associates of an introduced tree: endophytes of Siberian larch (Larix siberica). Unpublished
Endogone pisiformis	X58724	Simon, L., Lalonde, M. and Bruns, T.D. (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl. Environ. Microbiol. 58 (1), 291-295
Engyodontium album	AF049147	No publications
Fungal endophyte MUT 585	AF503563	Girlanda, M., Ghignone, S. and Luppi, A.M. (2002) Diversity of sterile root-associated fungi of two Mediterranean plants. New Phytol. 155 (3), 481-498
Lasallia rossica	AF088238	Stenroos, S.K. and DePriest, P.T. (1998) SSU rRNA phylogeny of cladoniiform lichens. Am. J. Bot. 85, 1548-1559
Leotiomycete sp. G2-5-S70	AY190266	Kauhanen, M., Vainio, E.J., Niemela, P. and Hantula, J. Microfungal associates of an introduced tree: endophytes of Siberian larch (Larix siberica). Unpublished
Massaria platani	AF164363	Liew, E.C., Aptroot, A. and Hyde, K.D. (2000)Phylogenetic significance of the pseudoparaphyses in Loculoascomycete taxonomy. Mol. Phylogenet. Evol. 16 (3), 392-402
Phoma herbarum	AY293777	Sullivan, R.F., Bischoff, J.F. and White, J.F. Jr. What is Phoma? Unpublished
Phoma sp.201	AY293774	Sullivan, R.F., Bischoff, J.F. and White, J.F. Jr. What is Phoma? Unpublished
Pleospora rudis	U00975	Berbee, M.L. and Taylor, J.W. Dating the evolutionary radiations of the true fungi. Canadian Journal of Botany (1993) (unpublished)
Pseudoplectania nigrella	AF104345	Harrington, F.A., Pfister, D.H., Potter, D., Donoghue, M. (1999) Phylogenetic studies within the Pezizales. I. 18S rRNA sequence data and classification. Mycologia 91 (1), 41-50

Table 3.3 cont.

Species	Ac. No.	Publications
Pyronema domesticum	U53385	Landvik, S., Egger, K.N. and Schumacher, T. (1997) Towards a subordinal classification of the Pezizales (Ascomycota): phylogenetic analyses of SSU rRNA sequences. Nord. J. Bot. 17, 403-418
Raciborskiomyces longisetosum	AY016351	Lumbsch, H.T. and Lindemuth, R. (2001) Major lineages of Dothideomycetes (Ascomycota) inferred from SSU and LSU rRNA sequences. Mycol. Res. 105, 901-908
Setosphaeria monoceras	AY016352	Lumbsch, H.T. and Lindemuth, R. (2001) Major lineages of Dothideomycetes (Ascomycota) inferred from SSU and LSU rRNA sequences. Mycol. Res. 105, 901-908
Sordariomycete sp. *pgp-hsf*	AF292054	Mucciarelli, M., Scannerini, S., Bertea, C. and Maffei, M. (2002) An ascomycetous endophyte isolated from *Mentha piperita* L.: biological features and molecular studies. Mycologia 94 (1), 28-39
Talaromyces flavus	M83262	Berbee, M.L. and Taylor, J.W. (1992) Two ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. Mol. Biol. Evol. 9 (2), 278-284
Termitomyces sp. *Ol*	AB051893	Katoh, H., Shinzato, N., Maekawa, K., Miura, T. and Matsumoto, T. Two genetic types of symbiotic fungi cultivated by the macrotermitine termite Odontotermes formosanus (Isoptera: Termitidae) in the Ryukyu Archipelago. Unpublished
Trematosphaeria hydrela	AF164376	Liew, E.C., Aptroot, A. and Hyde, K.D. (2000) Phylogenetic significance of the pseudoparaphyses in Loculoascomycete taxonomy. Mol. Phylogenet. Evol. 16 (3), 392-402
Ulocladium botrytis strain 3539	AF548106	Wu, Z., Tsumura, Y., Blomquist, G. and Wang, X.-R. (2003) 18S rRNA gene variation among common airborne fungi, and development of specific oligonucleotide probes for the detection of fungal isolates. Appl. Environ. Microbiol. 69 (9), 5389-5397
Uncultured ascomycete clone AT2-4	AF530541	Lopez-Garcia, P., Philippe, H., Gail, F. and Moreira, D. (2003) Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge. Proc. Natl. Acad. Sci. U.S.A. 100 (2), 697-702
Nectria lugdunensis	AY231639	Braha, B., Krauss, G. and Krauss, G.-J. Phylogenetic relation of *Heliscus lugdunensis* based on nSSU sequence comparison. Unpublished
Pyrenophora tritici-repentis	U42486.1	Berbee, M.L. (1996) Loculoascomycete origins and evolution of filamentous ascomycete morphology based on 18S rRNA gene sequence data. Mol. Biol. Evol. 13 (3), 462-470
Uncultured soil fungus clone Pent 3.5	AY163417	Hunt, J., Boddy, L. and Rogers, H.J. An evaluation of 18S rRNA approaches for the study of fungal diversity in grassland soil. Unpublished
Westerdykella cylindrica	AY016355	Lumbsch, H.T. and Lindemuth, R. (2001) Major lineages of Dothideomycetes (Ascomycota) inferred from SSU and LSU rRNA sequences. Mycol. Res. 105, 901-908
Westerdykella dispersa	U42488	Berbee, M.L. (1996) Loculoascomycete origins and evolution of filamentous ascomycete morphology based on 18S rRNA gene sequence data. Mol. Biol. Evol. 13 (3), 462-470

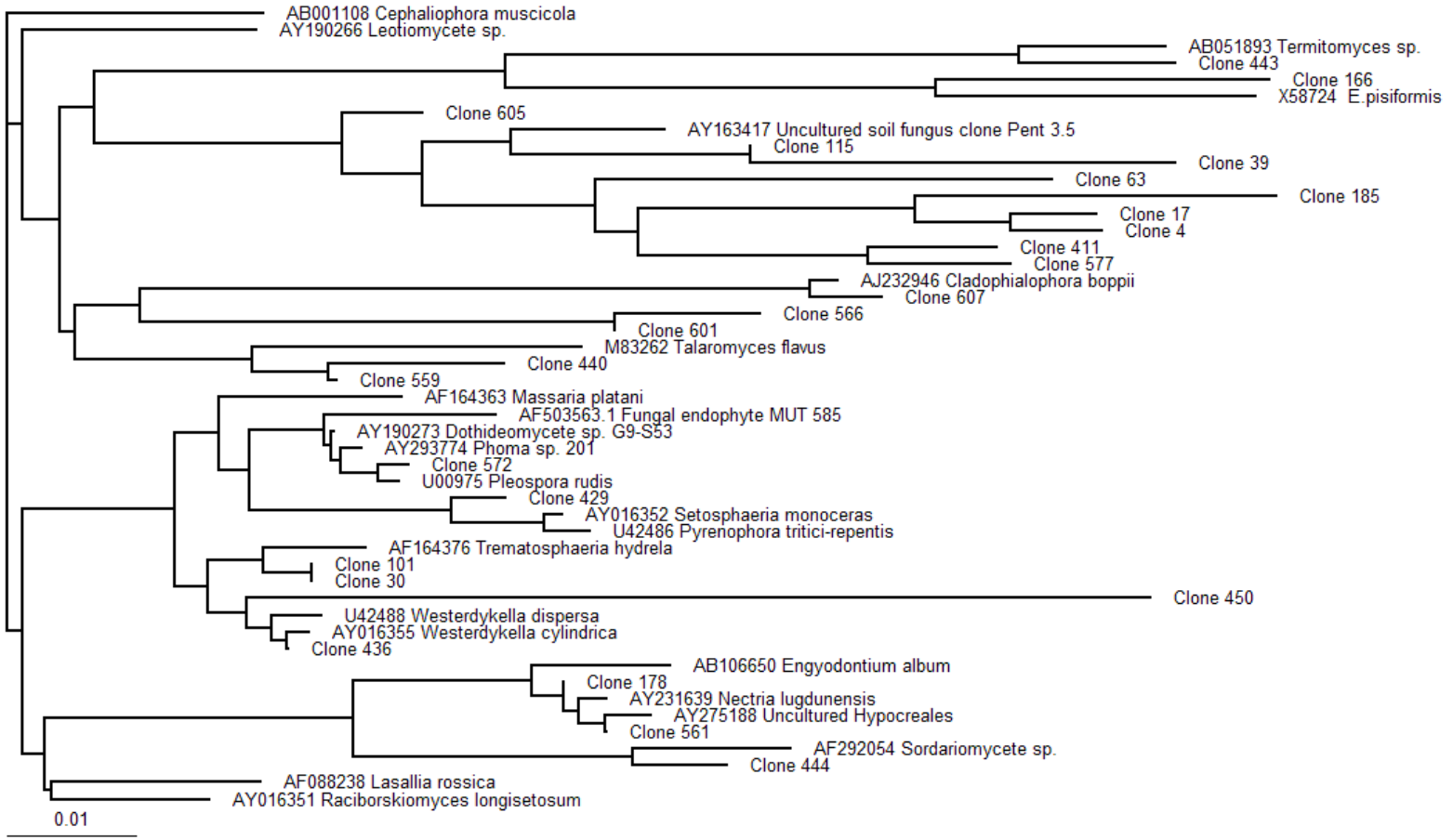


Figure 3.1: Phylogenetic tree showing the 18S rRNA relationship of selected fungal clones (Group2) isolated from healthy wheat roots to known sequences.

3.4 Discussion

Sequencing of the 18S rRNA clones identified 29 different fungal species present in the roots of wheat from four different field soils. All the fungi detected, except for three, belong to the *Ascomycota* phylum, which previously have been found to be the predominant fungal endophyte phyla in all plants tested (Ernst *et al.*, 2003). The primers used to amplify the 18S rRNA gene sequences were designed by Smit *et al.* (1999) for analysis of the fungal species present in the wheat rhizosphere. The primer set EF4-Fung5 was shown to detect a high coverage of *Ascomycota* and a lower coverage of *Basidiomycota* and only 40% of *Zygomycota* (Smit *et al.*, 1999). The EF4-Fung5 primers detected a range of fungi from the *Ascomycota* and *Zygomycota* phyla in the wheat rhizosphere. Only two species, *Pleospora rudis* and *Pleospora herbarum*, were also detected endophytically from healthy wheat roots in our study. However, it is recognised that different soil composition and indigenous microflora significantly affects the endophytic population as shown in chapter two.

Of the different species detected, a number have been previously identified as plant pathogens. Two pathogens of wheat were identified; *Pyrenophora tritici-repentis* is the causal agent of tan spot. Tan spot is an economically significant disease worldwide with reported yield losses of 2% to 40% (Ciuffetti *et al.*, 1997). Species from the *Clathrospora* genera have been known to cause disease in wheat. *Clathrospora pentamera* causes Platyspora leaf spot. *Clathrospora diplospora* was also detected, a species about which little is known. Pathogens that infect canola or rapeseed (*Brassica napus* L.) were also detected; the species *Leptosphaeria maculans* (anamorph is *Phoma lignum*) which is in the order of *Dothideomycete* causes

Blackleg disease of Brassica. Two *Phoma* species, the teleomorph *Pleospora rudis* and a *Dothideomycete* species were identified by 18S rRNA gene sequencing. Other pathogens detected include *Sordariomycete* sp., *Massaria platani* which causes disease in Sycamore and *Setosphaeria monoceras,* the causal agent of leaf blight in the genus *Echinochloa*.

The fungus *Talaromyces flavus,* was also detected. This fungus has been shown to control soilborne pathogens including *Sclerotinia sclerotiorum* (McLaren *et al.,* 1994), *Rhizoctonia solani* (Boosalis, 1956) and *Verticillium dahliae* (Fravel *et al.,* 1987; Stosz *et al.,* 1996; Murray *et al.,* 1999). A commercially available product, Protus WG®, consisting of *Talaromyces flavus* spores is used for management of *Verticillium dahliae, Verticillium albo-atrum* and *Rhizoctonia solani* in tomato, cucumber, strawberry and oilseed rape (Dufour, 2001). *Talaromyces flavus* was detected in the Swedes Flat soil with the mixed microbial inoculant NutriLife 4/20™. This commercial inoculant contains four predatory fungal species but *T. flavus* was not one of them. It is interesting that while a number of known phytopathogens were detected within the healthy wheat root, there were no symptoms of disease. It may be that these pathogens are not at the sexual stage where pathogenicity occurs or in significant numbers to causes disease. It is also possible that the spread of the phytopathogens are being controlled by other endophytes. A number of endophytic actinobacteria capable of controlling fungal pathogens of wheat including *Rhizoctonia solani*, *Pythium* sp. and *Gaeumannomyces graminis* var. *tritici*, *in vitro* and *in planta* have been isolated from healthy wheat roots in our laboratory previously (Coombs and Franco, 2003a).

The other fungal species detected previously have been identified as inhabitants of plant matter or soil. *Endogone pisiformis* forms structures similar to vesicles and arbuscules of versicular-arbuscular mycorrhizal (VAM) fungi (Berch and Fortin, 1983). *Cephaliophora muscicola* has been found in moss and forest debris in New Zealand and leaf mould in Japan (Barron, 1990). *Aleuria aurantia* has been found in disturbed soil beside woodland paths (Wood and Stevens, 2004). *Cladophialophora boppii, Ulocladium botrytis* and *Nectria lugdunensis* have been found in rotten plant material. *Raciborskiomyces longisetosum* has been isolated from the rhizosphere of maize and *Pyronema domesticum* in burnt soil and cotton gauze. It is probable that these fungi inhabiting the soil or dead plant material are able to penetrate and colonise healthy wheat tissue. This is the first time these species have been identified as endophytes of wheat. One clone was identified as matching to a fungal endophyte (Fungal endophyte MUT 585) which has not been characterised.

The sequencing of this limited number of fungal 18S rRNA clones has revealed a number of previously known fungal phytopathogens living within the healthy wheat tissue without causing apparent disease symptoms, a number of fungal species previously identified in soil or plant material but not as endophytes, a known fungal biocontrol agent and others species without a known relationship with plants. To further characterise such fungi, isolation would need to be performed. This was not pursued in this thesis as the course of research focused further on the molecular interactions with the endophytic actinobacteria and plants.

Chapter Four: Induction of Defence Pathways of *Arabidopsis thaliana* by Endophytic Actinobacteria.

4.1 Introduction

Plants have developed a non-specific mechanism of defence that can provide long term protection against a broad number of pathogens. Systemic Acquired Resistance (SAR) occurs by prior inoculation with a necrotizing pathogen, salicylic acid (SA) or chemical analogues and is characterised by an early increase in endogenously synthesised SA and the enhanced production of pathogenesis-related (PR) proteins, specifically PR-1, PR-2 and PR-5 (Uknes *et al.*, 1992).

In addition to the SAR pathway the Jasmonate (JA)/Ethylene (ET) pathway functions in a SA-independent manner and leads to the production of antimicrobial compounds that confer resistance to a number of pathogens. JA and ET signalling leads to the synthesis of the PR proteins PR-3, PR-4, the hevein-like protein, Hel, and the plant defensin PDF1.2 (van Loon and van Stein, 1999).

Non-pathogenic rhizobacteria have been shown to induce resistance in plants that is phenotypically similar to SAR. This has been termed Induced Systemic Resistance (ISR) and it is believed the pathway is distinct to the SAR pathway but is mediated by a JA/ET pathway. It does not involve the expression of PR proteins but requires the function of the NPR1 protein (van Wees *et al.,* 1999). Figure 4.1 shows the pathways proposed in Arabidopsis.

Endophytic actinobacteria isolated from healthy wheat plants in our laboratory have been shown to enhance the disease resistance in wheat when applied as a seed coating. Pot trials with wheat demonstrated that a number of endophytic actinobacteria strains were capable of enhancing resistance to the fungal pathogens *Gaeumannomyces graminis* var. *tritici* (Ggt), *Rhizoctonia solani* and *Pythium* spp.

(Coombs, 2002). Actinobacteria are prolific producers of antibiotics which may account for the disease resistance, though it is possible the endophytes are capable of activating a systemic defence response similar to the ISR mediated by rhizobacteria.

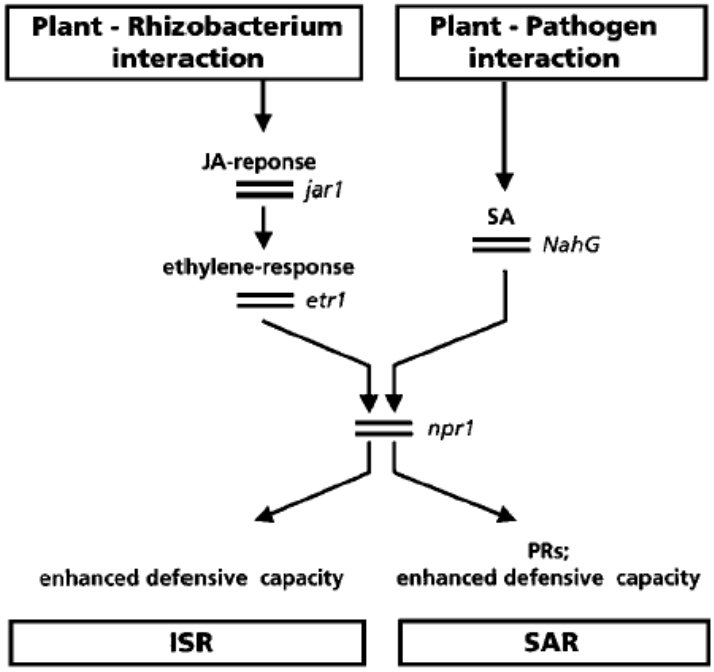


Figure 4.1: The basic pathways proposed for the ISR and SAR interactions in *Arabidopsis thaliana* (Pieterse *et al.,* 1998).

Real-time RT-PCR is a highly sensitive technique that enables detection and quantification of PCR products. The fluorescent label SYBR Green-1 was used to measure the formation of PCR products in this study. SYBR Green-1 binds all double-stranded DNA molecules and emits a fluorescent signal which has an excitation and emission maxima of 494nm and 521nm, respectively. The fluorescent signal was measured and relative quantification used to determine the level of gene induction. As products are amplified, the cycle number at which an arbitrary

fluorescence threshold is reached is termed C_t, or cycle threshold. The relationship between C_t and abundance of initial template is inverse.

As the defence pathways have been extensively studied in Arabidopsis, it was used as a model plant to determine the mechanism by which the endophytic actinobacteria enhance disease resistance. While SAR is thought to occur in wheat the pathways have not been well defined. Real-time RT-PCR was used to determine if genes in the SAR (*PR-1* and *PR-5*) and JA/ET (*PDF1.2* and *Hel*) pathways of Arabidopsis were induced by the addition of the endophytic actinobacteria.

4.2 Materials and Methods

4.2.1 Cultivation of *Arabidopsis thaliana*

Arabidopsis thaliana (ecotype Columbia-0) plants were grown in 9 cm petri dishes on half strength Murashige and Skoog (MS) salt medium (Sigma-Alrich) with 0.8% Bacto-agar (Bacto Laboratories, Liverpool, Australia) as the gelling agent when the plants were grown on plates with a horizontal orientation or 0.8% phytagel agar (Sigma-Aldrich) if grown on plates with a vertical orientation. Seeds were surfaced-sterilised by immersing in 70% ethanol for 1 min followed by 1 ml 12% sodium hypochlorite (as Domestos® bleach) for 10 min. The seeds were then rinsed twice in sterile 2% sodium thiosulphate to remove the residual chlorine (Miche and Balandreau, 2001) and a minimum of 5 times with sterile H_2O to remove all traces of sodium hypochlorite. Seeds were placed onto ½ MS salt medium and the plates were sealed with micropore tape and placed at 4°C overnight to achieve even germination. Plants were then transferred to a light box with a 9 hr light cycle with the temperature ranging from 20 ± 4°C.

4.2.1.1 Chemical Treatment of Arabidopsis Plants

Markers of the SAR pathway, *PR-1* and *PR-5*, expression can be induced by SA and its synthetic counterparts (Uknes *et al.*, 1992). Six to eight week old Arabidopsis plants were sprayed with 5 mM SA and harvested at 6 and 24 hrs. Genes in the JA/ET pathways such as *PDF1.2* and *Hel* can be induced by the application of JA or the methyl derivative, methyl jasmonate (MeJA), and ET or its pre-cursor, 1-

aminocyclopropane-1-carboxylic acid (ACC). Six to eight week old plants were sprayed with 50 µM MeJA or 0.01% ACC.

4.2.1.2 Endophytic Actinobacteria Cultures

The endophytic actinobacteria cultures listed in table 4.1 were maintained on half-strength potato dextrose agar (½ PDA) or oatmeal agar (Appendix 1). All the actinobacteria used in this study were previously isolated from healthy wheat roots in our laboratory.

Table 4.1: Endophytic actinobacteria cultures used in this study.

Culture No.	GenBank Ac. No.	Actinobacteria Culture Name
EN2	AY148073	*Microbispora* sp.
EN27	AY148075	*Streptomyces* sp.
EN28	AY148076	*Streptomyces* sp.
EN43	AY291589	*Micromonospora* sp.
EN46	AY148081	*Nocardioides albus*

4.2.1.3 Inoculation of Arabidopsis Seeds with Endophytic Actinobacteria

The endophytic actinobacteria listed in Table 4.1 were grown on ½ PDA or oatmeal agar. Plates were incubated at 27°C for 3 to 10 days until complete sporulation had occurred. Spores were harvested by scraping off the plate with a sterile loop and placing in 2 ml sterile H_2O. This spore suspension was used to inoculate surface sterilised Arabidopsis seeds. A 10 µl drop of the spore suspension, or H_2O for control plants, was placed onto the seed and left in the laminar flow cabinet so the H_2O could evaporate. Plates were sealed with micropore tape and kept

at 4°C overnight to break dormancy before being transferred to the light box.

4.2.1.4 Inoculation of Arabidopsis Plants with Endophytic Actinobacteria Culture Filtrates

The culture filtrates of the endophytic actinobacteria listed in Table 4.1 were used to inoculate sterile Arabidopsis seeds. The culture filtrate was prepared by inoculating 50 ml of pre-inoculum medium (Appendix 1) in 250ml Erlenmeyer flasks with a loop of the actinobacteria spores removed from a culture plate. The pre-inoculum medium was grown at 27°C for 3 days with 150 rpm shaking in a rotor shaker. Five percent (v/v) of the pre-inoculum culture was used to inoculate 50 ml of FLO26 or FL031 medium (Appendix 1) in 250 ml Erlenmeyer flasks. The cultures were incubated at 27°C with 150 rpm shaking on a rotor shaker. After 8 days of growth the cultures were transferred to sterile 50 ml falcon tubes and centrifuged at 4,000*g* for 5 min. The culture broth was removed and filtered through a 0.8 µm Millipore filter to remove bacterial cells. The cell pellet was resuspended in 20 ml 15% glycerol and stored at -20°C. The filtrate was used to treat the roots of 6-8 weeks old Arabidopsis plants.

4.2.2 Arabidopsis Pathogen Cultures

Erwinia carotovora subsp. *carotovora* (Ecc) originally isolated from leek was grown on ½ PDA at 27°C for 3 to 4 days. The bacterial cells were scraped off one plate and transferred to 30 ml sterile saline. The bacterial suspension was vortexed to

break up any clumps and the CFU counted by the Miles and Misra technique (Miles and Misra, 1938). *Fusarium oxysporum* originally isolated from cucumber was maintained on ½ PDA. Based on partial 18S rRNA gene sequencing the closest relative is *Fusarium oxysporum* f. *vasinfectum*, the primary cause of Fusarium wilt in cotton. A plug of the culture was used to inoculate 50 ml Sabouraud broth (Appendix 1) in 250 ml Erlenmeyer flasks and incubated at 27°C for 3 days with 150 rpm in a rotary shaker and the CFU was counted by the Miles and Misra method (Miles and Misra, 1938). A 1×10^8 CFU.ml^{-1} *F. oxysporum* suspension used to inoculate 6-8 week Arabidopsis plants.

The Ecc and *F. oxysporum* were kindly provided by the Horticultural Pathology and Nematology Group at the South Australian Research and Development Institute.

4.2.2.1 Inoculation of Arabidopsis with Pathogens

Six to eight week old endophyte-inoculated and untreated Arabidopsis plants grown on vertical plates on ½ MS salt medium were challenged with the fungal pathogen *Fusarium oxysporum*. The *F. oxysporum* suspension (1-3.5 × 10^8 CFU.ml^{-1}) was applied directly to the roots of the plants which were harvested 3 days post challenge.

The bacterial pathogen *Erwinia carotovora* subsp. *carotovora* (Ecc) suspension (1-3 × 10^8 CFU.ml^{-1}) was used to challenge endophyte-inoculated and untreated 6-8 week old Arabidopsis plants grown on ½ MS salt medium with Bacto-

agar as the gelling agent. The Ecc was applied by pressure infiltration using a needle-less syringe and 0.9% saline applied to control plants. The plants were harvested 3 days post challenge, which was chosen on the basis of a time-course of the disease progression and expression of defence genes for Ecc (Aguilar *et al.,* 2002) and *F. oxysporum* (Epple *et al.,* 1995).

4.2.3 Harvesting Arabidopsis Plant Material

The Arabidopsis plant material was harvested by carefully removing the plants from the medium and immediately freezing in liquid nitrogen. Two to three Arabidopsis plants were frozen in each eppendorf tube and stored at -80°C for RNA extraction.

4.2.4 Analysis of Gene Expression by Real-Time RT-PCR

4.2.4.1 Total RNA Extraction

Total RNA was extracted from 100-200 mg of snap frozen Arabidopsis tissue using Trizol (Invitrogen, Australia). The plant tissue was placed in a pre-chilled mortar with 600 µl of Trizol and ground into fine particles. The plant/Trizol mixtue was transferred to an eppendorf tube and kept on ice until all samples had been ground. Another 400 µl of Trizol was added and the samples kept at RT for 5 min. 200 µl of chloroform was added for every 1 ml of Trizol and the samples vigorously shaken for 15 seconds. The samples were held at RT for 3 min then centrifuged at 12,000*g* for 15 min at 4°C. The aqueous layer was transferred to an eppendorf tube

with 250 µl of high salt precipitation solution (0.8 M sodium citrate, 1.2 M NaCl) and 250 µl isopropanol. Samples were precipitated at RT for 10 min followed by centrifugation at 4°C for 10 min at 12,000*g*. The supernatant was removed carefully to prevent the pellet from being dislodged. The pellet was washed by adding 1 ml 75% ethanol and vortexed briefly. Samples were centrifuged at 4°C at 7,000*g* for 5 min. The ethanol was discarded and the pellet air dried for 10 min. The RNA was resuspended in 30 µl H_2O. Quantification of RNA was performed in duplicate using GeneQuant Pro RNA/DNA calculator (Amersham Biosciences) and integrity was checked on 1.5%-2.0% agarose gels.

4.2.4.2 Removal of DNA from Total RNA

Contaminating DNA was removed from RNA preparations using RQ-1 RNase-free DNase-1 (Promega). In a volume of 50 µl 10 µg RNA, 1X DNase buffer and 1 unit of DNase-1 was incubated at 37°C for 30 min. The RNA was re-extracted by adding 100 µl of Trizol and vortexing then adding 20 µl chloroform. The contents were mixed by shaking vigorously for 15 seconds. The samples were kept at RT for 3 min and then centrifuged at 4°C at 12,000*g* for 15 min. The aqueous phase was transferred to a sterile eppendorf tube and 84 µl of isopropanol added. The sample was gently mixed and allowed to precipitate at RT for 15 min. The sample was then centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was removed and the pellet washed by adding 1 ml 75% ethanol followed by vortexing and centrifugation at 7,500*g* for 3 min at 4°C. All ethanol was removed and the pellet air-dried for 10 min. The RNA pellet was dissolved in 10 µl H_2O. The RNA was quantified in triplicate

using the GeneQuant Pro RNA/DNA calculator (Amersham Biosciences) and integrity was checked on 1.5%-2.0% agarose gels. The efficiency of DNase treatment was checked by PCR using *Hel* primers (Table 4.2). The PCR was set up as per section 4.2.4.5 with the SYBR Green-1 dye omitted and 2 µl of DNA-free RNA as the experimental template and cDNA as the positive control.

4.2.4.3 Reverse Transcription of Total RNA to cDNA

Omniscript reverse transcriptase (Qiagen) was used to convert the DNA-free RNA into cDNA. The cDNA was produced according the manufacturer's instructions using 1 µg RNA pooled from nine plants, except Oligo dT primers (50 $ng.\mu l^{-1}$) were used in place of random hexamers and the 93°C inactivation step was omitted. The cDNA was diluted 1:4 with sterile H_2O and 2 µl cDNA used per 10 µl PCR reaction.

4.2.4.4 Real-Time RT-PCR Primers

The Arabidopsis housekeeping gene *Actin* was used to normalise the experimental genes. Primers designed by Charrier *et al.* (1996) were used as the *Actin2* and *Actin8* genes are simultaneously amplified and are suitable for real-time RT-PCR. The primers for the genes, hevein-like protein (*Hel*), plant defensin gene *PDF1.2*, putative pathogenesis-related *PR-1* gene and the putative thaumatin gene (*PR-5*) were designed using the primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers were then analysed for dimers, hairpins and cross dimers using the netprimer program available online

(http://primerdesign.com/netprimer/netprlaunch/netprlaunchorg.html). All primers were prepared by Geneworks (Adelaide, Australia). Table 4.2 lists the primer sequences for the target genes. The PCR products were sequenced to confirm correct amplification of the target gene. Sequences were compared to online databases using the BLAST program located at the NCBI (www.ncbi.nlm.nih.gov). The standard blastn (nucleotide-nucleotide) algorithm was used with the default settings (Altschul *et al.*, 1997).

Table 4.2: Primers for real-time RT-PCR

Target Gene	Accession No.	Primer Sequence	Product Size	Ref
Actin2 /Actin8	U41998 U42007	F: 5' GGT AAC ATT GTG CTC AGT GGT GG 3' R: 5' AAC GAC CTT AAT CTT CAT GCT GC 3'	120 bp	1, 2
Hel	NM_111344	F: 5' CAA GTG TTT AAG GGT GAA GA 3' R: 5' CGG TGT CTA TTT GAT TGA AC 3'	118 bp	3
PDF1.2	AY133787	F: 5' CTG CTC TTG TTC TCT TTG CT 3' R: 5' GTG TGC TGG GAA GAC ATA 3'	164 bp	3
PR-1	AY117187	F: 5' GCC TTA CGG GGA AAA CTT A 3' R: 5' CTT TGG CAC ATC CGA GTC T 3'	160 bp	3
PR-5	AY059114	F: 5' CGG AAA CGG TAG ATG TGT AAC 3' R: 5' GTT GAG GTC AGA GAC ACA GCC 3'	216 bp	3

Reference 1: An *et al.* (1996)
Reference 2: Charrier *et al.* (2002)
Reference 3: This study

4.2.4.5 Real-Time RT-PCR

The *Actin2/Actin8, PDF1.2, Hel* and *PR-5* transcripts were amplified using the primer sequences in Table 4.2. All reagents, including the cDNA, used in the real-time RT-PCR were aliquoted for single use to ensure no variation between runs.

For all samples, duplicate reactions with a final volume of 10 µl consisted of: 10 ng of forward and reverse primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.4X SYBR Green-1 (Molecular Probes, Eugene, U.S.A), 1X Qiagen PCR Buffer; 0.25 units of

Hot Star Taq (Qiagen) and H_2O to 8 µl and 2 µl cDNA per reaction.

Amplification of *Actin*, *Hel*, *PDF1.2* and *PR-5* products was performed in the Rotorgene 2000 (Corbett Research, Sydney, Australia) with the following thermal cycle profile: 95°C - 15 min, (94°C - 20 sec, 54°C – 20 sec, 72°C – 20 sec) × 35 cycles.

For amplification of *PR-1* the following thermal cycle profile was used: 95°C - 15 min, (94°C - 20 sec, 56°C – 20 sec, 72°C – 20 sec) × 40 cycles.

Products were then melted by heating from 65°C to 92°C over 5 min. This will produce a melt curve which enables the detection of a mixture of amplification products. The data was acquired on the SYBR-green channel with the gain set at 7, and for both quantitation and melt analysis a 'light' digital filter was utilised to smooth raw fluorescence readings. Melt-curve analysis was carried out with the dF/dT threshold set above the fluorescence background at a value of 1 for detection of single melt-products. Amplification products were also run on 1.5%-2.0% agarose gels to check correct product size and the absence of primer dimer.

4.2.4.6 Real-Time RT-PCR Primer Amplification Efficiency

The amplification efficiency of each primer set was determined using serial dilutions of purified PCR products. PCR products for each gene were amplified as per section 4.2.4.5 and purified with the UltraClean PCR Purification kit (MoBio, California, U.S.A). Serial 10-fold dilutions of the purified PCR products were made and 2µl used as the template in the real-time RT-PCR which was performed as per

4.2.4.5. The purified PCR products were assigned arbitrary copy numbers in the Rotorgene 2000 software to reflect the serial 10-fold dilutions (Rajeevan *et al.,* 2001). A standard curve was generated using the Rotorgene 2000 software based on the least-squares linear regression method with the C_t values plotted against the $\log_{10}$(copy number). The slope of this standard curve was used to determine amplification efficiency (E) using the calculation $E = 10^{(-1/slope)}$.

4.2.4.7 Relative Quantification of Gene Expression (QPCR)

Cycle threshold (C_t) values, were obtained from raw fluorescence data using quantitation analysis options in the Rotorgene 2000 software (Corbett Research, Sydney Australia). The fluorescence threshold was set at 0.03, dynamic tube normalisation was applied to correct for background fluorescence in raw data and the first five cycles were ignored. The cycle threshold values were used to calculate the fold induction of the target gene in each sample. The comparative C_t or Delta-Delta-C_t method that takes into account differences in amplification efficiencies of the target transcript and the housekeeping transcript used to calculate relative gene expression.

The Delta-Delta-C_t ($\Delta\Delta$-C_t) equation (Equation 4.1) developed by Livak and Schmittgen (2001) compares the C_t value of the target sequence compared to the reference sample which is normalised against the expression of a housekeeping transcript where it is constant under all treatments. Normalisation against a housekeeping transcript accounts for differences in RNA integrity, uneven input of RNA into reverse transcription and the efficiency of reverse transcription. Typically

β-actin, *β-tublin* and *GAPDH* sequences are used as they are relatively stable under most conditions although it has been shown some factors can alter the expression (Thellin *et al.*, 1999). The Arabidopsis *β-actin* transcripts were used for normalisation in this study as stated previously in 4.2.4.4.

Equation 4.1: $R = 2^{-\{[Ct_{TU} - Ct_{TC}] - [Ct_{HU} - Ct_{HC}]\}}$

Ct_{TU}: C_t value for the target sequence

Ct_{TC}: C_t value for the target sequence reference sample

Ct_{HU}: C_t value for the endogenous housekeeping sequence

Ct_{HC}: C_t value for the endogenous housekeeping sequence reference sample

This equation assumes that the amplification efficiencies of the target sequence and housekeeping sequence are 2.0. If the amplification efficiencies are not 2.0 there will be an overestimation of the gene expression. The equation (Equation 4.2) developed by Pfaffl (2001) incorporates differential amplification efficiencies and, therefore, this equation was used to calculate the relative fold induction of the genes of interest.

Equation 4.2: $$R = \frac{E_T^{\Delta Ct_T[C-U]}}{E_H^{\Delta Ct_H[C-U]}}$$

ET: amplification efficiency for the target sequence

EH: amplification efficiency for the housekeeping sequence

ΔCt_T[C-U]: difference in C_t value for the target sequence and the reference sample and the unknown sample

ΔCt_H[C-U]: difference in C_t for the housekeeping sequence and the reference sample and the unknown sample.

The RT-PCR results are presented as means of duplicate QPCR where the RNA was pooled from nine plants. Two independent experiments were conducted to validate the results.

4.2.5 Microscopy

Electron microscopy of the *Micromonospora* sp. EN43 treated Arabidopsis roots were performed by Dr. Margaret McCully and Dr. Cheng Huang at CSIRO Plant Industry, Canberra using a scanning cryoelectron microscope JSM-4600 as per 2.2.5. Sample preparation and sectioning was performed by Dr. Cheng Huang as per McCully *et al.* (2000).

4.3 Results

4.3.1 Real-Time RT-PCR Standards and Validation

The designed primers amplified the transcripts of interest with no visible primer dimer formation when the products were separated on an agarose gel. Figure 4.2 shows the PCR products of the five different transcripts *Actin*, *PR-1*, *PR-5*, *PDF1.2* and *Hel*. Sequencing confirmed the PCR products were from the message of interest. The amplification efficiency of each primer set was determined and is shown below in Table 4.3. The standard curves are show in Appendix six.

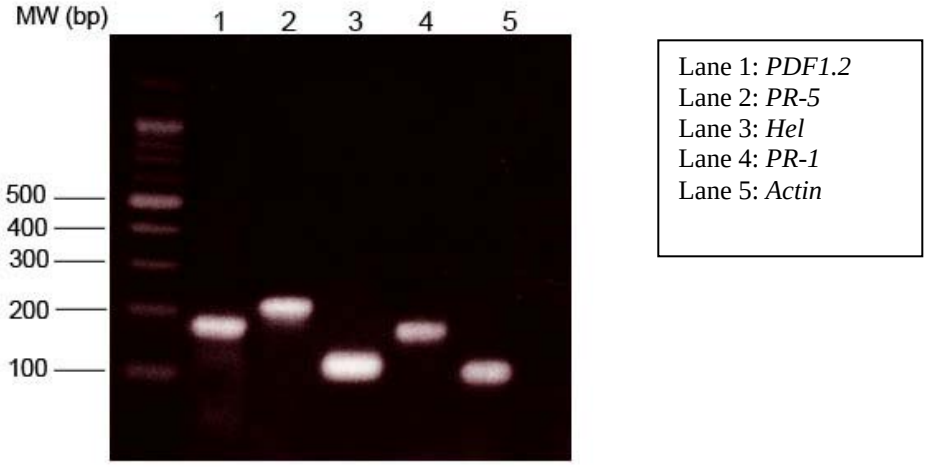


Figure 4.2: PCR products (10 µl) of *PDF1.2*, *PR-5*, *Hel*, *PR-1* and *Actin* run on a 1.8% agarose gel.

Table 4.3: Amplification efficiency of primer sets for the Arabidopsis *Actin*, *PR-1*, *PR-5*, *PDF1.2* and *Hel* transcripts.

Transcripts	Amplification Efficiency
Actin	1.61
PR-1	1.64
PR-5	1.69
PDF1.2	1.78
Hel	1.89

The standard deviation and coefficient of variation (CV – percentage ratio of standard deviation to the mean) was determined for each run. Samples that had an intra-run CV (CV between PCR replicates) higher than 4% were repeated; however this occurred rarely and in the majority of experiments the CV was under 2%. The inter-run CV ranged from under 1% up to 10%. Table 4.4 shows an example of the intra- and inter-run CV for the *Actin* transcript. Table 4.5 shows the CV for four transcripts, *Actin*, *PDF1.2*, *Hel* and *PR-5* for three replicate cDNAs. This was done to confirm the cDNA synthesis reaction was consistent.

Table 4.4: The coefficient of variation (CV) between PCR replicates (intra-run CV) and between runs (inter-run CV) for the *Actin* transcript.

cDNA Sample	**C_t Run 1**	**Intra Run CV**	**C_t Run 2**	**Intra Run CV**	**Inter Run CV**
Col-0 Untreated	21.62	0.22	21.01	1.29	2.02
	21.30		20.63		2.26
EN27 Inoculated + Fusarium Root Sample	22.04		21.26		2.55
	21.85	0.14	21.17	0.30	2.24
EN27 Inoculated + Fusarium Leaf Sample	21.60		21.07		1.76
	21.75	0.11	21.54	1.56	0.69
Col-0 + Fusarium Day 1	21.23		21.32		0.39
	21.89	0.47	21.33	0.03	1.83
Col-0 + Fusarium Day 2	22.14		22.51		1.17
	23.68	1.08	23.34	2.56	1.02
Col-0 + Fusarium Day 3	21.11		20.64		1.59
	22.39	0.91	21.52	2.95	2.80
Col-0 + Fusarium Day 4	22.01		21.09		3.02
	22.94	0.66	22.12	3.37	2.57
EN27 Inoculated + Fusarium Day 1	22.77		22.05		2.27
	23.21	0.32	22.53	1.52	2.10
EN27 Inoculated + Fusarium Day 2	21.66		21.59		0.23
	23.00	0.94	21.89	0.98	3.50
EN27 Inoculated + Fusarium Day 3	22.74		22.13		1.92
	22.99	0.18	22.57	1.39	1.30
EN27 Inoculated + Fusarium Day 4	22.74		22.4		1.07
	22.66	0.05	23.12	2.24	1.42

Table 4.5: Coefficient of variation between three cDNA replicates for four transcripts *Actin*, *PR-5*, *PDF1.2*, and *Hel*.

cDNA Sample	*Actin* C_t	*PR-5* C_t	*PDF1.2* C_t	*Hel* C_t
EN2 cDNA 1	24.46	30.82	26.39	18.83
EN2 cDNA 2	24.14	29.95	25.97	18.43
EN2 cDNA 3	24.15	29.53	25.42	18.28
Average	24.25	30.10	25.93	18.51
Standard deviation	0.18	0.66	0.49	0.28
CV	**0.74**	**2.18**	**1.88**	**1.53**

4.3.2 Induction of Gene Expression in the SAR and JA/ET Pathways using Chemical Elicitors

Validation of the real-time RT-PCR technique as a method for quantifying the induction of the SAR genes, *PR-1* and *PR-5*, and the JA/ET genes, *PDF1.2* and *Hel*, was performed by applying SA, MeJA and ACC to 6-8 week old Arabidopsis plants. The fold induction of these transcripts compared to an untreated control was then determined using the real-time RT-PCR technique. Table 4.6 shows the fold induction of each transcript after treatment.

Table 4.6: Up-regulation of the *PR-1*, *PR-5*, *PDF1.2* and *Hel* transcripts after treatment with SA, MeJA and ACC and normalised against *Actin*. The yellow highlighted boxes indicate a significant induction compared to the untreated plant.

Treatment	Time after treatment	*PR-1*	*PR-5*	*PDF1.2*	*Hel*
Untreated	-	1.00	1.00	1.02	1.02
SA	6 hrs	948	3.88	1.02	1.57
SA	24 hrs	1468	1.09	1.22	4.55
MeJA	24 hrs	1.37	0.26	69.13	8.35
ACC	24 hrs	2.00	0.54	11.06	6.14

The application of SA to the Arabidopsis plant triggers the SAR pathway and as a result the *PR-1* and *PR-5* transcripts are increased (Uknes *et al.*, 1992). *PR-1* was strongly induced by SA at 6 (948-fold) and 24 hours (1468-fold), but was not induced by MeJA or ACC. The *PR-5* transcript was induced by SA approximately 4-fold at 6 hours but this was transient and no induction could be detected at 24 hours. The *PR-5* transcript was reduced with the application of MeJA and ACC but this was not considered significant.

In Arabidopsis JA and ET stimulate the up-regulation of the pathogenesis-related genes *PR-3*, *PR- 4*, *Hel* and *PDF1.2* (van Loon and van Stein, 1999). *Hel* has previously been shown to be induced strongly by ET and to a lesser extent SA (Potter *et al.*, 1993). In this study the application of 5mM SA induced the *Hel* transcript 4.5-fold whereas an 8-and 6-fold induction was detected when MeJA and ACC were applied, respectively.

Previously it has been reported that the *PDF1.2* transcript can be induced with ET and MeJA but not by SA or INA (Manners *et al.*, 1998). In this study the application of MeJA resulted in the 69-fold induction of the *PDF1.2* transcript. The ET precursor, ACC, was also able to induced *PDF1.2* but to a lesser extent with only an 11-fold induction. *PDF1.2* transcription was not able to be induced by SA.

These results correlate with previous studies and this validates the real-time RT-PCR relative quantitation approach to study gene expression changes in *Arabidopsis thaliana*.

4.3.2 The Effect of Endophytic Actinobacteria on Key Genes in the SAR and JA/ET Pathways

Inoculation of the Arabidopsis seeds with actinobacteria endophytes was performed to determine if induction of genes in the SAR and JA/ET pathways occurred. The seed was inoculated to ensure the endophyte had maximum opportunity to colonise the plant as the seed germinated. Plants were harvested after approximately 7 weeks of growth and the changes in gene expression analysed by

Real-time RT-PCR and the results presented in Figure 4.3.

Inoculation with *Streptomyces* sp. EN27 resulted in a 19-fold induction of the *PR-1* transcript. The *Streptomyces* sp. EN28 strain was able to induce *PR-1* 4-fold, *PR-5* 3-fold and *PDF1.2* 23-fold. Inoculation with *Nocardioides albus* EN46 resulted in a 6-fold induction of *PR-1* and 3-fold induction of *PR-5*. *Micromonospora* sp. EN43 did not show an ability to induce gene expression.

4.3.3 The Effect of *Erwinia carotovora* subsp. *carotovora* on the Induction of Key Genes in the SAR and JA/ET Pathways after Pre-Treatment with Endophytic Actinobacteria

Inoculation of the Arabidopsis seed with the endophytic actinobacteria was performed at the time of sowing. Plants treated with endophytic actinobacteria and untreated controls were challenged with the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* (Ecc) after approximately seven weeks of growth.

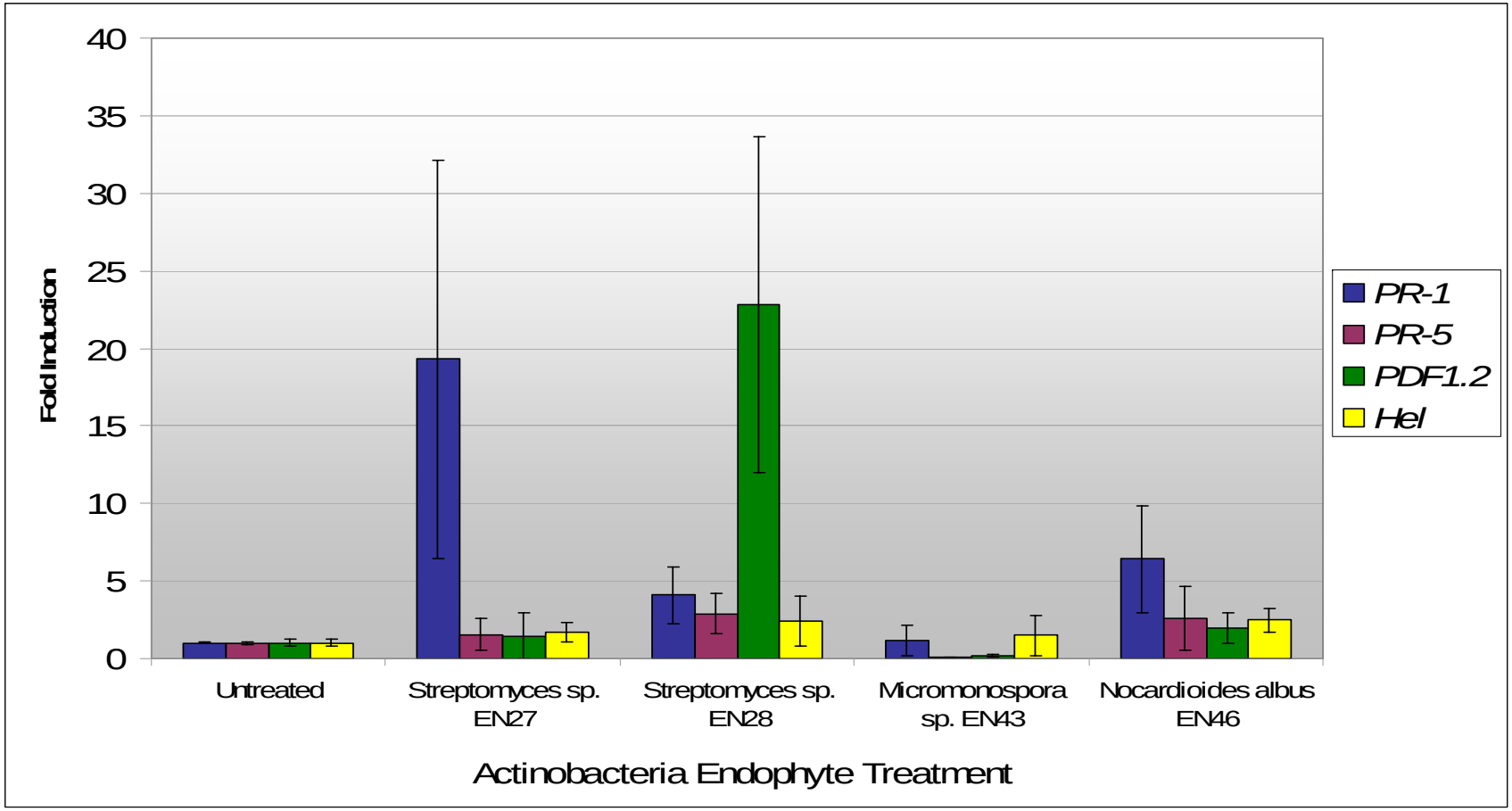


Figure 4.3: Induction of genes in the SAR and JA/ET pathways in seven week old Arabidopsis (Col-0) plants pre-inoculated with actinobacteria endophytes *Streptomyces* sp. EN27, *Streptomyces* sp. EN28, *Micromonospora* sp. EN43 and *Nocardioides albus* EN46 (n=9).

Three days after infection the untreated plant showed severe disease symptoms of soft rot, whereas the endophyte treated plants showed a normal appearance. The changes in gene expression in these plants were analyzed by real-time RT-PCR and the results are presented in Figure 4.4.

Analysis of the untreated plants infected with Ecc showed the JA/ET pathway was strongly up-regulated with increased *PDF1.2* and *Hel* gene expression. After challenge with Ecc, the *PDF1.2* transcript was significantly induced in all the endophytic actinobacteria treated plants. The *PR-1* transcript was induced approximately at the same level in the EN27-treated infected plant and the untreated infected plant, whereas in the EN28- and EN46-treated plants the expression of the *PR-1* gene was very low. The EN27-, EN28- and EN46-treated plants resulted in the induction of *Hel* at half the level of the untreated infected plant.

The EN43-treated Arabidopsis showed a different pattern of gene expression after Ecc infection compared to treatment with the other actinobacteria endophytes. As with the other endophytes the *PDF1.2* transcript was strongly induced 400-fold. However, unlike with the other endophytes the *PR-1* and *Hel* transcripts were induced, 188-fold and 185-fold, respectively. This is 17- and 3-times over the level detected in the untreated infected plants, respectively.

No significant changes in *PR-5* gene expression were detected in any actinobacteria endophyte-treated plants challenged with Ecc.

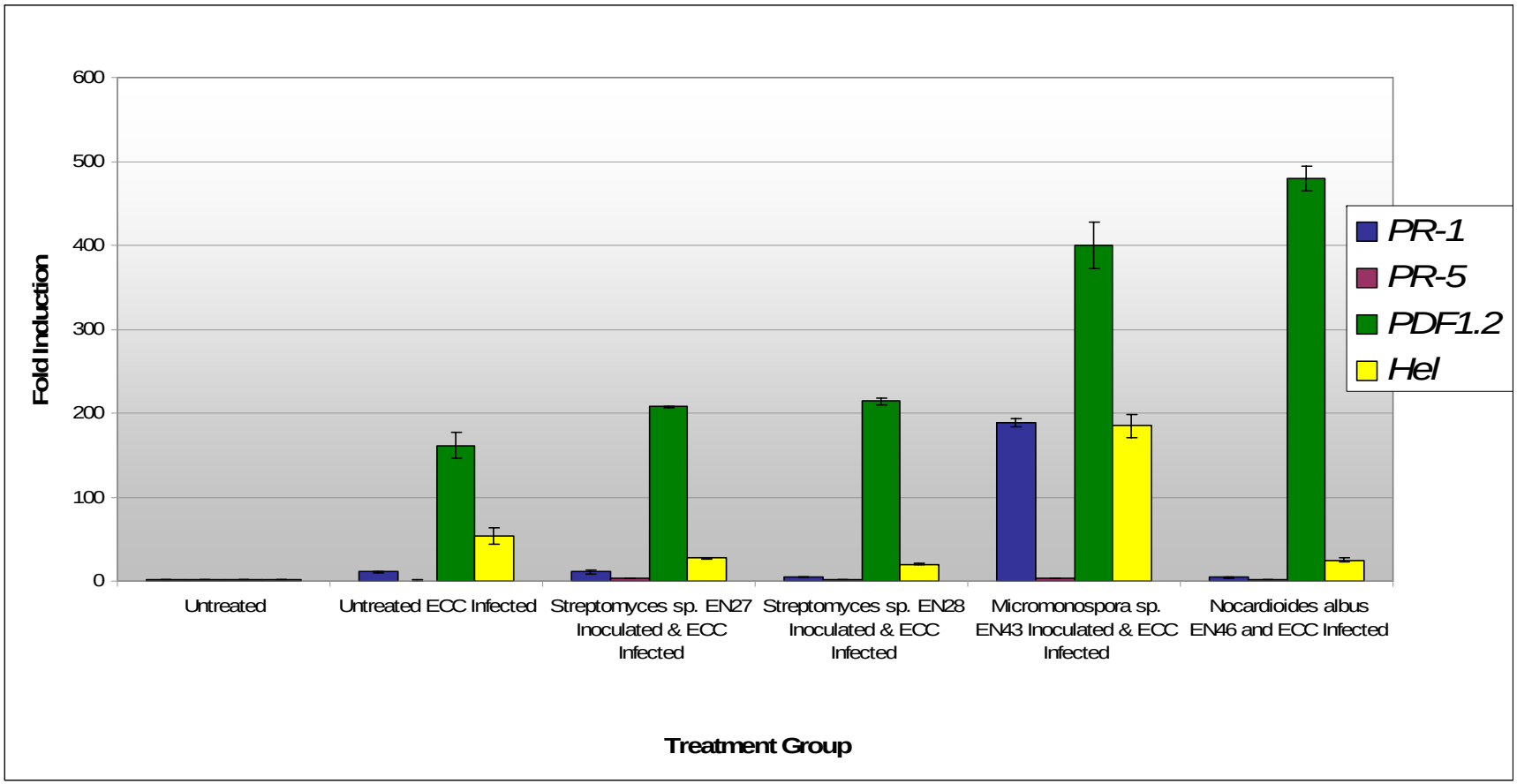


Figure 4.4: Induction of genes in the SAR and JA/ET pathways in seven week old Arabidopsis (Col-0) plants pre-inoculated with actinobacteria endophytes *Streptomyces* sp. EN27, *Streptomyces* sp. EN28, *Micromonospora* sp. EN43 and *Nocardioides albus* EN46 or untreated plant 3 days after challenge with *Erwinia carotovora* subsp. *carotovora* (n=9).

4.3.4 The Effect of *Fusarium oxysporum* on the Induction of Key Genes in the SAR and JA/ET Pathways after Pre-Treatment with Endophytic Actinobacteria

Arabidopsis seeds were coated with the endophytic actinobacteria spores and challenged with the fungal pathogen *F. oxysporum*. Three days after infection the untreated plant showed symptoms of disease (yellowing leaves) which could not be seen in the endophyte treated plants. The changes in gene expression of these plants were analyzed by real-time RT-PCR and the results are presented in Figure 4.5.

Infection of Arabidopsis with *F. oxysporum* resulted in the induction of genes in both the SAR and JA/ET pathways to some degree. The *PR-1* and *PR-5* transcripts were induced to a higher degree when the plant was inoculated with an endophyte. The highest level of gene expression change was detected in pathogen-infected plants treated with EN28. The *PR-1* and *PR-5* transcripts were induced 2138-fold and 44-fold which is 48- and 13-times the level in the untreated infected plant, respectively. The *PDF1.2* transcript was induced in all endophyte-treated plants but only EN27 and EN46 induced the transcript significantly above the level in the untreated plants, 2.5 and 1.5 times abovle the level detected in the infected untreated control, respectively. The *Hel* transcript was induced to a higher degree in only the EN28- and EN46-treated plants, approximately 2.1 and 1.8 times above the level in the untreated plants, respectively.

The roots and leaves of Arabidopsis plants treated with EN27 and infected with *F. oxysporum* were separated and the gene expression analysed independently in the roots and leaves, the results are presented in Figure 4.6. The *PR-1* transcript could

only be detected in the leaves with an 850-fold induction detected. Likewise with the *PDF1.2* transcript, an 84-fold induction was detected only in the leaves. The *PR-5* and *Hel* transcripts were induced both in the roots and leaves. The *PR-5* transcript was induced 4-fold in the roots and 9-fold in the leaves and *PDF1.2* 17-fold in the roots and 32-fold in the leaves. This indicated that the induced resistance by the endophytes was indeed systemic.

4.3.5 The Effect of Endophytic Actinobacteria Culture Filtrate on SAR and JA/ET Gene Expression

The actinobacteria culture filtrates were used to treat the Arabidopsis plants and the gene expression changes were analysed by real-time RT-PCR (Figure 4.7). Two different media were used to culture the actinobacteria; a simple (FL026) and a complex (FL031) medium. The cultures were harvested after 8 days as this has allowed for secondary metabolite production. The application of the culture filtrates from the EN27 and EN28 cultures did not result in any significant gene induction. When the EN43 FL026 culture filtrate was applied, the *PR-1* transcript was induced 9-fold and the *PR-5* transcript 16-fold after 24 hours indicating activation of the SAR pathway. Futhermore, when EN43 was cultured in the complex medium FL031 the JA/ET pathway was more strongly induced. The *PDF1.2* transcript was induced 24-fold and the *Hel* transcript 8-fold after 24 hours. There was a significant increase in the *PR-1* transcript at 5-fold but no induction of *PR-5*. The only other endophyte that induced a significant change in the expression of the tested genes was EN46 when cultured in the complex medium. The *PDF1.2* transcript was induced 8-fold.

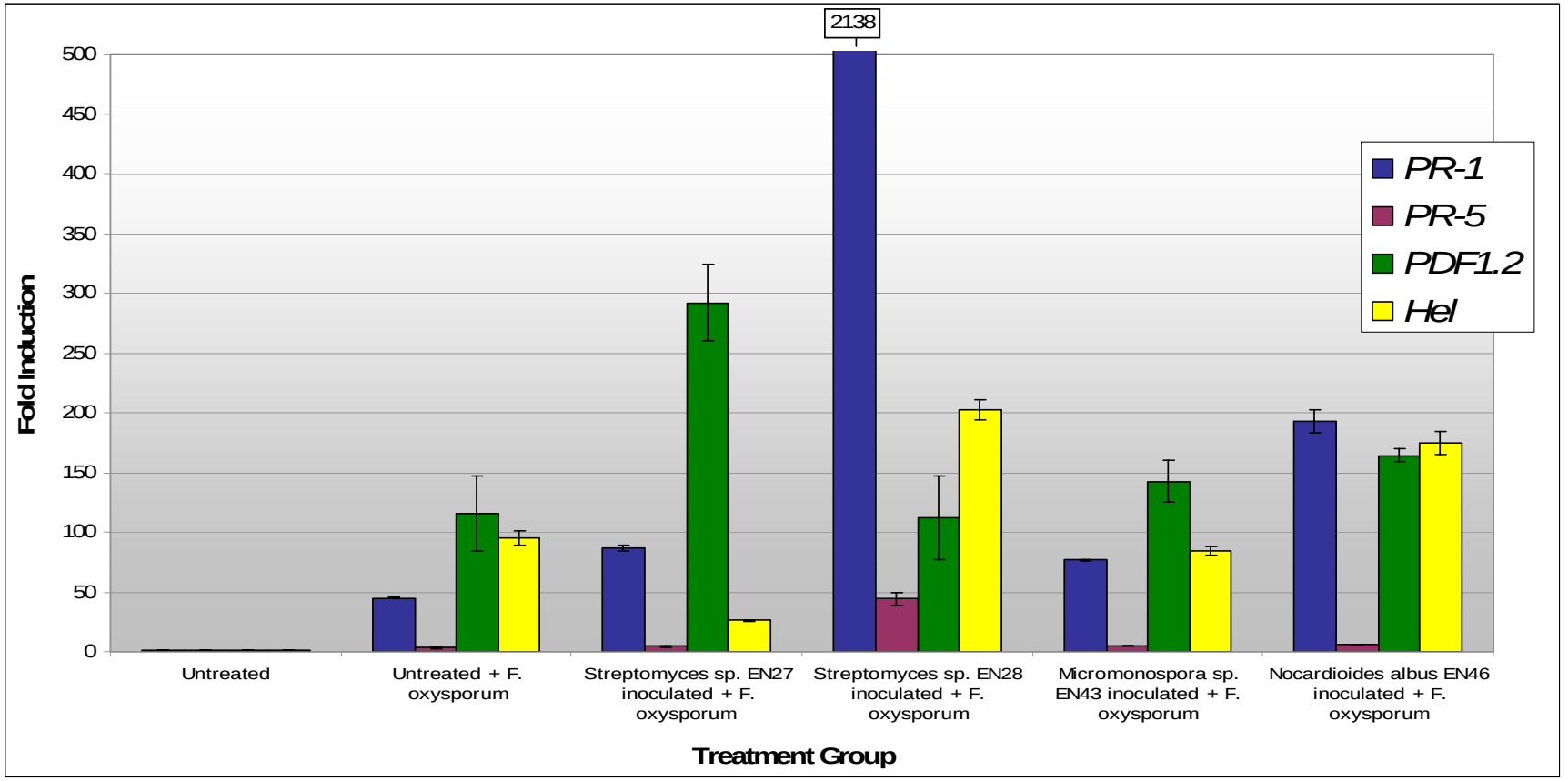


Figure 4.5: Induction of genes in the SAR and JA/ET pathways in seven week old Arabidopsis (Col-0) plants pre-inoculated with Actinobacteria endophytes *Streptomyces* sp. EN27, *Streptomyces* sp. EN28, *Micromonospora* sp. EN43 and *Nocardioides albus* EN46 and untreated plants 3 days after *Fusarium oxysporum* challenge (n=9).

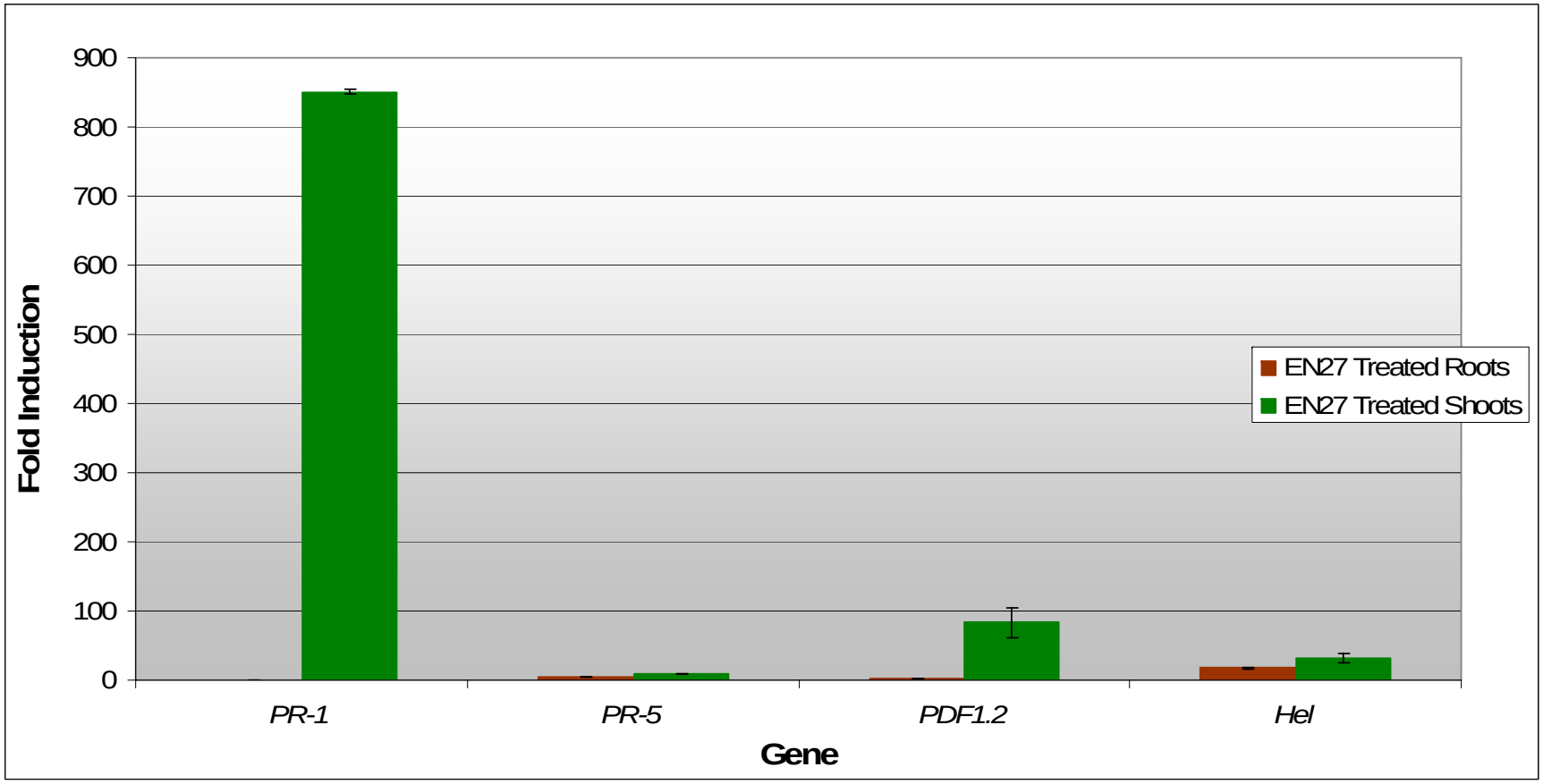


Figure 4.6: Induction of genes in the SAR and JA/ET pathways in seven week old *Streptomyces* sp. EN27 inoculated Arabidopsis (Col-0) plants 3 days after *Fusarium oxysporum* infection (n=9).

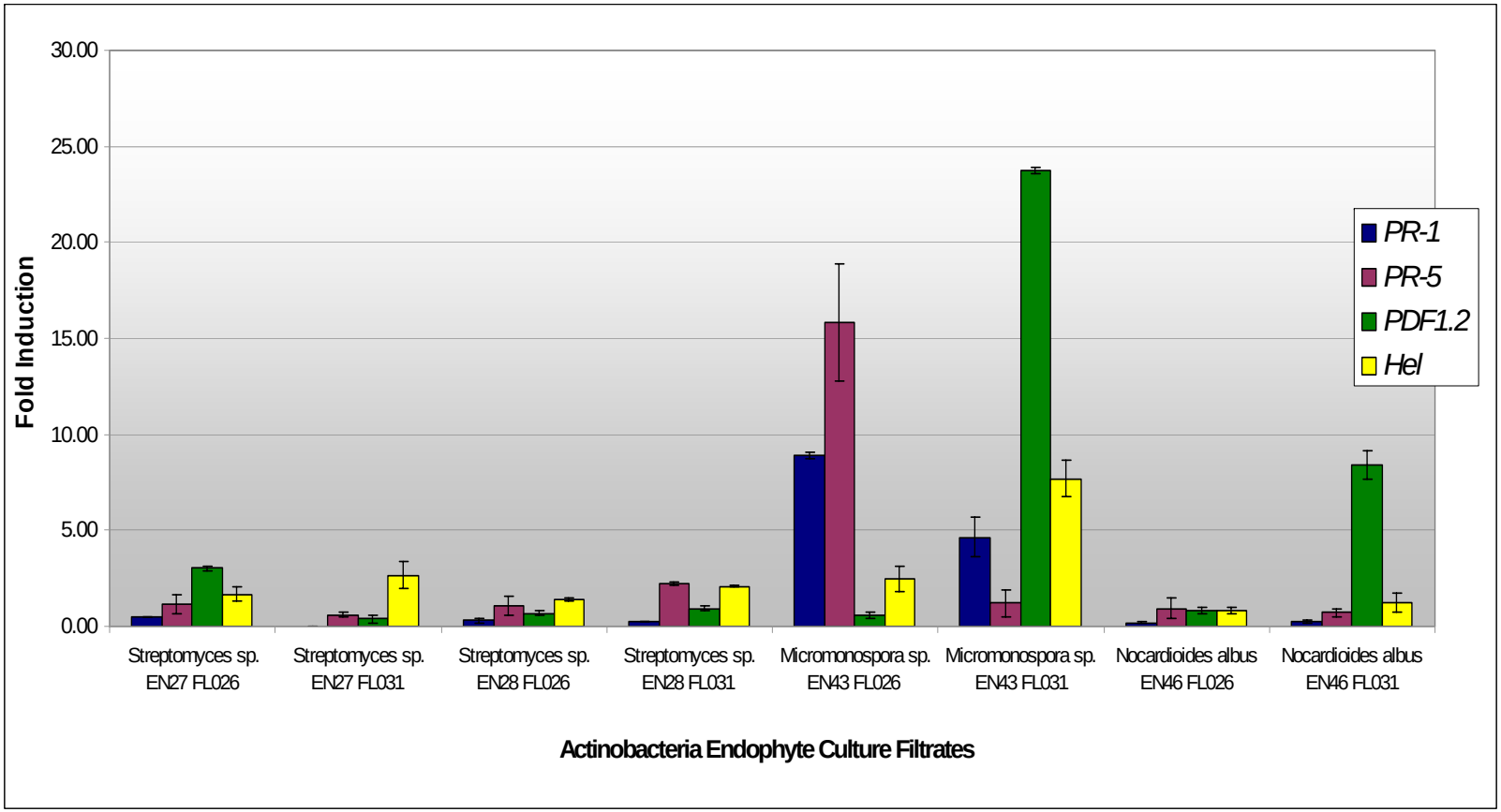


Figure 4.7: The effect of culture filrates of Actinobacteria endophytes, *Streptomyces* sp. EN27, *Streptomyces* sp. EN28, *Micromonospora* sp. EN43 and *Nocardioides albus* EN46, grown in two different media, FL026 and FL031, on genes in the SAR and JA/ET pathways in seven week old *Arabidopsis thaliana* (Col-0) (n=9).

4.3.6 Visualisation of *Micromonospora* sp. EN43 in Arabidopsis

Confirmation of endophyte colonisation of Arabidopsis was achieved by scanning cryoelectron microscopy (CryoSEM). Figure 4.8 shows this endophytic colonisation of *Micromonospora* sp. EN43.

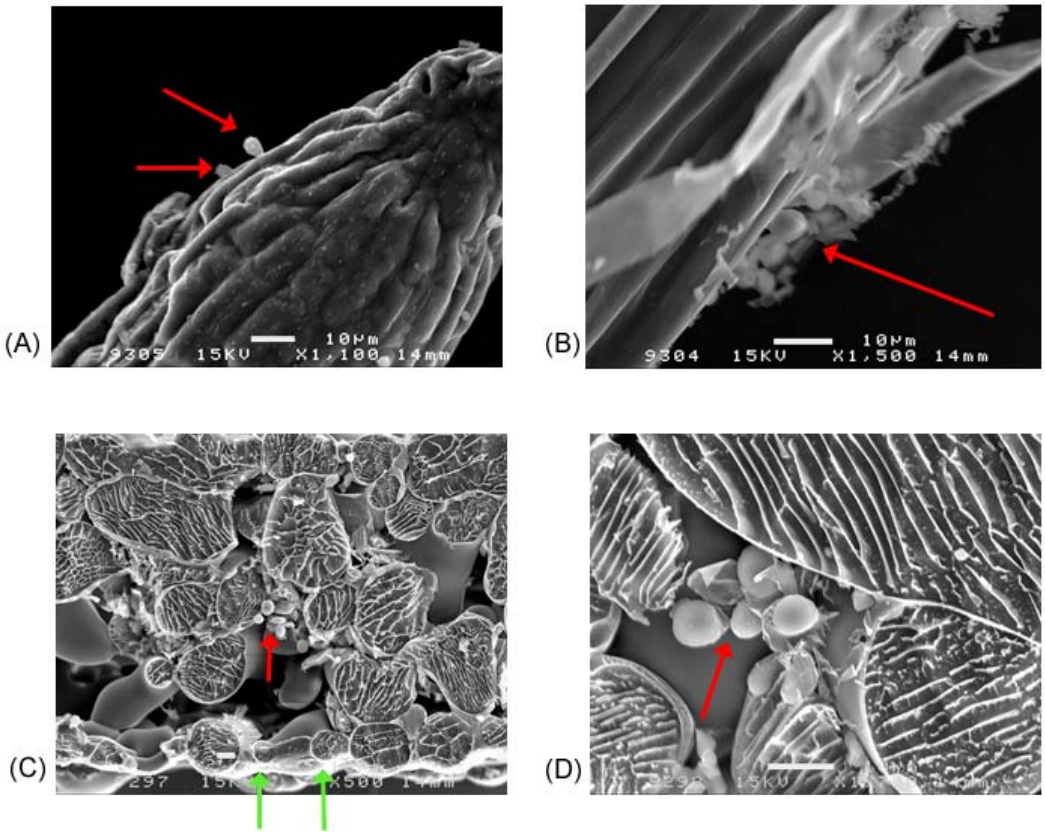


Figure 4.8: Endophytic colonisation of a five week old *Arabidopsis thaliana* (Col-0) plant grown from a seed inoculated with *Micromonospora* sp. EN43. (A) Spores of EN43 present on the surface of the root tip. (B) Spores of EN43 on the surface the root. (C & D) EN43 spores in between leaf cells after entry through stomatal guard cell. Red arrows indicate spores. Green arrows guard cells. White bars denote 10 µm.

The electron microscope images show the spores of *Micromonospora* sp. EN43 colonising the surface of the Arabidopsis roots and root tip. Entry into the root may occur through small cracks or through lateral branching sites. The spores of *Micromonospora* sp. EN43 were also present within the leaf. Entry into the leaf appears to be occurring through stomatal openings as the spores are present in the intercellular spaces.

4.4 Discussion

Plant pathogen resistance can occur via complex pathways requiring salicylic acid and/or jasmonic acid and ethylene signalling. Inoculation of the Arabidopsis plants with selected endophytic actinobacteria stimulated the plant defence pathways. All Arabidopsis plants appeared healthy when inoculated with the endophytic actinobacteria and in some cases plant growth was increased. Three out of the four endophytic actinobacteria were capable of activating the plant defence pathways in the absence of a pathogen. The individual strains EN27 and EN28 resulted in a different pattern of gene induction. The *Streptomyces* sp. EN27 resulted in induction of the *PR-1* gene, whereas *Streptomyces* sp. EN28 induced *PDF1.2* to a higher degree than for the *PR-1* and *PR-5* transcripts. This would indicate that EN27 is capable of priming the SAR pathway whereas EN28 appears to prime the JA/ET pathway. The *Streptomyces* sp. strains EN27 and EN28 are highly similar to each other morphologically and the 16S rRNA gene sequences are 94% similar to each other; however it is well known that different strains can produce a different range of secondary metabolites (Hopwood, 2003). The *Nocardioides albus* EN46 species appears to prime the SAR pathway as there was induction of the *PR-1* and *PR-5* transcripts. The *Micromonspora* sp. EN43 was unable to up-regulate plant defence genes in either the SAR or JA/ET pathways.

The SAR pathway is normally activated by necrotic pathogens either as a part of the hypersensitive response or as a symptom of disease and the JA/ET pathway is triggered by infection with biotrophic pathogens (Dong and Durrant, 2004; Thaler *et al.*, 2004; Glazebrook, 2005). It is possible that the plant is recognising the

endophytic actinobacteria as minor pathogens due to the internal colonisation and therefore switching on defence genes in preparation for attack.

The activation of plant defence pathways would be an effective method for the endophyte to protect its niche. When defence pathways are activated it is associated with changes in cell wall composition which strengthens the plant cell wall and further inhibits pathogen invasion (Hammerschmidt, 1999; Heil and Bostock, 2002). Endophytic colonisation can also result in structural changes in host plant cells. Benhamou *et al.* (1996) demonstrated that colonisation of pea (*Pisum sativum* L.) roots with *Bacillus pumilis* strain SE34 resulted in an enhanced resistance to *Fusarium oxysporum* f. sp. *pisi*. The colonised plant reacted by strengthening the epidermal and cortical cell walls and deposition of newly formed barriers beyond the infection sites. In non-endophyte colonised roots the pathogen was able to multiply abundantly whereas in endophyte-colonised roots the pathogen was restricted to the epidermis and outer cortex. Therefore, once the actinobacteria endophytes have colonised the plant, they may be effectively preventing colonisation by other endophytes and protecting their niche by inducing structural changes and, in turn, preventing pathogen invasion.

The endophytic actinobacteria were able to induce expression of defence genes in the absence of a pathogen. This is in contrast to the ISR triggered by *Pseudomonas fluorescens* WCS417r. ISR induced by *P. fluorescens* WCS417r did not result in the induction of SA-responsive genes *PR-1, PR-2,* and *PR-5*, the ET- and JA-responsive genes *ChiB, Hel, PDF1.2,* and the jasmonate-inducible genes *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2*, locally in the roots or systemically in the leaves (van Wees *et al.*, 1999). When challenged with the bacterial pathogen *Pseudomonas*

syringae pv. *tomato* the *Atvsp* gene (encoding vegetative storage protein) was induced, but not other jasmonate-responsive genes when analysed using the northern blotting technique (van Wees *et al.,* 1999; van Wees *et al.,* 2000). However, when analysed by microarray ISR induced by *P. fluorescens* WC417r and challenged with *Pseudomonas syringae* pv. *tomato* DC3000, resulted in a 2.5-fold induction of *PDF1.2* compared to the control (Verhagen *et al.,* 2004). Therefore, it was concluded that *P. fluorescens* WC417r was 'priming' the plant defence pathways where inoculation alone with this bacterium does not induce the plant defence response but when attacked by a pathogen the defence response is quickly mounted inducing systemic resistance (Conrath *et al.,* 2002). In contrast, the selected endophytic actinobacteria used in this study activated the plant defence genes in the absence of a pathogen and this may result in more effective 'priming' of the defence pathways.

The Arabidopsis plants inoculated with endophytic actinobacteria were challenged with pathogens to determine if induced resistance did occur and if it correlated with further up-regulation of the plant defence genes. The first pathogen used was the non-specific bacterial plant pathogen *Erwinia carotovora* subsp. *carotovora* (Ecc) which causes soft-rot disease in a wide range of hosts and can cause serious economic losses in crops (Aguilar *et al.,* 2002). This pathogen secretes hydrolytic enzymes including pectinases which degrades the plant cell wall and releases oligogalacturonides which can elicit plant defence responses (Norman *et al.,* 1999). Infection of Arabidopsis with *E. carotovora* has been shown to induce the local and systemic expression of genes that are mainly responsive to ET or JA (eg. *Hel, ChiB, PDF1.2,* and *Atvsp*) (Norman-Setterblad *et al.,* 2000). This was confirmed by real-time RT-PCR with a 161-fold induction of the *PDF1.2* transcript and 54-fold

induction of the *Hel* transcript in the untreated plant infected with Ecc. Inoculation with actinobacteria endophytes resulted in an enhanced level of resistance as the plants appeared normal three days after Ecc challenge compared to the untreated plants which exhibited disease symptoms. This resistance correlated with an enhanced level of *PDF1.2* transcript induction above the level detected in the untreated infected control. Colonisation with EN43 also resulted in an increase in the *PDF1.2* transcript compared to the untreated infected plants; however there was also an increase in *Hel* and the *PR-1* transcript. *Micromonospora* sp. EN43 appears to be able to induce resistance in a manner similar to *P. fluorescens* WC417r. *Micromonospora* sp. EN43 was the only endophyte that did not induce the SAR or JA/ET genes in the absence of a pathogen; however, it still had the ability to prime the SAR and JA/ET pathways enabling a stronger defence response upon pathogen invasion.

Resistance to Ecc mediated by EN27, EN28 and EN46 occurred via the JA/ET pathway whereas EN43-mediated resistance occurred via both the JA/ET and SAR pathways. The SAR and ISR pathways have been shown to be additive. van Wees *et al.* (2000) demonstrated in Arabidopsis the SAR and ISR pathways resulted in 40% to 60% reduction in disease symptoms of *Pseudomonas syringae* pv. *tomato*, respectively. When ISR and SAR genes were expressed simultaneously disease reduction was enhanced up to 80%. Therefore, as *Micromonospora* sp. EN43 has the ability to activate both the SAR and JA/ET pathways, it may be a good biocontrol agent for protection against bacterial and fungal pathogens.

The selected endophytic actinobacteria enhanced expression of genes in the JA/ET pathway in Ecc-infected Arabidopsis. When the plants were infected with the

soil-borne fungus, *Fusarium oxysporum*, a different pattern of gene expression was induced.

Induced resistance to *F. oxysporum* mediated by the endophytic actinobacteria occurred primarily by the SAR pathway with the JA/ET genes expressed at a lower level. The *PR-1* and *PR-5* genes were all expressed at a higher level in the plants inoculated with the endophytic actinobacteria, whereas the JA/ET genes, *PDF1.2* and *Hel*, were not induced above the level in the untreated plants for all endophyte treatments. Previously it has been shown that infection of Arabidopsis with *F. oxysporum* induces the thionin gene *Thi2.1* and over-expression of this gene results in further resistance (Epple *et al.,* 1997). Thionins are defence-related proteins that are toxic for gram-negative bacteria, fungi, yeasts, and various mammalian cell types (Garcia-Olmedo *et al.,* 1989; Epple *et al.,* 1995; Thevissen *et al.,* 1996). Berrocal-Lobo and Molina (2004) showed that the ET response protein ERF1, a transcriptional factor, is also involved in mediating resistance to *F. oxysporum* in Arabidopsis. It was shown that constitutive expression of *ERF1* results in *F. oxysporum* resistance but requires intact JA, ET and SA signalling pathways. Berrocal-Lobo and Molina (2004) also showed the induction *PR-1* transcript 4 days after infection and by 8 days both *PR-1* and *PDF1.2* were induced. This indicates that activation of both the SAR and JA/ET pathways by *F. oxysporum* has been detected before. The endophytic actinobacteria, though, have the ability to further up-regulate both the SAR pathway and JA/ET pathway defence genes resulting in enhanced resistance and, as stated previously, when both pathways are in operation a stronger protection may be manifested.

Induction of the SAR and JA/ET genes is thought to occur via a lipid-derived

signal that can be transported through the phloem (Maldonado *et al.*, 2002). Since actinobacteria are prolific producers of antibiotics and secondary metabolites, including antifungal agents (Nolan and Cross, 1988), further investigation was performed to determine if the priming of the defence pathways was a result of a secreted secondary metabolite(s) or cell factor(s). The culture filtrates of the endophytic actinobacteria were applied to the plant and the gene expression analysed 24 hours after application.

Induction of SAR and JA/ET genes was only detected with the *Micromonospora* sp. EN43 and *Nocardioides albus* EN46 culture filtrates. Interestingly, the culture filtrate of EN43 grown in a simple medium resulted in the induction of the SAR pathway but when grown in a complex medium the JA/ET pathway was activated. This would suggest secondary metabolites which are affected by environmental conditions is responsible for the activation of the defence pathways.

Previously it was shown by Ryu *et al.* (2004) that volatile organic compounds (VOCs) from *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a were able to induce ISR in Arabidopsis and reduce the disease severity of Ecc. It has also been found that the culture filtrate of *Erwinia carotovora* triggers local and systemic induction of defence-related genes in tobacco and in Arabidopsis, as well as enhanced systemic resistance to the pathogen (Vidal *et al.*, 1998; Norman-Setterblad *et al.*, 2000). Isolating the actinobacteria endophyte compound responsible for the activation of the defence genes could lead to the identification of a biochemical biological control agent. However, it is likely that the physical interaction of the endophyte and host plant is needed for the full range of defence responses to be activated.

The endophytic actinobacteria appear to be able to ‘prime’ both the SAR and

JA/ET pathways, up-regulating genes in either pathway depending on the infecting pathogen. The pathway, that is triggered, is largely dependent on the infecting pathogen. It has been suggested that resistance to biotrophs, which feed on living tissue, occurs via the SAR pathway and necrotrophs, which kill the host tissue for a food source, occurs via the JA/ET pathway (Thaler *et al.*, 2004; Glazebrook, 2005). This was also found in this study where the necrotrophic bacterial pathogen Ecc activated the JA/ET pathway and the biotrophic fungal pathogen *F. oxysporum* activated largely the SAR pathway but also the JA/ET pathway to some degree. It has also been reported that differential induction of the pathogenesis-related genes by different pathogens is known to occur (Thomma, 2001).

Colonisation of the host plant with the endophytic actinobacteria leads to a mutually beneficial interaction. It is possible that 'priming' of the plant defence pathways leads to structural changes in the plant cell walls protecting the niche of the colonising endophyte and in turn reducing pathogen invasion. If pathogen attack does occur the plant defence pathways are already activated allowing for a stronger and quicker defence response by the induction of the pathogenesis-related genes.

Chapter Five: Dissection of the Arabidopsis Defence Pathways Induced by *Streptomyces* sp. EN27.

5.1 Introduction

Streptomyces spp. are saprophytic, filamentous actinobacteria that predominately inhabit the soil and spend the majority of their life-cycle as semi-dormant spores (Mayfield *et al.*, 1972). *Streptomyces* spp. have been widely studied mainly due their ability to produce a wide range of antibiotics and secondary metabolites (Kieser *et al.*, 2000).

Streptomyces sp. EN27 was isolated from a surface-sterilised, healthy wheat root. *Streptomyces* sp. EN27 is closely related to *Streptomyces caviscabies* and *Streptomyces setonii* based on 16S rRNA sequencing (Coombs and Franco, 2003a). Endophytic colonisation of wheat was confirmed by tagging the strain with enhanced green fluorescent protein (eGFP) and was observed to colonise wheat plants early in their development with colonisation of the embryo, endosperm and emerging radicle (Coombs and Franco, 2003b). Endophytic colonisation was also confirmed in this study (Chapter 2) by T-RFLP and electron microscopy. The *Streptomyces* sp. EN27 was applied as a coating on the wheat seed and the colonisation was shown to increase by approximately two-fold after 6 weeks of growth by T-RFLP (Conn and Franco, 2004b).

Inoculating wheat with *Streptomyces* sp. EN27 has been shown to promote growth and enhance disease resistance *in vitro* and *in planta*. Coombs (2002) demonstrated that in steamed soil inoculated with *Gaeumannomyces graminis* var. *tritici* (Ggt), the causative agent of ‘take-all’, there was a 27% disease reduction in *Streptomyces* sp. EN27-treated wheat plants compared to untreated controls. In field soil the *Streptomyces* sp. EN27-treated plants resulted in a 39% reduction in ‘take-all’

disease symptoms and a 35% reduction in disease cause by *Rhizoctonia solani*. Growth promotion was also observed in *Streptomyces* sp. EN27-treated plants with a 34% increase in dry root mass. The *Streptomyces* sp. EN27 was also found to contain a 13-kb plasmid pEN2701 (Coombs *et al.*, 2003). This plasmid was completely sequenced and 13 putative ORFs were identified.

Previously in this thesis (Chapter Four) it was shown that inoculation of *Arabidopsis thaliana* (Col-0) with *Streptomyces* sp. EN27 resulted in the 'priming' of the defence pathways and enhanced resistance to both *Erwinia carotovor*a subsp. *carotovora* (Ecc) and *Fusarium oxysporum*. The enhanced resistance to the pathogens was correlated with an up-regulation of defence genes in the JA/ET and SAR pathways. In order to analyse in more detail which steps in the SAR and JA/ET pathways are critical for the endophyte induced resistance, defence-compromised Arabidopsis mutants were used.

The defence-compromised mutants *NahG* (*salicylic acid degrading*), *npr1* (*non-expression of PR proteins*), *etr1-3* (*ethylene insensitive*) and *jar1* (*jasmonic acid insensitive*) seeds were inoculated with the *Streptomyces* sp. EN27 and challenged with Ecc and *F. oxysporum*. The expression of the SAR genes, *PR-1* and *PR-5*, and the JA/ET genes, *PDF1.2* and *Hel*, were analysed by real-time RT-PCR. Figure 5.1 outlines the Arabidopsis defence pathways and the highlighted boxes illustrate where the defence-compromised mutants are situated.

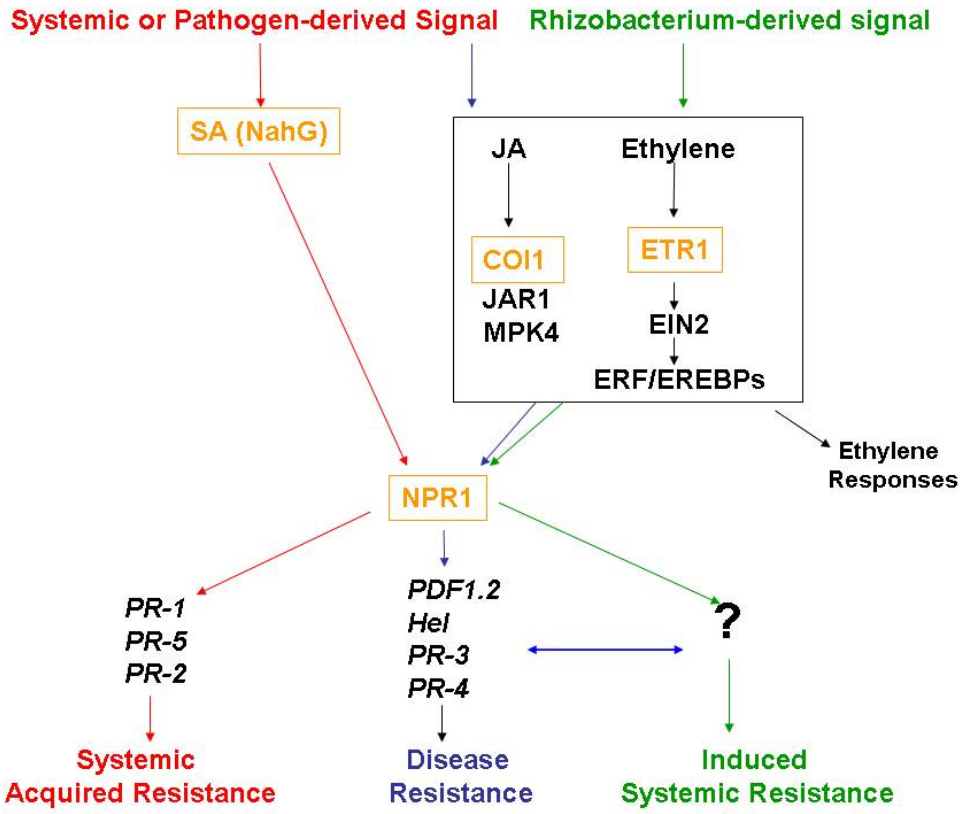


Figure 5.1: The Arabidopsis defence pathways activated either from a pathogen-derived or rhizobacterium-derived signal. The orange highlighted boxes represent the mutants that were used in this study.

5.2 Materials and Methods

5.2.1 Cultivation of *Arabidopsis thaliana* Col-0

Wild-type *Arabidopsis thaliana* Col-0 was cultivated as per 4.2.1.

5.2.1.1 Cultivation of *Arabidopsis thaliana* Mutant Lines

Arabidopsis mutant seeds were obtained from the Arabidopsis Biological Resource Centre (ABRC) and shown below in Table 5.1.

Table 5.1: Defence-compromised Arabidopsis mutant seeds obtained from ABRC.

Name	Accession No.	Background	Mutation	Phenotype
npr1-1	CS3726	Col-0	Polymorphism	Little expression of *PR* genes; unable to respond to various SAR-inducing treatments; increased susceptibility to pathogen infections; kanamycin resistant, carrying BGL2-GUS reporter; allelic to *nim1*
jar1-1	Salk 011510	Col-0	T-DNA insertion	An auxin-induced gene encoding a cytoplasmic localised phytochrome. A signaling component protein similar to the GH3 family of proteins. Losses of function mutants are defective in a variety of responses to JA.
etr1-3	CS3070	Col-0	Polymorphism	Formerly *ein1-1*, dominant mutation, root and hypocotyl elongation insensitive to ethylene, leaf chlorophyll content not reduced in ethylene, reduced ethylene binding activity, large rosette, delayed in bolting.

The *NahG* B15 seeds were kindly provided by Novartis, Research Triangle

Park, North Carolina, USA. The *NahG* B15 seeds contain the salicylate hydroxylase gene from *Pseudomonas putida* which catabolises the decarboxylation of SA to catechol. The *NahG* B15 line was produced by *Agrobacterium* mediated transformation of the Col-0 ecotype and is described in Lawton *et al.* (1995).

The *jar1-1* line contained a T-DNA insert which resulted in jasmonic acid insensitivity. The *jar1-1* seeds were surface sterilised and sown on ½ MS salt medium with 35 $\mu g.ml^{-1}$ kanamycin to select for plants containing the T-DNA insert. After breaking dormancy at 4°C overnight the plants were transferred to a biological containment area with a 16 hr light cycle. After four weeks transformants were selected and seedlings transplanted to a sand- perlite- peatmoss-soil mix. After 2 weeks a leaf sample was removed and immediately frozen in liquid nitrogen. The genomic DNA was extracted from the F1 progeny as per 5.2.1.1.1 and the T-DNA insert was confirmed by PCR using specific primers as per 5.2.1.1.2. This was repeated for the F2 progeny and this seedwas used for subsequent experiments.

5.2.1.1.1 Arabidopsis Genomic DNA Extraction

Arabidopsis genomic DNA was extracted from snap frozen leaves and, when possible, flower stem tissue by grinding in 300 µl of extraction buffer (140 mM Sorbitol, 220 mM Tris pH 8.0, 22 mM EDTA, 800 mM NaCl, 1% Sarkosyl and 0.8% CTAB) incubated at 65°C for 10 min. After incubation, 300 µl chloroform was added and the samples vortexed followed by centrifugation at maximum speed in a microfuge for 5 min at RT. The aqueous phase was collected and 200 µl of isopropanol added. The samples were mixed and left on ice for 10 min after which

samples were centrifuged at maximum speed for 10 min at RT. The supernatant was discarded and the pellet air dried. The pellet was resuspended in 100 µl of Tris-EDTA (10 mM Tris; 10 mM EDTA, pH 8.0) and 20 $\mu g.ml^{-1}$ of RNAse A added. The sample was incubated at 37°C for 30 min then precipitated with 0.1 volume of 3 M sodium acetate and 250 µl absolute ethanol and left of ice for 10 min. The DNA was pelleted by centrifugation at maximum speed for 10 min at RT. The pellet was washed with 70% ethanol and air dried. The pellet was resuspended in 100 µl H_2O.

5.2.1.1.2 T-DNA Insert PCR

The *jar-1* T-DNA insert was amplified using T-DNA specific primers. The primers were designed using the Salk Institute Genomic Analysis Laboratory T-DNA primer design program available online (http://signal.salk.edu/tdnaprimers.html).

Primer Label	Sequence	Product Size
JAR1_F	5' TCC ATC AAG CCT TGT ATT GCC A 3'	922 bp
JAR1_R	5' GCG TAA TGT CCT GGA TCT GTC G 3'	

The JAR1_F and JAR1_R primers were used in a 20 µl PCR reaction with the following contents: 20 ng forward and reverse primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1X Qiagen PCR Buffer, 0.5 units Hot Star Taq (Qiagen, Venlo, Netherlands), PCR grade H_2O to 20 µl and 2 µl of the genomic DNA.

The following thermal profile was followed: 94°C - 15 min, (94°C – 30 secs, 59°C - 1 min, 72°C - 1 min) × 35 cycles, 72°C - 10 min.

For each sample 5 µl to 10 µl of PCR product was run on 1.5%-2.0% agarose gels as per 2.2.3.7.

5.2.1.2 *Streptomyces* sp. EN27 and eGFP-tagged EN27 Culture

Streptomyces sp. EN27 was maintained on ½ strength potato dextrose agar (Appendix 1). The *Streptomyces* sp. EN27 strain transformed with *eGFP* using an 8.0-kb construct, pIJ8641, which contains the *eGFP* gene downstream of a strong constitutive ErmEp promoter, an apramycin resistant marker (aac(3)IV), an oriT/RK2 region, and a lambda phage chromosomal integration sequence (IntC31), was also used in this study (Coombs and Franco, 2003b).

The *Streptomyces* sp. EN27 expressing the *eGFP* gene was selected and maintained on ½ potato dextrose agar supplemented with 50 µg.ml^{-1} apramycin. A suspension of the *eGFP*-tagged *Streptomyces* sp. EN27 spores was made by scraping the spores from the agar plate and resuspending in 30 ml sterile saline. The spores were counted by the Miles and Misra (1938) technique on ½ PDA agar supplemented with 50 µg.ml^{-1} apramycin and adjusted to 1×10^8 CFU.ml^{-1}.

5.2.1.3 Inoculation of Arabidopsis Plants with *Streptomyces* sp. EN27 and the *eGFP*-tagged Strain

Wild-type *Arabidopsis thaliana* Col-0 and the mutant lines *NahG*, *npr1*, *jar1* and *etr1* were inoculated with spores of *Streptomyces* sp. EN27 and *Streptomyces* sp. EN27 containing the pIJ8641 construct. Wild-type and mutant seeds

were surface sterilised as per 4.2.1 and cultivated on ½ MS salt medium as per 4.2.1. The seeds were inoculated by placing 10 µl of a 1×10^8 CFU.ml^{-1} suspension of the spores onto the seed. The droplet of spores were left to dry on the seed before sealing the plate with micropore tape and placing at 4°C overnight. The plants were then transferred to a light box with a 9 hr light cycle.

5.2.2 Arabidopsis Pathogen Cultivation

The bacterial pathogen *Erwinia carotovora* subsp. *carotovora* (Ecc) and the fungal pathogen *Fusarium oxysporum* were cultivated as per 4.2.2.

5.2.2.1 Inoculation of Arabidopsis Plants with Pathogens

Wild-type and defence-compromised *Arabidopsis thaliana* mutants were inoculated with the pathogens Ecc and *F. oxysporum* as per 4.2.2.1.

5.2.3 Harvesting Arabidopsis Plant Material

The Arabidopsis plant material was harvested as per 4.2.3

5.2.4 Analysis of Gene Expression by Real-Time RT-PCR

Real-time RT-PCR was used to determine changes in expression of the SAR genes, *PR-1* and *PR-5*, and the JA/ET genes, *PDF1.2* and *Hel* and was performed as per 4.2.4. The total RNA was extracted as per 4.2.4.1. The removal of DNA from

total RNA extractions protocol was changed to the Turbo DNA-free method (Ambion, Cambridgeshire, UK) and performed as per the manufacturer's instruction. The DNA-free RNA was quantified in triplicate using the GeneQuant pro RNA/DNA calculator (Amersham Biosciences) and integrity was checked on 1.5%-2.0% agarose gels. The efficiency of DNase-1 treatment was checked by PCR as per 4.2.4.2. Reverse transcription of total RNA to cDNA was per 4.2.4.3. The Real-time RT-PCR primers sequences are outlined in 4.2.4.4 and the Real-time RT-PCR was performed as per 4.2.4.5.

5.2.5 Colonisation of Arabidopsis Wild-type (Col-0) and Mutant *NahG, npr1, etr1* and *jar1* Plants with *eGFP*-tagged *Streptomyces* sp. EN27

Changes in the colonisation level of *Streptomyces* sp. EN27 in the wild-type Arabidopsis and defence-compromised mutants *NahG*, *npr1*, *etr1* and *jar1* was determined by inoculating the wild-type and mutant seeds with *Streptomyces* sp. EN27 containing the pIJ8641 construct as per 5.2.1.3. After 6 weeks of growth single plants were removed from the plates at the base of the stem avoiding contact with inoculant on the medium and root material. The plant weight was recorded and then ground in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.4). Serial dilutions up to 10^{-8} were prepared and the CFU determined by the Miles and Misra (1938) technique on ½ PDA agar supplemented with 50 $\mu g.ml^{-1}$ apramycin to select for *Streptomyces* sp. EN27 containing the pIJ8641 construct.

5.3 Results

5.3.1 Colonisation of Wild-type (Col-0) and Defence-Compromised Arabidopsis Mutants with *eGFP*-tagged *Streptomyces* sp. EN27

Colonisation of *Streptomyces* sp. EN27 containing the pIJ8641 construct was monitored in the stems and leaves of wild-type and defence-compromised mutants Arabidopsis plants over an 8 week period and the results are shown in Figure 5.2

After 4 weeks of growth *Streptomyces* sp. EN27 colonisation was increased in the all the defence-compromised mutants in comparison to the wild-type. The highest increase in colonisation was detected in the *npr1* mutant (2.60-fold) followed by *NahG* (1.84-fold), *jar1* (1.28-fold) and *etr1* (1.70-fold). At 6 weeks the only mutant with a significant increase in *Streptomyces* sp. EN27 colonisation was *npr1* with a 1.51-fold increase compared to the Col-0 plants. After 8 weeks of growth the *Streptomyces* sp. EN27 colonisation decreased in all of the ecotypes. The only significant reduction was in the *npr1* mutant with a 0.57-fold decrease in comparison to the Col-0 plants. The *etr1* and *jar1* mutants did show a significant reduction in colonisation at week 8 compared to at week 6 with a 0.40-fold and 0.38-fold reduction in colonisation, respectively.

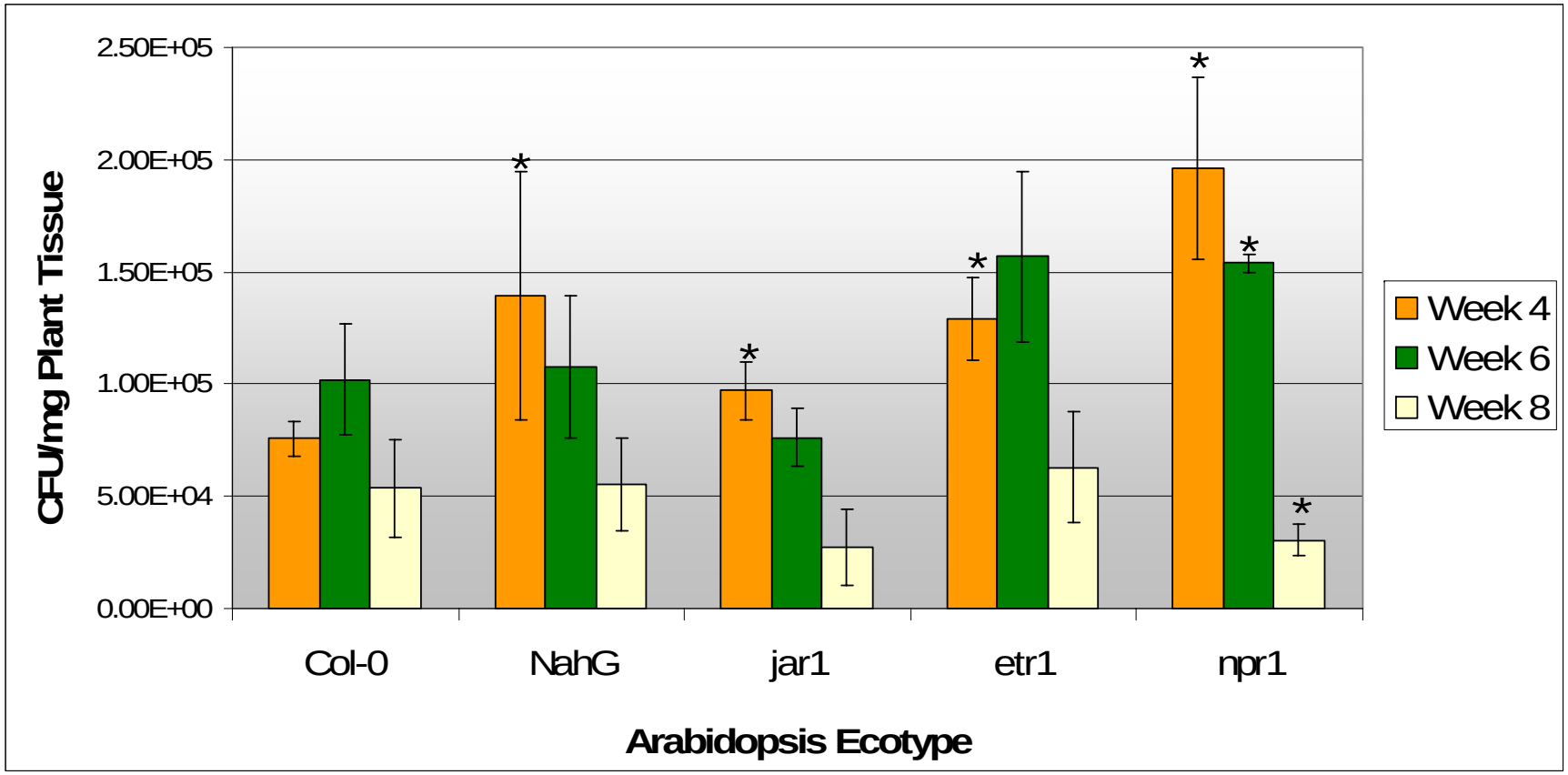


Figure 5.2: Re-isolation of *Streptomyces* sp. EN27 containing the pIJ8641 construct from the stems and leaves of 4, 6 and 8 week old wild-type Arabidopsis (Col-0) and defence-compromised mutants *NahG*, *jar1*, *etr1* and *npr1* plants (n=3). *significantly different in comparison to Col-0.

5.3.2 Expression of Defence Genes in Arabidopsis Defence-Compromised Mutants after Challenge with *Erwinia carotovora* subsp. *carotovora*

Infection of *Arabidopsis thaliana* Col-0 with Ecc results in the elevated expression of the JA/ET gene, *PDF1.2*, and treatment with *Streptomyces* sp. EN27 further enhanced the induction of the *PDF1.2* transcript and resistance to Ecc infection (4.3.3). The SAR mutants (*NahG* and *npr1*) and JA/ET mutants (*jar1* and *etr1*) were inoculated with *Streptomyces* sp. EN27 and infected with Ecc in order to determine if only the JA/ET pathways needs to be functioning for EN27-mediated Ecc resistance.

Inoculation of the SA-degrading mutant, *NahG*, with Ecc resulted in severe disease symptoms within the first 24 hours and by day three the plants had collapsed completely (Figure 5.3). There was no enhanced disease resistance in the EN27-inoculated plants (Figure 5.3), whereas in wild-type plants EN27 dramatically enhanced resistance to Ecc (Figure 5.4). As the *NahG* plants were so severely damaged by the infection the gene expression results may be unreliable.

As the *NahG* plants degrade SA there should be no *PR-1* or *PR-5* gene expression. Application of 5mM SA to the plant was unable to induce the *PR-1* or *PR-5* transcripts confirming the SA degrading status. No *PR-1*, *PR-5* and *PDF1.2* and only a 7-fold induction of *Hel* could be detected in the EN27-inoculated infected *NahG* plant. However, there was a low level of *PR-1* transcript in the untreated infected *NahG* plant with an 8-fold induction detected. No *PR-5* expression was detected; however, there was a significant induction of *PDF1.2* and *Hel*, 228-fold and

35-fold, respectively. The *PDF1.2* induction was approximately 3.5 times greater than the level in an infected Col-0 plant.

The mutant *npr1* was compromised in *PR* gene expression. Application of 5 mM SA was unable to induce *PR-1* or *PR-5* gene expression in the *npr1* plants. Inoculation of the *npr1* with Ecc resulted in disease symptoms comparable to infection in a wild-type plant. Treatment with EN27 enhanced the disease resistance though not to the extent seen in the wild-type plant (Figure 5.5).

Analysis of the gene expression in infected *npr1* plants revealed the *PR-1* transcript was elevated 62-fold. No *PR-5* induction was detected, but, the JA/ET pathway genes *PDF1.2* and *Hel* were strongly induced, 121-fold and 30-fold, respectively (Figure 5.6). In comparison, the EN27-treated *npr1* plants infected with Ecc resulted in a decreased level of *PR-1*, 21-fold, compared to the untreated infected *npr1* plant (Figure 5.6). The JA/ET pathway genes were induced more strongly; *PDF1.2* was induced 314-fold and the *Hel* transcript 45-fold. This level of induction was above the level detected in the untreated *npr1* plant.

The *jar1* and *etr1* mutants are defective in their perception to JA and ET, respectively. Application of 50 µM MeJA and 0.01% ACC to the *jar1* and *etr1* plants was unable to induce the *PDF1.2* and *Hel* gene expression. Inoculation of the *jar1* and *etr1* plants with Ecc resulted in disease symptoms comparable to infection in the wild-type plants (Figures 5.7 and 5.8). However, when inoculated with EN27 the *jar1* and *etr1* plants were resistant to Ecc. The gene expression was analysed and shown in Figure 5.9. In both the uninfected *jar1* and *etr1* plants the *PR-1* transcript was elevated to approximately 26-fold (compared to 10-fold in Col-0). When the *jar1* and *etr1* plants were treated with EN27 the *PR-1* transcript was further induced with a 43-

fold increase in the *etr1* plants and 141-fold induction in the *jar1* plants in the absence of Ecc infection. There was no significant induction of the *PR-5* transcript.

The JA/ET pathway genes were significantly induced in the *jar1* and *etr1* mutants three days after inoculation with Ecc. The *PDF1.2* transcript was induced 499-fold and 191-fold in the *etr1* and *jar1* infected plants, respectively. The *Hel* transcript was induced 4-fold and 37-fold in the *etr1*and *jar1* Ecc-infected plants, respectively. The induction was reduced in comparison to the Ecc-infected wild-type plants where *Hel* was induced 31-fold.

The *Streptomyces* sp. EN27-treated *jar1* and *etr1* plants were resistant to Ecc though the level of gene expression was lower than in the susceptible untreated *jar1* and *etr1* mutants. The *PDF1.2* transcript was induced 59-fold and 39-fold in the infected *jar1* and *etr1* plants, respectively. The *Hel* transcript was induced 9-fold and 6-fold in the infected *jar1* and *etr1* plants, respectively. This level of *PDF1.2* and *Hel* induction was significantly lower than the Ecc-infected EN27-treated wild-type where there was a 405-fold and 22-fold induction of *PDF1.2* and *Hel* respectively.

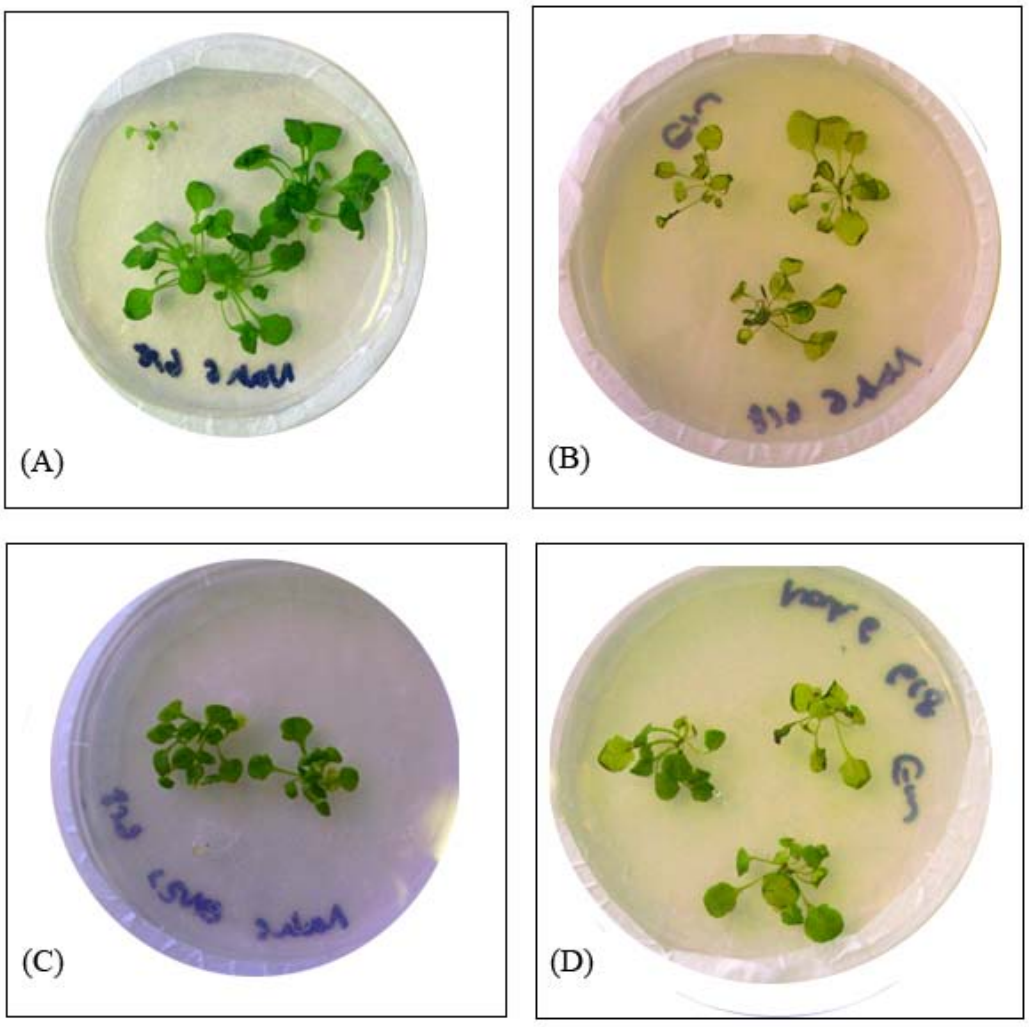


Figure 5.3: Defence-compromised *Arabidopsis thaliana* mutant *NahG* **A:** 7 week old *NahG* plants. **B:** 7 week old *NahG* 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3 × 10^8 CFU.ml^{-1}. **C:** 7 week old *NahG* plants grown from seed inoculated with 1 × 10^8 CFU.ml^{-1} *Streptomyces* sp. EN27 **D:** 7 week old *NahG* plants grown from seed inoculated with 1 × 10^8 CFU.ml^{-1} *Streptomyces* sp. EN27 and 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3 × 10^8 CFU.ml^{-1}.

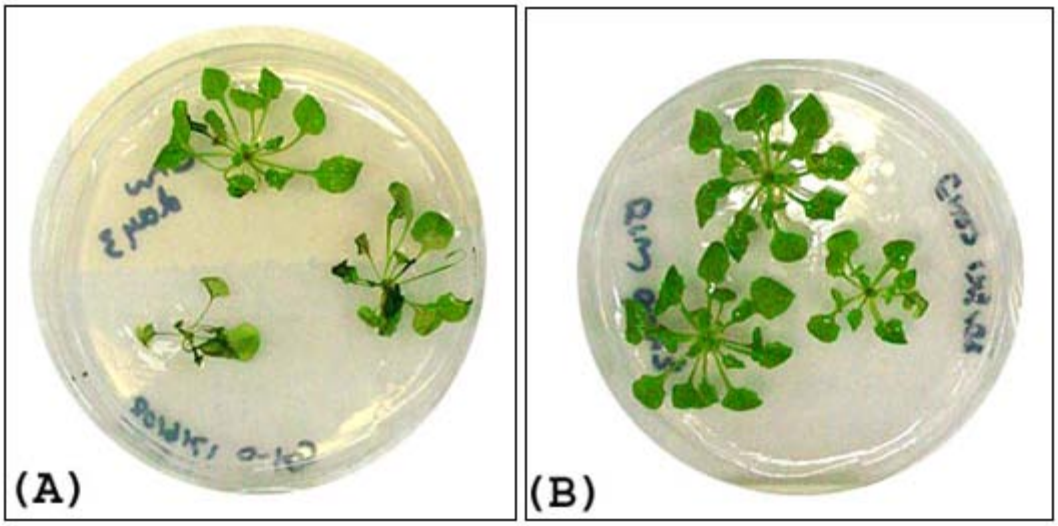


Figure 5.4: Wild-type *Arabidopsis thaliana* infected with *E. carotovora* subsp. *carotovora*. **A:** Wild-type Arabidopsis 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3×10^8 CFU.ml^{-1}. **B:** Wild-type Arabidopsis pre-inoculated with EN27 1×10^8 CFU.ml^{-1} 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3×10^8 CFU.ml^{-1}.

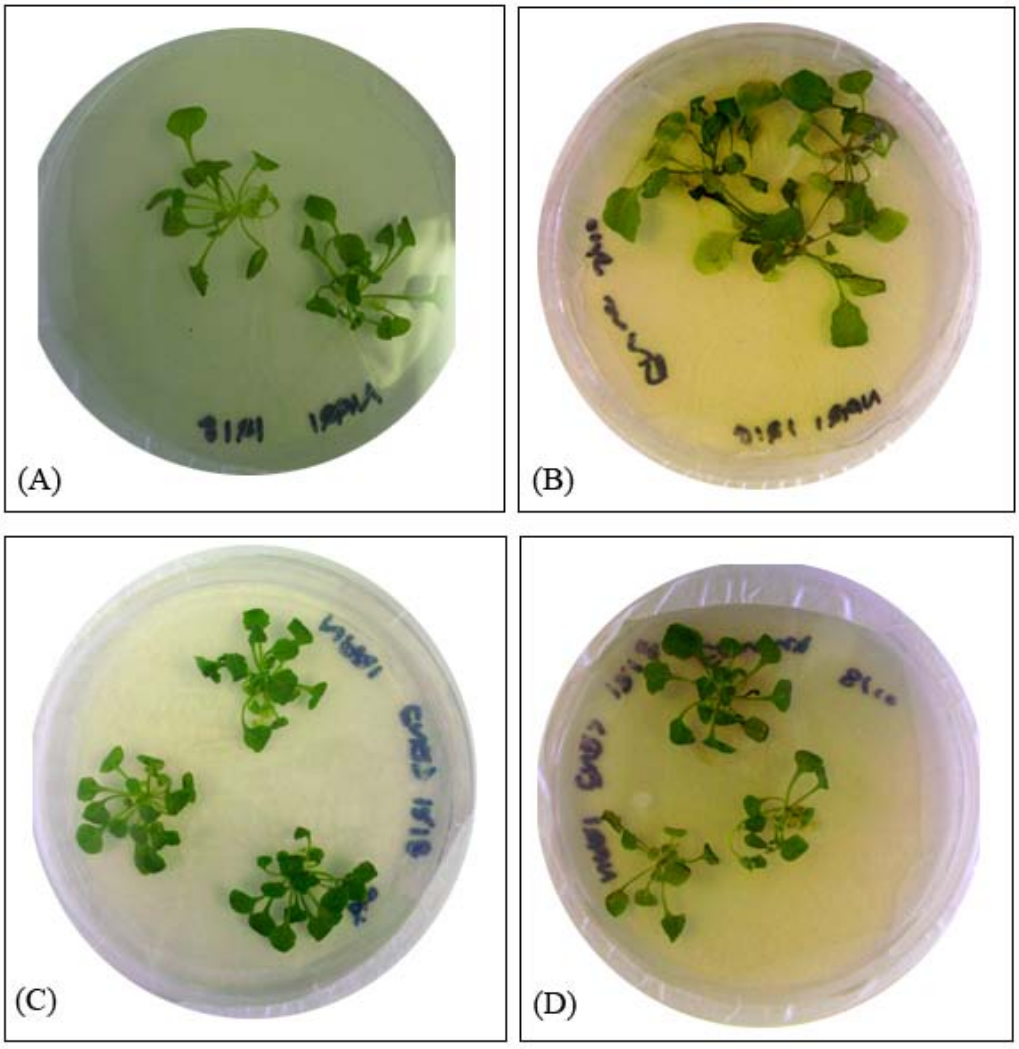


Figure 5.5: Defence-compromised *Arabidopsis thaliana* mutant *npr1*. **A:** 7 week old *npr1* plants **B:** Mutant *npr1* plants 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3×10^8 CFU.ml^{-1}. **C:** 7 week old *npr1* plants grown from seed inoculated with 1×10^8 CFU.ml^{-1} *Streptomyces* sp. EN27 **D:** 7 week old *npr1* plants grown from seed inoculated with 1×10^8 CFU.ml^{-1} *Streptomyces* sp. EN27 and 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3×10^8 CFU.ml^{-1}.

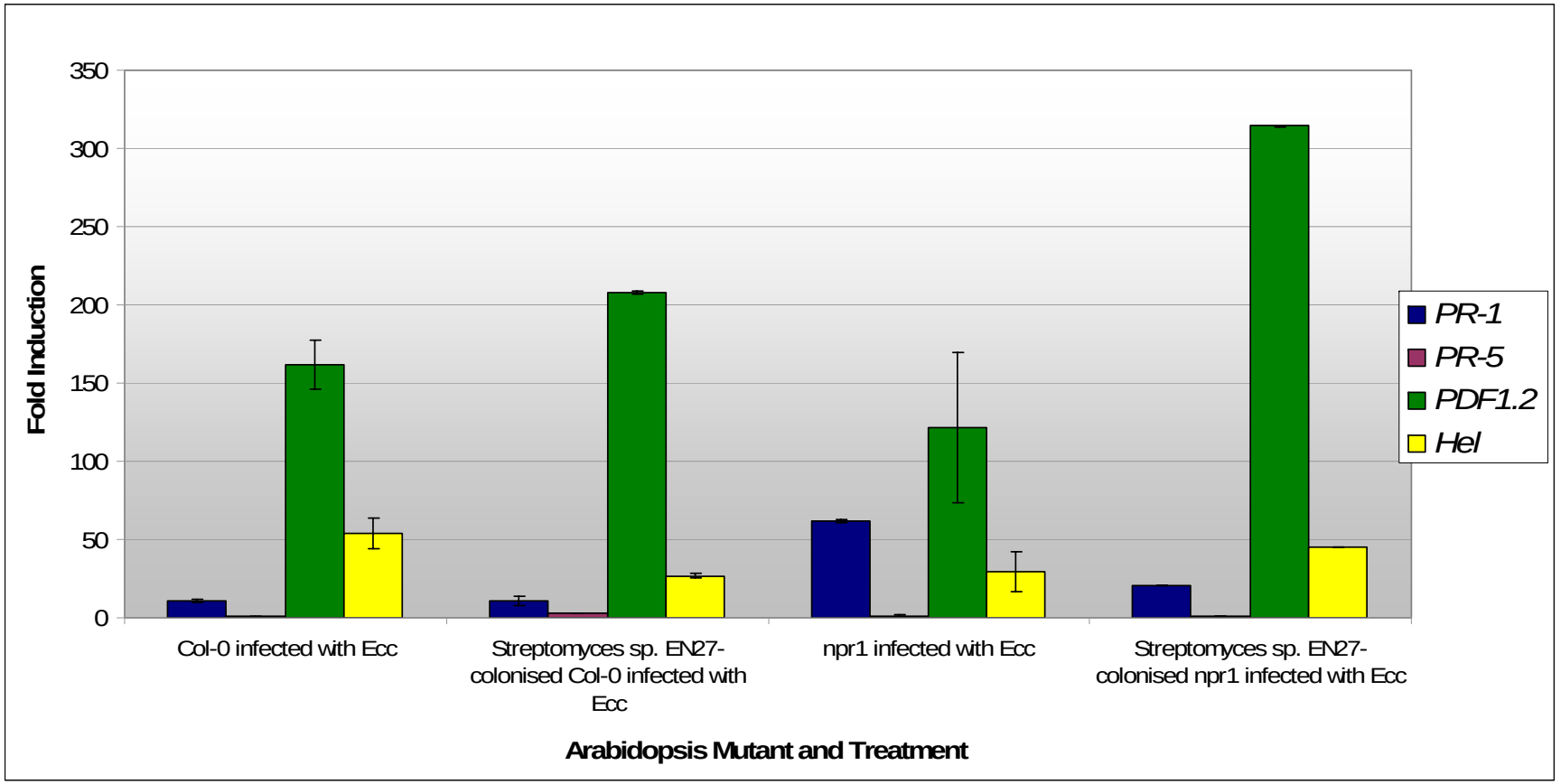


Figure 5.6: Fold induction of SAR pathway genes (*PR-1* and *PR-5*) and JA/ET pathway genes (*PDF1.2* and *Hel*) in the *npr1* mutant and Col-0 with and without *Streptomyces* sp. EN27 pre-treatment three days after infection with *Erwinia carotovora* subsp. *carotovora* (n=6).

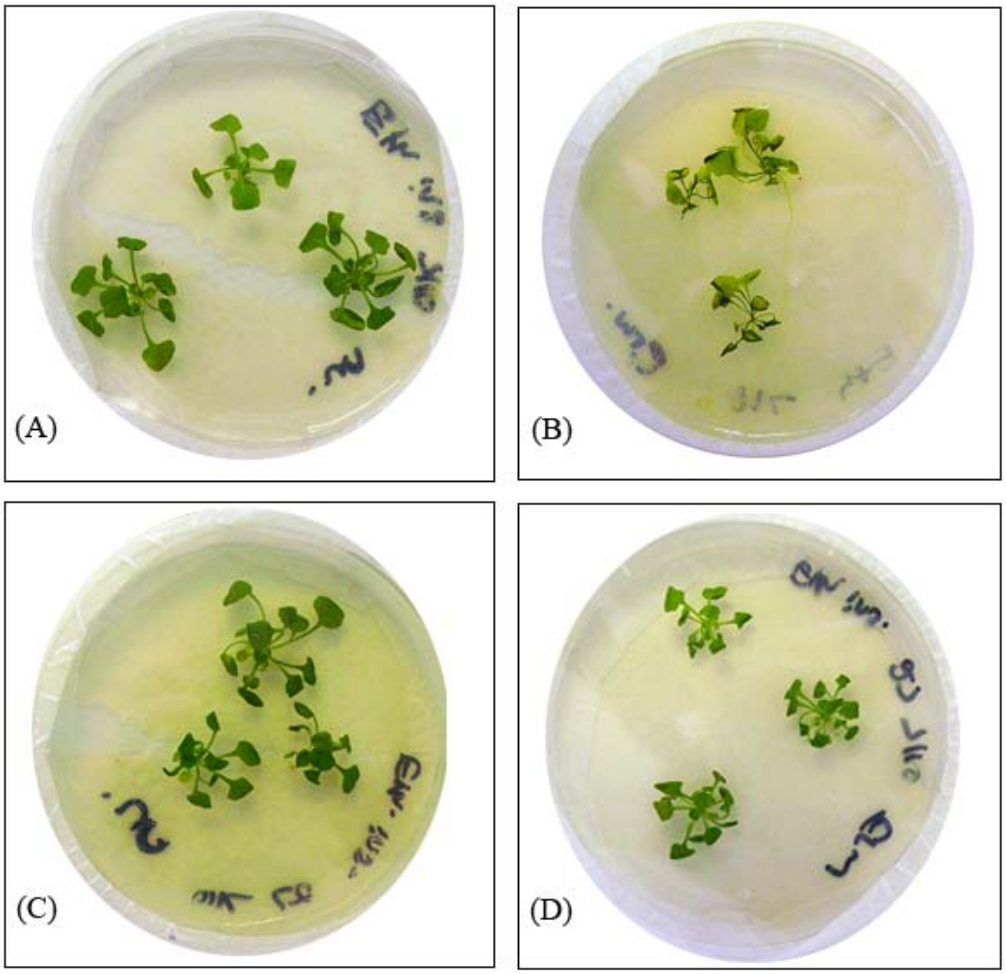


Figure 5.7: Defence-compromised *Arabidopsis thaliana* mutant *etr1*. **A:** 7 week old *etr1* plants **B:** Mutant *etr1* plants 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3×10^8 CFU.ml^{-1}. **C:** 7 week old *etr1* plants grown from seed inoculated with 1×10^8 CFU.ml^{-1} *Streptomyces* sp. EN27 **D:** 7 week old *etr1* plants grown from seed inoculated with 1×10^8 CFU.ml^{-1} *Streptomyces* sp. EN27 and 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3×10^8 CFU.ml^{-1}.

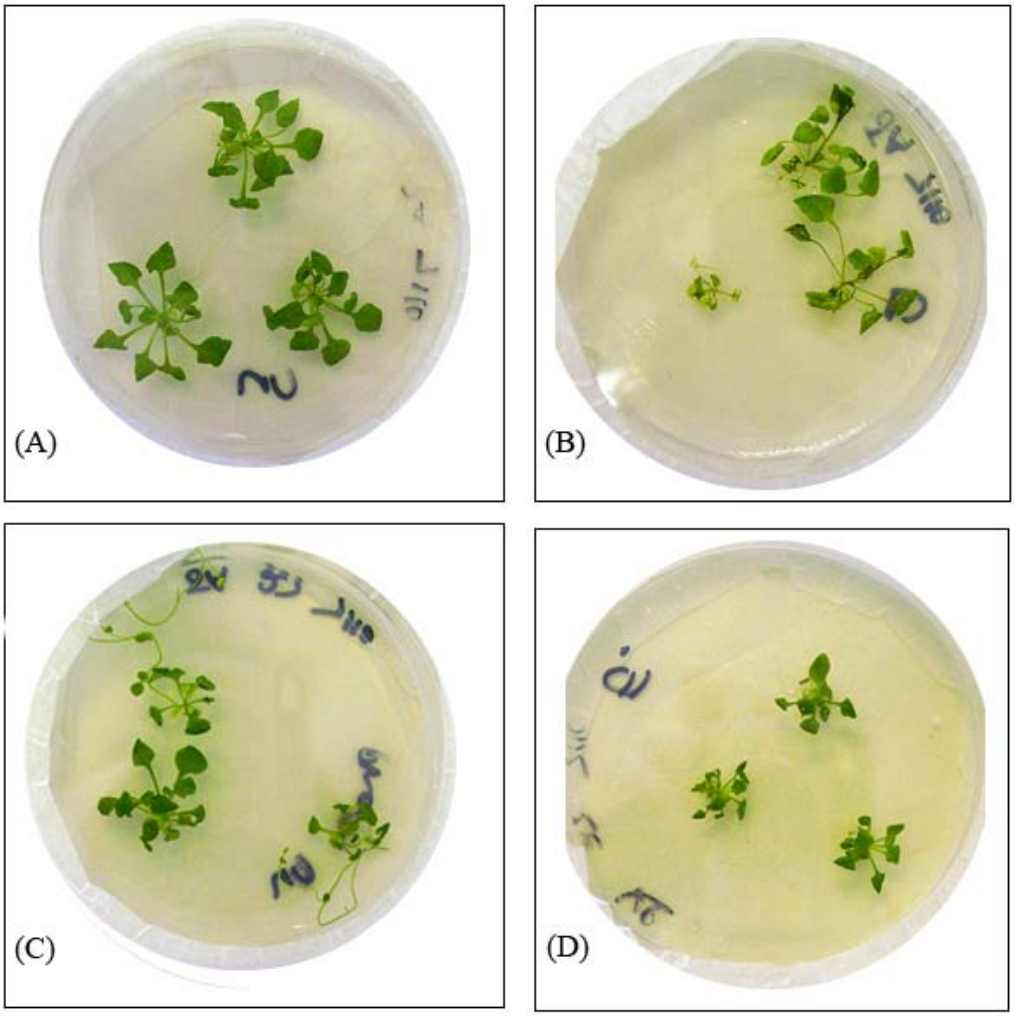


Figure 5.8 Defence-compromised *Arabidopsis thaliana* mutant *jar1*. **A:** 7 week old *jar1* plants **B:** Mutant *jar1* plants 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3×10^8 CFU.ml^{-1}. **C:** 7 week old *jar1* plants grown from seed inoculated with 1×10^8 CFU.ml^{-1} *Streptomyces* sp. EN27 **D:** 7 week old *jar1* plants grown from seed inoculated with 1×10^8 CFU.ml^{-1} *Streptomyces* sp. EN27 and 3 days after inoculation with *E. carotovora* subsp. *carotovora* 3×10^8 CFU.ml^{-1}.

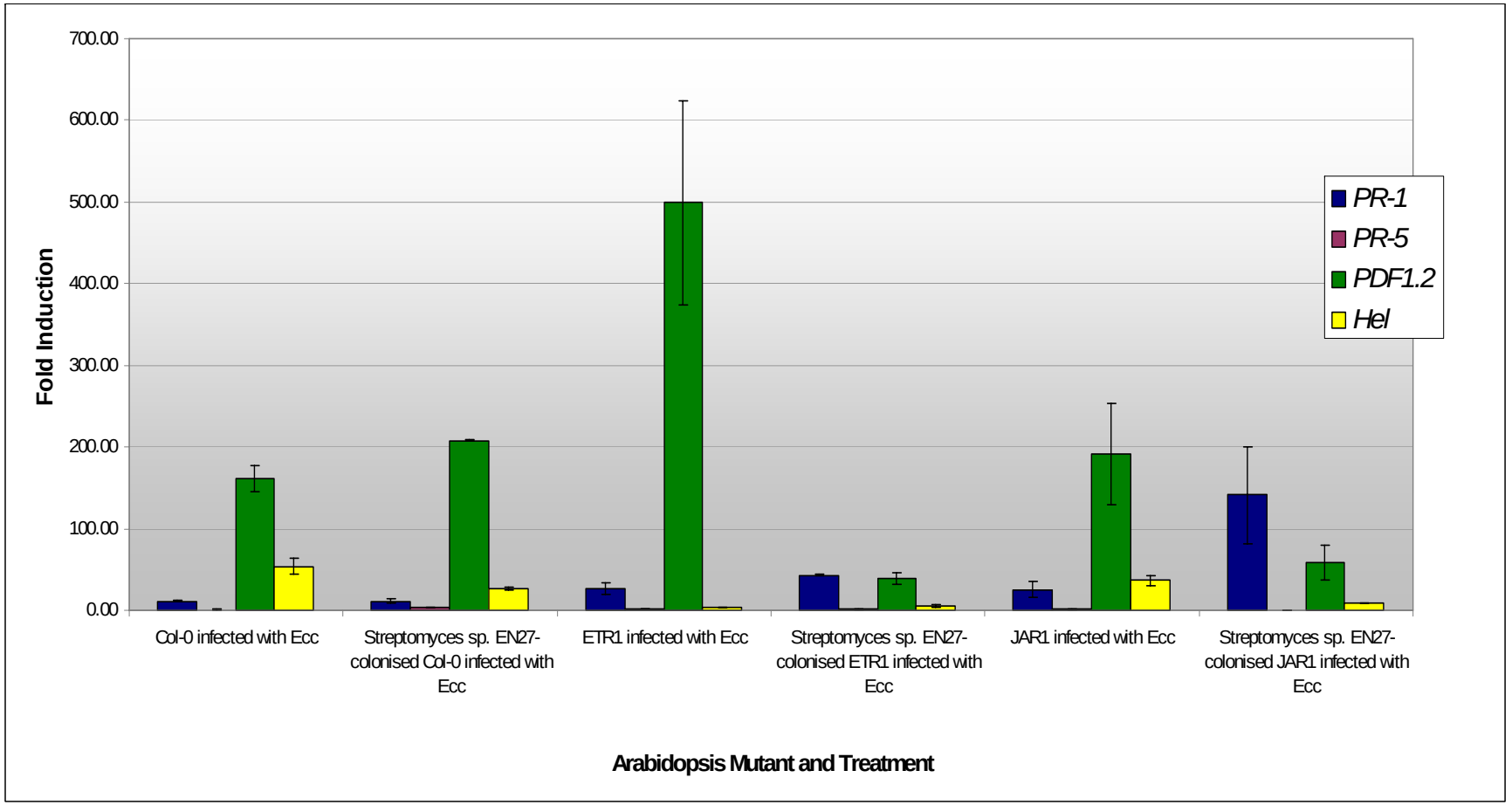


Figure 5.9: Fold induction of SAR pathway genes (*PR-1* and *PR-5*) and JA/ET pathway genes (*PDF1.2* and *Hel*) in the ethylene insensitive (*etr1*) and jasmonic acid insensitive (*jar1*) mutants with and without *Streptomyces* sp. EN27 pre-treament three days after infection with *Erwinia carotovora* subsp. *carotovora* (n=9).

5.3.3 Expression of Defence Genes in Arabidopsis Defence-Compromised Mutants after Challenge with *Fusarium oxysporum*

Inoculation of wild-type *Arabidopsis thaliana* with *Streptomyces* sp. EN27 resulted in the induction of genes in both pathways when challenged with *F. oxysporum*. The SAR mutants (*NahG* and *npr1*) and JA/ET mutants (*jar1* and *etr1*) were inoculated with EN27 and infected with *F. oxysporum* for three days and the gene expression analysed by Real-time RT-PCR.

Infection of the SA degrading mutant, *NahG* and the *npr1* mutant resulted in little expression of defence genes in both the SAR and JA/ET pathways and disease symptoms appeared earlier than in the infected wild-type. The same result was observed in EN27-treated mutants infected with *F. oxysporum*. The gene expression was analysed as shown in Figure 5.10.

There was minimal expression of the *PR-1* transcript in both the uninfected untreated and endophyte-treated *NahG* and *npr1* mutants compared to wild-type infected Arabidopsis. *F. oxysporum* induced *PR-5* expression in wild-type plants; however, no induction was detected in the *NahG* or *npr1* mutants. Compared to the induction detected in the wild-type infected plants the *PDF1.2* and *Hel* gene expression was also significantly decreased. *PDF1.2* induction in the untreated plant decreased from 116-fold to 26-fold and 29-fold in the *NahG* and *npr1* plants, respectively. This was further reduced in the EN27-treated plants with the *PDF1.2* transcript induced 18-fold and 3-fold, respectively.

F. oxysporum infection of the JA/ET mutants, *jar1* and *etr1*, resulted in a

considerably different gene expression profile (Figure 5.11) compared to the SAR mutants. Most noticeably the *PR-1* transcript was significantly induced in the *jar1* and *etr1* mutants (380-fold and 468-fold respectively) and was further enhanced in the EN27-treated plants (1188-fold and 663-fold respectively). The *PDF1.2* and *Hel* transcripts were significantly induced in the untreated *jar1* and *etr1* plants; however this level of reduction was reduced when the plants were inoculated with EN27 (Figure 5.11). The *PDF1.2* induction decreased from 440-fold to 96-fold in untreated and EN27-treated *jar1* plants, respectively. This trend was also seen in the *etr1* plants with the *PDF1.2* transcript decreasing from a 270-fold induction in the untreated plant to a 48-fold induction in the EN27-treated plant.

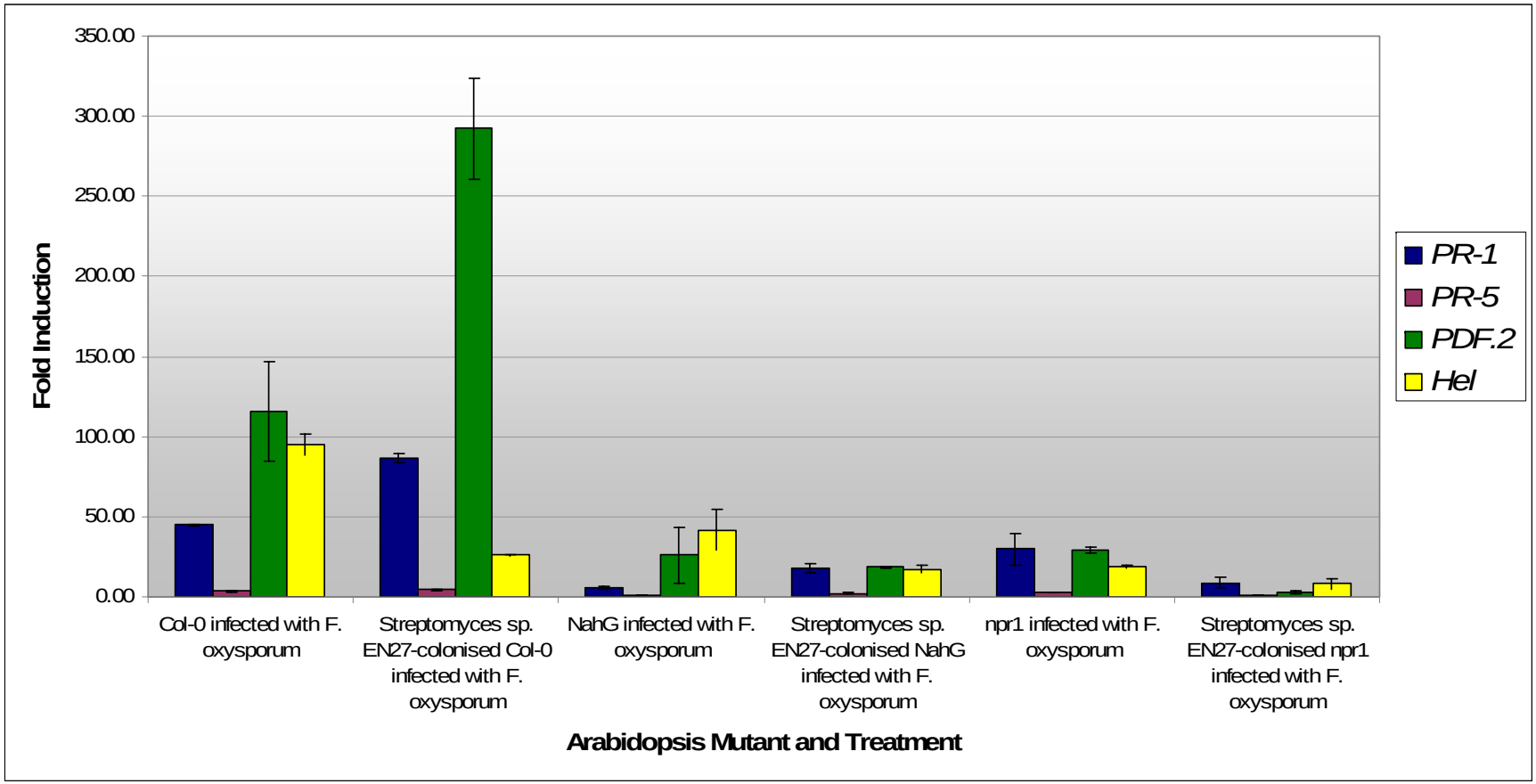


Figure 5.10: Fold induction of SAR pathway genes (*PR-1* and *PR-5*) and JA/ET pathway genes (*PDF1.2* and *Hel*) in the salicylic acid degrading (*NahG*) and PR-gene compromised (*npr1*) mutants with and without *Streptomyces* sp. EN27 pre-treatment three days after infection with *Fusarium oxysporum* (n=9).

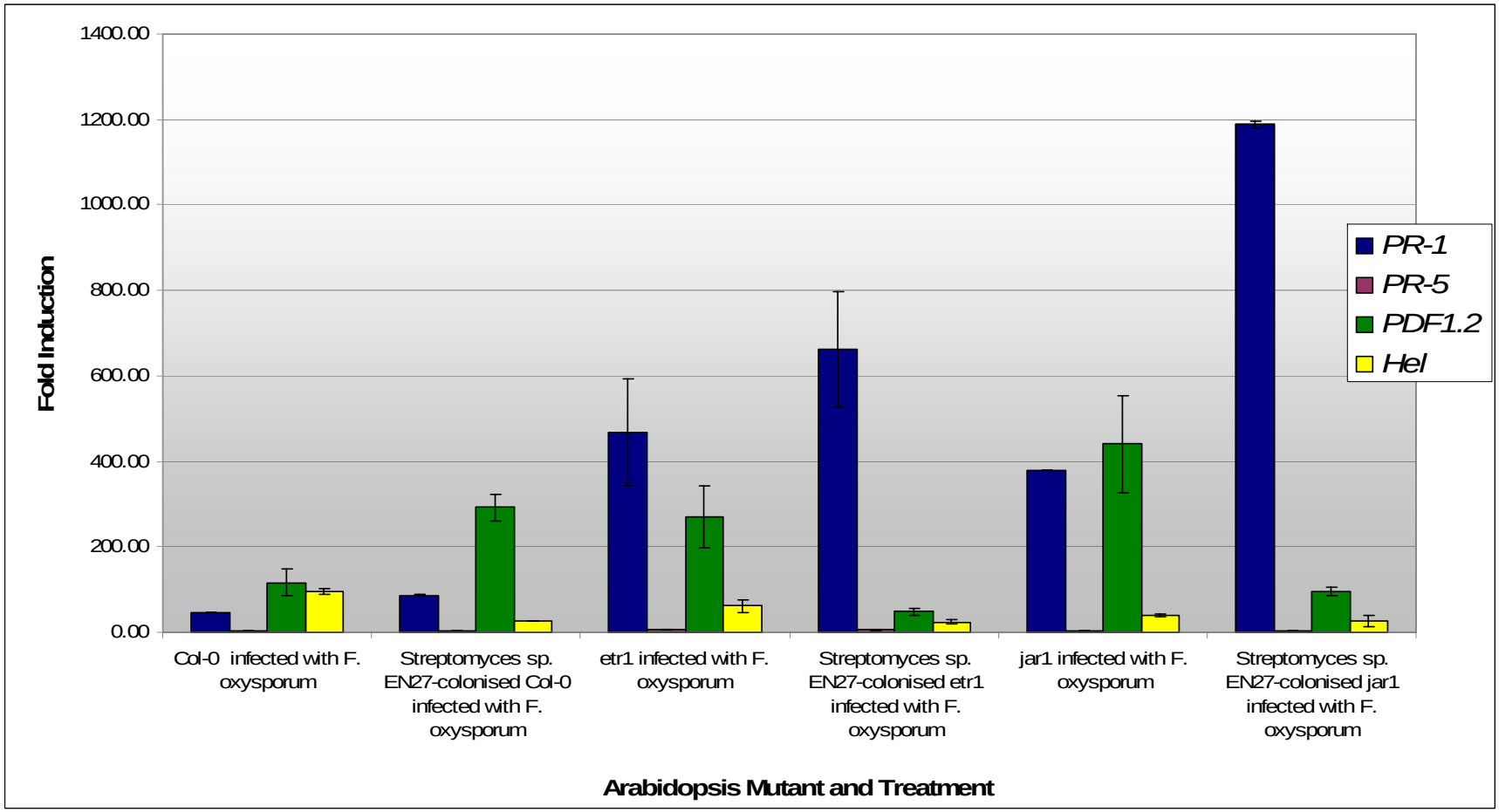


Figure 5.11: Fold induction of SAR pathway genes (*PR-1* and *PR-5*) and JA/ET pathway genes (*PDF1.2* and *Hel*), in the ethylene insensitive (*etr1*) and jasmonic acid insensitive (*jar1*) mutants with and without *Streptomyces* sp. EN27 pre-treatment three days after infection with *Fusarium oxysporum* (n=9).

5.4 Discussion

Plant defence pathways are activated in response to pathogen invasion, leading to the production of defence compounds and structural changes including the strengthening of the plant cell walls (Hammerschmidt, 1999; Heil and Bostock, 2002). Inoculation of Arabidopsis with the endophytic actinobacteria *Streptomyces* sp. EN27 activates the SAR defence pathway as shown by up-regulation of *PR-1* gene expression. While structural changes in the plant were not investigated it is possible they are occurring in response to *Streptomyces* sp. EN27 colonisation in the wild-type plants. The change in endophytic colonisation levels was investigated in Arabidopsis defence-compromised mutants. It was found that in comparison to the wild-type plants there was an enhanced level of *Streptomyces* sp. EN27 colonisation in the stems and leaves of the *NahG*, *npr1*, *etr1* and *jar1* mutants after four weeks of growth. It is possible that because of the compromised defence systems the plant structural changes are not occurring and therefore allowing more *Streptomyces* sp. EN27 to enter the plant and colonise the internal tissue. After six weeks of growth only the *npr1* mutant showed an enhanced level of *Streptomyces* sp. EN27 colonisation in comparison to the wild-type. The NPR1 protein has been shown to be vital for correct functioning of both the SAR and JA/ET pathways and therefore with a step in both pathways knocked-out plants would be more susceptible to pathogen attack and colonisation by endophytes.

By week 8 the *Streptomyces* sp. EN27 colonisation decreased in all plants but again only the *npr1* mutant showed a significant decrease in comparison to the wild-type plants. The decrease in *Streptomyces* sp. EN27 colonisation may be due to the

plants tripling in size and the actinobacteria not being able to spread rapidly throughout the internal tissue. A recent study by Iniguez *et al.* (2005) showed colonisation of *Medicago truncatula* by the maize endophyte *Klebsiella pneumoniae* 342 (Kp342) was enhanced in the defence-compromised ethylene-insensitive mutant. It was also shown that in the presence of increasing ACC concentrations, the endophytic colonisation of wheat roots by Kp342 was reduced. While in Arabidopsis it was shown that only SA-independent defence responses contribute to the restriction of Kp342 colonisation. Colonisation of the Arabidopsis plants with *Streptomyces* sp. EN27 was shown to activate the plant defence responses after 7 weeks (Chapter 4). Therefore after 8 weeks the activation of these defence pathways may also lead to the reduction in endophytic colonisation as seen in all the plant treatments.

Infection of wild-type Arabidopsis with the bacterial pathogen, *Erwinia carotovora* subsp. *carotovora* (Ecc), primarily activated the JA/ET pathway with strong up-regulation of the plant defensin gene *PDF1.2.* Colonisation with *Streptomyces* sp. EN27 was shown to further enhance the expression of the *PDF1.2* transcript and resulted in enhanced disease resistance as the plant had a normal appearance 3 days after infection. Norman-Setterblad *et al.* (2000) found resistance to Ecc occurred via two types of defence pathways in Arabidopsis. One pathway was found to be JA-dependent and inhibited by SA whereas the other pathway was ET- and JA-dependent but potentiated by SA.

In this study it was found that the *Streptomyces* sp. EN27-mediated resistance to Ecc required SA as the *NahG* mutants were unable to mount a defence response to Ecc infection. The *NahG* mutants were unable to accumulate SA as the *NahG* transgene, salicylate hydroxylase, converts SA to the inactive form catechol and

therefore are unable to express *PR-1* (Lawton *et al., 1*995). While SA is essential for EN27-mediated Ecc resistance, it occurred via a NPR1-independent pathway.

The NPR1 protein is initially found in oligomeric form in the cytoplasm, when SA increases the oligomers dissociate into monomers which allows nuclear localization (Mou *et al.,* 2003; Glazebrook, 2005). In the nucleus the monomers interact with the TGA family of basic domain/leucine zipper (bZIP) transcription factors (Zhang *et al.,* 1999; Zhou *et al.,* 2000) and are required for *PR-1* expression by SA (Kinkema *et al.,* 2000). It was found that in the *npr1* mutants infected with Ecc there was a significant level of *PR-1* induction. In the *npr1* mutants treated with *Streptomyces* sp. EN27 there was also *PR-1* induction but also *PDF1.2* and *Hel* induction and this correlated with Ecc resistance as the plants showed little symptom of disease. This indicates that a NPR1-independent pathway must have been functioning.

Shah *et al.* (1999) identified the dominant mutation *ssi1* (*suppressor of SA immunity*) where *PR-1, PR-2* and *PR-5* were expressed constitutively and the requirement for NPR1 was bypassed. SSI1 is thought to function as a switch modulating cross-talk between the SA and JA/ET–mediated defence pathways. Evidence for SA-dependent but NPR1-independent pathways for regulation of *PR-1* gene expression and resistance to bacterial pathogens has been reported previously (Bowling *et al.,* 1997; Clarke *et al.,* 1998; Rate *et al.,* 1999; Devadas *et al.,* 2002; Rairdan and Delaney, 2002).

Infection with Ecc was shown to trigger the JA/ET pathway in wild-type Arabidopsis and the *PDF1.2* transcript was further up-regulated upon EN27-treatment. Therefore, it would be anticipated that resistance mediated by

Streptomyces sp. EN27 would be abolished in plants defective in JA and ET signalling. However, it was found in the JA-insensitive and the ET-insensitive plants, *jar1* and *etr1*, respectively, inoculation with EN27 was still able to induce resistance to Ecc as the plant had a normal appearance following inoculation with Ecc.

JAR1 is predicted to belong to the acyl adenylate-forming firefly luciferase super-family encoding a JA-amino synthetase that forms conjugates between JA and amino acids, where the isoleucine conjugate may be the active form of JA (Staswick *et al.*, 2002; Staswick and Tiryaki, 2004). Mutations in *jar1* result in insensitivity to jasmonates. The ethylene receptor family of Arabidopsis consists of five members (ETR1, ETR2, ERS1, ERS2 and EIN4). Four dominant mutations in ETR1 (*etr1-1*, *etr1-2*, *etr1-3* and *etr1-4*) have been isolated and result in single amino acid changes within the hydrophobic domain of ETR1 which is involved in ethylene binding (Chang *et al.*, 1993).

Analysis of the gene expression in Ecc-infected *jar1* and *etr1* plants revealed that *PDF1.2* was still able to be expressed; indicating expression of *PDF1.2* can occur via the JA/ET pathway even when insensitive to either JA or ET. Expression of *PDF1.2* was enhanced in the *etr1* mutant containing the *etr1-3* mutation. This mutation only reduces ethylene sensitivity but does not eliminate it. Pennickx *et al.* (1996) also found that in the *etr1-3* mutant, *PDF1.2* could be induced upon infection with *Alternaria brassicicola*.

The resistance to Ecc-mediated by *Streptomyces* sp. EN27 in wild-type Arabidopsis was correlated with an increased induction of the JA/ET gene *PDF1.2*. However, in the *Streptomyces* sp. EN27-treated *jar1* and *etr1* mutants resistance to Ecc was observed but the expression of *PDF1.2* and *Hel* were significantly reduced.

Instead the SAR pathway gene *PR-1* was increased in comparison to the untreated plants. It is possible that JA/ET mutants inoculated with *Streptomyces* sp. EN27 are able to detect the JA/ET pathway is defective and enhance resistance by triggering the SAR pathway as an alternative. The analysis of SAR and JA/ET mutants has led to the finding that resistance to Ecc mediated by *Streptomyces* sp. EN27 occurs via a NPR1-independent pathway. This requires SA though JA and ET signalling are not essential for resistance.

The previous results indicated that resistance to *F. oxysporum*, mediated by *Streptomyces* sp. EN27, occurred primarily through the SAR pathway with the JA/ET pathway activated to a lesser extent. Infection of the two SAR mutants, *NahG* and *npr1*, with *F. oxysporum* and analysis of the gene expression revealed that expression of the SAR pathway genes *PR-1* and *PR-5* were significantly reduced as were the JA/ET pathway genes *PDF1.2* and *Hel*. Therefore, SA and the NPR1 protein are essential for *F. oxysporum* resistance.

Analysis of the JA-insensitive and ET-insensitive mutants infected with *F. oxysporum* revealed JA/ET signalling is not required to mount a defence response. In fact *PR-1* gene expression was enhanced in the *jar1* and *etr1* mutants and *Streptomyces* sp. EN27-treatment further induced *PR-1* expression. Interestingly, the *PDF1.2* gene was able to be expressed in the *jar1* and *etr1* mutants but when treated with *Streptomyces* sp. EN27 the induction was again significantly reduced as was the case with the Ecc infection.

Streptomyces sp. EN27 contains a 13kb plasmid (pEN2701). One of the eleven identified open reading frames (ORFs) on this plasmid shows 46% amino acid similarity to a ethylene-responsive element binding factor (ERF) (GenBank

Accession Number: BAA31525). The ERF contains the AP2, DNA-binding domain which is found in plant transcription regulators such as APETALA2 and EREBP (ethylene responsive element binding protein). In EREBPs the domain specifically binds to the 11 bp GCC box of the ethylene response element (ERE), a promoter element essential for ethylene responsiveness and found in several pathogenesis-related (PR) gene promoters (Buttner and Singh, 1997; Onate-Sanchez and Singh, 2002). In Arabidopsis, ERF proteins are involved in mediating responses to dehydration, salt, and cold stress, abscisic acid and ethylene (Onate-Sanchez and Singh, 2002). Ethylene Response Factor 1 (ERF1) can be rapidly induced by ET or JA and synergistically by both hormones (Lorenzo *et al.,* 2003). Four ERF genes were shown to be induced in Arabidopsis when infected with *Pseudomonas syringae* pv *tomato* (Onate-Sanchez and Singh, 2002) and overexpression of ERF1 was shown to enhance resistance to *F. oxysporum* (Berrocal-Lobo *et al.,* 2002; Berrocal-Lobo and Molina, 2004). It has been proposed the members of the ERF family are involved in cross-talk between the SAR and JA/ET pathways (Onate-Sanchez and Singh, 2002; Lorenzo *et al.,* 2003).

Infection with Ecc activates the JA/ET pathway and, in wild-type plants treated with *Streptomyces* sp. EN27, the *PDF1.2* gene is induced strongly. In EN27-treated *jar1* and *etr1* mutants there was minimal *PDF1.2* expression, which was anticipated as signaling in the JA/ET pathway was compromised. However, the *PDF1.2* gene was still significantly induced in the JA/ET mutants without EN27-treatment when infected with Ecc. This same phenomenon was observed when the JA/ET mutants were infected with *F. oxysporum*. However, when the JA/ET mutants were inoculated with *Streptomyces* sp. EN27 and infected with either Ecc or *F.*

oxysporum there was a strong shift to the SAR pathway and *PR-1* expression was significantly enhanced. It appears treatment with *Streptomyces* sp. EN7 enabled detection of the defective JA/ET pathway and switching to the functioning SAR pathway. It is possible that the putative ERF contained on the pEN2701 plasmid may play a role in this pathway cross-talk.

In conclusion, analysis of the SAR and JA/ET mutants revealed that EN27-mediated resistance to Ecc occurs via a NPR1-independent pathway and requires SA whereas EN27-mediated resistance to *F. oxysporum* occurs via a NPR1-dependent pathway and requires SA but JA and ET signalling were not essential for resistance to either pathogens.

Chapter Six: Major Findings and Future Directions

6.1 Major Findings of the Project

The aim of this research project was to investigate the molecular interactions of endophytic actinobacteria strains previously isolated in our laboratory. As these isolates enhanced growth and disease resistance in wheat understanding the interactions of these endophytes within the plant and mechanism of disease resistance was needed in order for their use as an effective biological control agent.

The major findings of this research were:

- Wheat roots are inhabited by a diverse range of endophytic actinobacteria which is influenced by the number of microorganisms supported in the soil.
- The diversity and level of colonisation of endophytic actinobacteria in wheat roots are negatively affected by the application of non-adapted mixed microbial inoculants to the soil but not by single endophyte inoculants.
- The wheat root is inhabited by a diverse range of fungal endophytes which had not been previously reported to have an endophytic association.
- The endophytic actinobacteria 'primed' the Systemic Acquired Resistance and Jasmonic Acid/Ethylene defence pathways of *Arabidopsis thaliana*.
- Endophytic actinobacteria enhanced resistance to *Erwinia carotovora* subsp. *carotovora* via the JA/ET pathway and *Fusarium oxysporum* primarily via the SAR pathway.
- *Streptomyces* sp. EN27-mediated resistance to *E. carotovora* subsp. *carotovora* via a NPR1-independent SA-depdenent pathway, whereas, resistance to *F. oxysporum* was mediated by a NPR1-dependent SA-dependent pathway.

Throughout the life of a plant it will have a constant interaction with microbial communities. Some microorganisms are deleterious to the plants health while others live in a mutually beneficial relationship. A proportion of rhizobacteria known as the plant growth promoting rhizobacteria (PGPR) have the ability to enhance plant growth and enhance disease resistance (Bloemberg and Lugtenberg, 2001). The exploitation of the beneficial processes by the PGPR has been attempted for many years and has had varied success. Many PGPR have shown the ability to work as biological control agents in controlled laboratory conditions but fail when taken into the field. This has been attributed mainly to the inability to compete with indigenous microorganisms (Sturz and Nowak, 2000). However it has been found that microorganisms also live inside the internal plant and this has led to the investigation of endophytic microorganisms as biocontrol agents.

A number of endophytic actinobacteria were isolated from healthy wheat roots in our laboratory previously and have ability to improve the growth of wheat and enhance resistance to fungal diseases both *in vitro* and *in planta* (Coombs, 2002; Coombs *et al.*, 2004). The mechanisms behind this interaction were further investigated in this study. The endophytic actinobacteria population present in the roots of wheat was investigated using a culture-independent technique (T-RFLP). Wheat roots were found to be inhabited by a diverse range of actinobacteria that is significantly affected by the soil. Soils that support a higher level of indigenous microflora resulted in a higher actinobacterial diversity and level of colonisation (Conn and Franco, 2004a). In contrast the addition of non-indigenous microorganisms in the form of a commercial inoculant had a negative impact on the level of actinobacteria colonisation and diversity (Conn and Franco, 2004b). Mixed

microbial inoculants contain bacterial and/or fungal strains chosen on their ability to improve the plant's health through different mechanisms such as nitrogen-fixation or antibiotic production. In theory these types of products should work, but often they do not provide any benefit and this may be due to the disruption of the natural endophytic population. This study has provided evidence for why the addition of a number of non-indigenous microbes to the soil do not provide the benefits they theoretical should. The addition of these non-indigenous microbes can out-compete the natural microorganisms that are capable of colonising the plant tissue and exhibiting positive effects.

In contrast the addition of a single endophyte inoculant did not disrupt the natural endophytic population. The endophytic actinobacteria inoculated into the wheat plant increased in colonisation approximately 3-fold by six weeks. The implication is that the endophytes are not dominating the endophytic environment and do not need to colonise in high numbers to exhibit beneficial effects. The endophytic actinobacteria isolated in our laboratory improved the plant growth and/or disease resistance when re-inoculated back into wheat indicating some role in maintaining the plants health.

The use of endophytic actinobacteria as biocontrol agents may have a better chance of success in the field as endophytic colonisation provides a more protected environment in comparison to the highly competitive rhizosphere and they do not need to colonise the plant tissue at a high level which in turn means the natural endophytic population is not disrupted.

Like bacterial endophytes, fungal endophytes have been shown to be beneficial to plant health. While the number of clones sequenced was limited, 29

different fungal species were identified in the roots of wheat grown in four different field soils. All fungal species, except for three, belonged to the Ascomycota phylum, which has previously been shown to be the predominant fungal endophyte phyla (Ernst *et al.,* 2003). For the majority of fungal species detected an endophytic relationship with wheat had not been described before. Many fungi are known to interact with the plant host providing a mutally beneficial symbiosis as is the case with the AM fungi. Endophytic fungi may be able to enhance the plants growth or enhance disease resistance in a similar manner to the endophytic actinobacteria. Understanding the metabolic capability of each individual fungal species will help define its role in the host plant. Therefore the isolation and characterisation of such fungal endophytes would provide greater insight into the role of endophytic fungi.

The inoculation of selected endophytic actinobacteria into wheat plants has been shown to enhance the disease resistance to a number of wheat fungal pathogens (Coombs, 2002; Coombs *et al.,* 2004). The molecular mechanism behind this interaction was investigated using the laboratory model plant, Arabidopsis. Inoculation of Arabidopsis with selected endophytic actinobacteria activated the Arabidopsis plant defence pathways as there was up-regulation of genes in the SAR or JA/ET pathways. The endophytic actinobacteria were able to further up-regulate the defence genes in either pathway depending on the infecting pathogen and this correlated to an enhanced resistance. The level of resistance and gene induction varied among the selected endophytic actinobacteria. The culture filtrates of the endophytic actinobacteria used in this study were applied to the Arabidopsis plants. The culture filtrates from *Micromonospora* sp. EN43 was able to induce a low level of gene expression in either the SAR or JA/ET pathways depending on the culture

medium. This indicates there is a secreted metabolite(s) or cell factor(s) that is able to activate the plant defence pathways which needs to be further investigated. The compound(s) responsible for this induction needs to be isolated. This would then allow for further manipulation and the use of the compound(s) in replace of the bacterial treatment.

The methods designed in this study provide a way to screen further endophytic actinobacteria for the ability to induced systemic resistance and activate plant defence genes in response to pathogen invasion. This may lead to the identification of a superior biological control agent.

The ability of the selected endophytic actinobacteria to activate the Arabidopsis plant defence pathways and enhance disease resistance provides a model for the how disease resistance mediated by the endophytes is occurring in wheat. The systemic acquired resistance and jasmonic acid/ethylene defence pathways have not been conclusively identified in wheat. A PR-1 like protein has been indentified as have wheat chemically induced (*WCI*) and wheat induced resistance (*WIR*) genes (Gorlach *et al.,* 1996; Molina *et al.,* 1999). Further work needs to be performed to translate the information from Arabidopsis to wheat.

Endophtyic actinobacteria have the potential to be successful biocontrol agents. Coating wheat seeds with the spores of actinobacteria endophytes allows effective colonisation without the isolates dominating in the plant tissue. This has been shown to be important as disruption of the natural population may be detrimental to the plant health. Selected endophytic actinobacteria are able to significantly enhance plant growth through the production of phytohormones (Coombs, 2002). The rapid establishment of roots increase the seedlings ability to

anchor to the soil and obtain water and nutrients while minimizing the opportunity for pathogen infection. A number of strains are able to produce antimicrobials which may have the ability to control a variety of pathogens (Coombs, 2002). However, it was also shown in this study that endophytic actinobacteria can activate plant defence pathways inducing systemic resistance which may lead to the protection against a broad number of pathogens.

Appendix One: Media Recipes and Common Molecular Biology Reagents

A.1 Media Recipes

Actinobacteria Pre-Inoculation Medium: glucose 15 $g.L^{-1}$, soya bean meal 15 $g.L^{1}$, corn steep liquor 5 $g.L^{-1}$, NaCl 5 $g.L^{-1}$, $CaCO_3$ 2 $g.L^{-1}$; pH 7.2

FL026 Medium: glucose 20 $g.L^{-1}$, soya bean meal 10 $g.L^{-1}$, $CaCO_3$ 4 $g.L^{-1}$, $CoCl_2.6H_2O$ 1 $mg.L^{-1}$; pH 7.2

FL031 Medium: glycerol 15 $g.L^{-1}$, glucose 5 $g.L^{-1}$, pharmamedia 20 $g.L^{-1}$, yeast extract 5 $g.L^{-1}$, KH_2PO_4 1 $g.L^{-1}$, $MgSO_4.7H_2O$ 0.5 $g.L^{-1}$, $CaCO_3$ 2 $g.L^{-1}$, $CuSO_4.5H_2O$ 7 $mg.L^{-1}$, $FeSO_4.7H_2O$ 1 $mg.L^{-1}$, $MnCl_2.4H_2O$ 8 $mg.L^{-1}$, $ZnSO_4.7H_2O$ 2 $mg.L^{-1}$; pH 7.2

Hoaglands Solution:

Solution A: $Ca(NO_3)2.4H_2O$ 165.3 $g.L^{-1}$, $(CH_2.N(CH_2COOH)_2)_2FeNa$ 7.341 $g.L^{-1}$

Solution B: KH_2PO_4 40.8 $g.L^{-1}$, K_2SO_4 43.6 $g.L^{-1}$, $MgSO_4.7H_2O$ 49.3 $g.L^{-1}$, NH_4NO_3 8 $g.L^{-1}$, pH 6.5 +/- 0.2 with 2N nitric acid.

Trace elements were added individually into Solution B

$MnSO_4.4H_2O$	1.032 g
H_3BO_3	248 mg

$CuSO_4.5H_2O$	25 mg
$(NH_4)_6MO_7O_{24}.4H_2O$	17.6 mg
$ZnSO_4.7H_2O$	230 mg

Add 5ml of solution A and 5ml solution B to 1L of sterile H_2O.

King's B Medium: glucose 10 $g.L^{-1}$, proteose peptone 20 $g.L^{-1}$, K_2HPO_4 1.5 $g.L^{-1}$, $MgSO_4.7H_2O$ 1.5 $g.L^{-1}$, agar 15 $g.L^{-1}$

Media was made up to 1L with RO H_2O before autoclaving.

Luria Broth (LB): tryptone 10 $g.L^{-1}$, sodium chloride 10 $g.L^{-1}$, yeast extract 5 $g.L^{-1}$, pH 7.0

The media was made up to 1L with RO H_2O and the pH adjusted to 7.0 before autoclaving. For LB agar plates, 15 $g.l^{-1}$ of agar was added before autoclaving. When selecting for pGEM T-vector transformants 2 mls of 50 $mg.ml^{-1}$. Ampicillin was added to the media once it was cooled to approximately 60°C. After the plates had been poured and dried sufficiently 100 µl of 100 mM IPTG and 20 µl of X-gal (50 $mg.ml^{-1}$) was spread over the plate and left to absorb.

Mannitol Soy (MS) Medium: mannitol 20 $g.L^{-1}$, soy flour 20 $g.L^{-1}$, agar 15 $g.L^{-1}$. The media was made up to one litre with RO H_2O and autoclaved. If an antifungal was agent was needed the media was cooled to approximately 60°C and 2 ml 20 $mg.ml^{-1}$ cyclohexamide was added.

Nutrient Agar: peptone 2.5 $g.L^{-1}$, lab lemco (beef extract) 0.5 $g.L^{-1}$, yeast extract 1.0 $g.L^{-1}$, NaCl 2.5 $g.L^{-1}$, agar 15 $g.L^{-1}$

The media was made up to 1L with RO H_2O before autoclaving. When antifungal agents were require the media was cooled to approximately 60°C and 2 ml 25 $mg.ml^{-1}$ cyclohexamide was added. For ¼ strength nutrient agar one quarter of the reagents was added to 1L of RO H_2O.

Oatmeal Agar: oatmeal 20 $g.L^{-1}$, yeast extract 1.5 $g.L^{-1}$, agar 15 $g.L^{-1}$; pH 7.2

The oatmeal was added to 300 ml distilled H_2O and boiled for 15 min. Strain this mixture through a cheese cloth then add the yeast extract and agar. Adjust pH to 7.2 then bring the volume to 1L with RO H_2O and autoclave.

Potato Dextrose Agar (PDA): Full strength PDA consisted of 39 $g.L^{-1}$ PDA and 15 $g.L^{-1}$ of agar to which 1L of RO H_2O was added before autoclaving. Half strength PDA consisted of 19.5 $g.L^{-1}$ of PDA with 15 $g.L^{-1}$ agar which was made up to 1L with RO H_2O before autoclaving.

Sabouraud Broth: glucose 20 $g.L^{-1}$, soy peptone 10 $g.L^{-1}$; pH 5.6 +/- 0.2

SOC Medium: tryptone 20 $g.L^{-1}$, yeast extract 5 $g.L^{-1}$, 10 mM NaCl, 4 mM KCl, 20 mM glucose, 20 mM Mg^{2+} added as a salt

The tryptone, yeast extract, NaCl and KCl were added to 98 ml RO H_2O and autoclaved. Once cooled the 2 M Mg^{2+} and glucose were added to the medium

through a 0.2 μm filter.

Yeast Malt Extract (YME) Agar: malt extract 10 $g.L^{-1}$, yeast extract 4 $g.L^{-1}$, glucose 4 $g.L^{-1}$, agar 15 $g.L^{-1}$; pH 7.3

Media was made up to 1L with RO H_2O and then the pH adjusted to 7.3 before autoclaving.

A.2 Common Molecular Biology Reagents

5X TBE: Tris-Base 54 $g.L^{-1}$, Boric Acid 27.5 $g.L^{-1}$, 0.5 M EDTA (pH 8.0) 20 $ml.L^{-1}$

6X Agarose Gel Loading Buffer: Bromophenol Blue 0.25%, Boric Acid 0.25%, Sucrose 40%.

CTAB/NaCl: 700 mM NaCl, 275 mM CTAB

NAP Buffer: 124 mM $Na_2HPO_4.H_2O$

Phosphate Buffer: 0.06 M KH_2PO_4, 0.06 M K_2HPO_4; pH 7.6

Saline: 0.9% (w/v) NaCl

Tris-EDTA: 10 mM Tris, 10 mM EDTA, pH 8.0

Appendix Two: T-RFLP Raw Data

Table A.2.1 : 16S rRNA gene sequence TRFs obtained with the restriction enzyme *Hinf*I, *Hha*I and *Mbo*I for the roots of wheat grown for six weeks in field soils obtained from Swedes Flat with and without NutriLife 4/20™ (n=6).

HinfI	Minus NutriLife 4/20™	Plus NutriLife 4/20™	HhaI	Minus NutriLife 4/20™	Plus NutriLife 4/20™	MboI	Minus NutriLife 4/20™	With NutriLife 4/20™
35	+	+	36	+	-	34	+	+
39	+	+	39	-	+	39	+	+
41	+	-	41	+	-	43	+	+
44	+	+	44	+	+	49	+	+
49	-	-	49	+	+	53	+	+
53	+	+	53	+	+	57	-	+
57	-	+	58	-	-	63	+	+
61	-	-	61	-	-	70	+	+
63	+	+	63	+	+	72	-	+
70	+	+	70	+	+	80	-	+
80	+	+	81	+	+	82	+	+
82	+	+	85	+	+	89	+	+
84	+	+	89	+	+	91	-	-
89	+	+	91	-	-	95	+	+
92	-	-	94	+	+	99	-	+
94	+	+	97	-	+	105	-	-
97	-	+	99	-	+	113	-	-
99	-	+	106	-	+	118	+	-
105	-	-	113	-	-	128	+	-
112	-	-	120	-	-	133	+	+
128	+	+	127	+	-	147	-	+
133	+	+	133	+	+	156	+	-
147	+	+	147	+	+	158	+	-
167	-	+	175	-	-	162	+	+
174	+	+	178	+	+	166	-	+
176	+	+	190	-	-	173	+	+
178	+	+	213	+	+	175	-	-
180	+	+	227	-	-	178	-	+
188	-	+	239	-	+	214	-	-
213	+	+	258	-	-	258	-	-
236	+	+	279	-	-			
258	-	-	321	-	-			
279	-	-	348	-	-			
284	-	-	368	-	-			
321	-	-	372	-	-			
348	-	-	385	+	+			
369	-	-	387	+	+			
407	-	-	410	+	+			
411	-	-	412	+	+			
			414	+	+			
			416	-	-			
			418	+	-			
			420	-	+			

Table A.2.2: Endophytic actinobacteria population and the TRF matches in the TAP-T-RFLP database for wheat roots grown Swedes Flat soil with a microbial inoculant.

*Hinf*I	*Hha*I	*Mbo*I	Phylum	Family	Genus	Min.	Max.
177	421	159	*Actinobacteria*	*Streptosporangiaceae*	*Planomonospora*	0.12	2.82
178	421	160	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*	0.12	2.82
			Actinobacteria	*Nocardioidaceae*	*Nocardioides*		
236	419	788	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*	0.12	0.75
176	419	788	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*	0.12	0.75
176	419	517	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*	0.12	0.75
178	420	160	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*	0.12	2.82
177	420	159	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*	0.12	2.82
51	420	159	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*	0.12	4.83
174	410	35	*Actinobacteria*	*Microbacteriaceae*	*Microbacterium*	0.09	2.08
			Actinobacteria	*Mycobacteriaceae*	*Mycobacterium*		
			Actinobacteria	*Microbacteriaceae*	*Agromyces*		
			Actinobacteria	*Gordoniaceae*	*Gordonia*		
			Actinobacteria	*Geodermatophilaceae*	*Geodermatophilus*		
			Actinobacteria	*Dietziaceae*	*Dietzia*		
			Actinobacteria	*Corynebacteriaceae*	*Corynebacterium*		
			Actinobacteria	*Microbacteriaceae*	*Agromyces*		
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*		
174	410	51	*Actinobacteria*	*Nocardioidaceae*	*Kribbella*	0.20	15.69
176	411	35	*Actinobacteria*	*Geodermatophilaceae*	*Geodermatophilus*	0.09	2.08
175	411	35	*Actinobacteria*		clone ACE-33 (genus unknown)	0.09	2.08
			Actinobacteria	*Mycobacteriaceae*	*Mycobacterium*		
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*		
			Actinobacteria	*Micrococcaceae*	*Micrococcus*		
175	386	35	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*	0.09	1.17
174	385	35	*Actinobacteria*	*Microbacteriaceae*	*Curtobacterium*	0.09	1.17
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*		
			Actinobacteria	*Mycobacteriaceae*	*Mycobacterium*		
			Actinobacteria	*Microbacteriaceae*	*Leifsonia*		
			Actinobacteria	*Nocardiaceae*	*Nocardia*		
			Actinobacteria	*Dermabacteraceae*	*Brachybacterium*		
174	386	35	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*	0.09	1.17
175	412	35	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*	0.09	2.08
176	412	35	*Actinobacteria*	*Nocardiaceae*	*Rhodococcus*	0.09	2.08
			Actinobacteria	*Gordoniaceae*	*Gordonia*		
			Actinobacteria	*Microbacteriaceae*	*Microbacterium*		
			Actinobacteria	*Rubrobacteraceae*	*Rubrobacter*		
			Actinobacteria	*Nocardioidaceae*	*Pimelobacter*		
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*		
			Actinobacteria	*Geodermatophilaceae*	*Geodermatophilus*		
			Actinobacteria	*Nocardiaceae*	*Nocardia*		
178	414	160	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*	0.30	2.82
					Total	2.18	50.78

Table A.2.3: 16S rRNA fragments sizes obtained with the restriction enzyme *Hinf*I for the roots of wheat inoculated with or without an endophyte and grown for six weeks in soil obtained from Haslam.

HinfI	Uninoculated	EN2-Inoculated	EN27-Inoculated	EN46-Inoculated
34	+	+	+	+
39	+	+	+	+
45	+	+	+	+
49	+	+	-	+
53	+	+	+	+
58	+	+	+	+
62	+	+	+	+
63	+	+	+	+
67	-	-	+	-
70	+	+	+	+
76	-	+	+	-
81	+	+	+	+
84	+	+	+	+
88	+	+	+	+
91	+	+	+	+
95	+	+	+	+
97	+	+	+	+
100	+	+	+	+
112	+	+	+	-
126	+	+	+	+
132	+	+	+	+
147	+	+	+	+
163	+	+	+	+
167	+	+	+	+
176	+	+	+	+
178	+	+	+	+
180	+	+	+	+
189	+	+	+	+
201	+	+	+	+
214	+	+	-	+
219	+	-	-	-
232	-	-	+	-
237	+	+	+	-
241	-	+	+	+
348	+	+	-	+
354	+	-	-	-
369	+	+	+	+

Table A.2.4: 16S rRNA fragments sizes obtained with the restriction enzyme *Hha*I for the roots of wheat inoculated with or without an endophyte and grown for six weeks in soil obtained from Haslam.

*Hha*I	Uninoculated	EN2-Inoculated	EN27-Inoculated	EN 46-Inoculated
34	-	-	-	+
39	+	+	+	+
42	+	-	-	-
45	+	+	+	+
49	+	+	+	+
53	+	+	+	+
58	+	+	+	+
63	+	+	+	+
70	+	+	+	+
74	-	+	-	-
77	-	-	+	-
81	+	+	+	+
83	+	+	+	+
88	+	+	+	+
91	+	+	-	-
95	+	+	+	+
97	+	+	+	-
100	-	+	+	+
106	-	+	-	+
120	-	-	+	-
112	+	-	-	-
126	+	+	+	+
132	+	+	+	+
147	+	+	+	+
162	+	-	+	-
175	+	+	+	+
190	+	+	+	+
200	-	+	-	-
214	+	+	+	+
227	-	-	+	-
279	+	+	-	-
332	+	-	-	-
348	+	+	+	-
369	+	-	+	-
387	+	+	+	-
398	+	+	+	-
412	+	+	+	+
413	+	+	+	+
416	+	-	-	+
420	+	+	+	+
422	+	-	+	+
470	+	-	-	+

Table A.2.5: 16S rRNA fragments sizes obtained with the restriction enzyme *Mbo*I for the roots of wheat inoculated with or without an endophyte and grown for six weeks in soil obtained from Haslam.

*Mbo*I	Uninoculated	EN2-Inoculated	EN27-Inocuated	EN 46-Inoculated
34	+	+	+	-
36	-	+	+	+
39	+	+	+	+
45	+	+	+	+
49	+	+	+	+
53	+	+	+	+
58	+	+	+	+
61	+	+	+	+
64	+	+	+	+
67	-	-	+	-
70	+	+	+	+
77	-	-	+	-
81	+	+	+	+
82	+	+	+	+
84	+	+	+	+
88	+	+	+	+
92	+	+	+	+
95	+	+	+	+
97	+	+	+	+
100	-	+	+	-
112	+	+	+	-
132	+	-	-	+
134	+	-	+	+
153	+	-	+	+
158	+	+	+	+
162	+	+	+	+
163	-	+	+	+
166	-	-	+	+
173	+	+	+	+
175	+	+	-	+
183	+	-	+	-
214	+	-	+	-
218	+	-	+	-
284	+	-	+	-
287	+	-	-	-

Table A.2.6: Actinobacteria endophytes present in the roots of wheat grown from an uninoculated seed for six weeks in soil obtained from Haslam.

*Hinf*I	*Hha*I	*Mbo*I	Phylum	Family	Genus
176	419	162	*Actinobacteria*		*Microbispora.*
178	421	160	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*
			Actinobacteria	*Nocardioidaceae*	*Nocardioides*
178	420	160	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	419	788	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
369	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
178	414	160	*Actinobacteria*	*Pseudonocardiaceae*	*Thermocrispum*
35	414	160	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*
177	413	159	*Actinobacteria*	*Pseudonocardiaceae*	*Saccharomonospora*
			Actinobacteria	*Actinomycetaceae*	*Actinomyces*
			Actinobacteria	*Actinosynnemataceae*	*Saccharothrix*
			Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium*
			Actinobacteria	*Thermomonosporaceae*	*Thermomonospora*
178	413	160	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	413	35	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*
51	413	159	*Actinobacteria*	*Micrococcaceae*	*Arthrobacter*
176	412	158	*Actinobacteria*	*Nocardiaceae*	*Rhodococcus*
			Actinobacteria	*Promicromonosporaceae*	*Promicromonospora*
			Actinobacteria	*Brevibacteriaceae*	*Brevibacterium*
			Actinobacteria	*Frankiaceae*	*Frankia*
			Actinobacteria	*Micrococcaceae*	*Arthrobacter*
			Actinobacteria	*Sanguibacteraceae*	*Sanguibacter*
			Actinobacteria	*Actinosynnemataceae*	*Lentzea*
			Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium*
			Actinobacteria	*Pseudonocardiaceae*	*Saccharomonospora*
			Actinobacteria	*Nocardiaceae*	*Nocardia*
			Actinobacteria	*Micrococcineae*	*Micrococcaceae*
			Actinobacteria	*Williamsiaceae*	*Williamsia*
176	412	35	*Actinobacteria*	*Nocardiaceae*	*Rhodococcus*
			Actinobacteria	*Gordoniaceae*	*Gordonia*
			Actinobacteria	*Nocardioidaceae*	*Pimelobacter*
			Actinobacteria	*Geodermatophilaceae*	*Geodermatophilus*
			Actinobacteria	*Nocardiaceae*	*Nocardia*
			Actinobacteria	*Microbacteriaceae*	*Microbacterium*
175	411	35	*Actinobacteria*	*Frankineae*	clone ACE-33 (genus unknown)
			Actinobacteria	*Mycobacteriaceae*	*Mycobacterium*
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*
			Actinobacteria	*Micrococcaceae*	*Micrococcus*

Table A.2. 6 cont.

*Hinf*I	*Hha*I	*Mbo*I	Phylum	Family	Genus
175	411	157	*Actinobacteria*	*Brevibacteriaceae*	*Brevibacterium*
			Actinobacteria	*Promicromonosporaceae*	*Cellulosimicrobium*
			Actinobacteria		*Candidatus Microthrix*
			Actinobacteria		*Sarraceniospora*
			Actinobacteria	*Frankiaceae*	*Frankia*
			Actinobacteria	*Nocardiaceae*	*Parvopolyspora*
			Actinobacteria	*Micrococcaceae*	*Arthrobacter*
			Actinobacteria	*Actinosynnemataceae*	*Lechevalieria*
			Actinobacteria	*Actinosynnemataceae*	*Lentzea*
			Actinobacteria	*Actinomycetaceae*	*Actinobaculum*

Table A.2.7: Additional actinobacteria present in the roots of wheat grown for six weeks from an uninoculated seed in soil obtained from Haslam.

*Hinf*I	*Hha*I	*Mbo*I	Phylum	Family	Genus
232 235	470	215 218	*Actinobacteria*	*Streptosporangiaceae*	*Microbispora.*
51	423	160	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
177	421	159	*Actinobacteria*	*Streptosporangiaceae*	*Planomonospora*
178	420	160	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
177	420	159	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*
51	420	159	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
175	420	157	*Actinobacteria*	*Streptosporangiaceae*	*Streptosporangiaceae*
236	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
236	419	788	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	419	517	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*
51	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	418	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
235	418	157	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
236	418	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	418	758	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
174	418	156	*Actinobacteria*	*Streptosporangiaceae*	*Planobispora*
175	418	157	*Actinobacteria*		*Actinomyces*
178	416	160	*Actinobacteria*		Uncultured bacterium TAYNAYA-20 (genus unknown)
234	416	156	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
35	414	160	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*
177	413	35	*Actinobacteria*	*Geodermatophilaceae*	*Geodermatophilus*
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*
177	413	159	*Actinobacteria*	*Pseudonocardiaceae*	*Saccharomonospora*
			Actinobacteria	*Actinomycetaceae*	*Actinomyces*
			Actinobacteria	*Actinosynnemataceae*	*Saccharothrix*
			Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium*
			Actinobacteria	*Thermomonosporaceae*	*Thermomonospora*
178	413	160	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
235	411	157	*Actinobacteria*		Soil actinomycete (*Kitatsatospora*)
			Actinobacteria	*Streptomycetaceae*	*Kitasatospora*
180	398	160	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*
176	387	158	*Actinobacteria*	*Micrococcaceae*	*Arthrobacter*
			Actinobacteria	*Nocardiaceae*	*Nocardia*
			Actinobacteria	*Kineosporiaceae*	*Kineococcus-like* bacterium
			Actinobacteria	*Pseudonocardiaceae*	*Streptoalloteichus*
			Actinobacteria	*Micromonosporaceae*	*Actinoplanes*
			Actinobacteria	*Micromonosporaceae*	*Spirilliplanes*
176	387	35	*Actinobacteria*	*Nocardiaceae*	*Nocardia*
175	387	157	*Actinobacteria*	*Micromonosporaceae*	*Actinoplanes*
			Actinobacteria	*Actinosynnemataceae*	*Actinosynnema*

Table A.2.7 cont.

			Actinobacteria	*Corynebacteriaceae*	*Corynebacterium*
			Actinobacteria	*Pseudonocardiaceae*	*Saccharomonospora*
			Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium*
			Actinobacteria	*Kineosporiaceae*	*Kineococcus-like* bacterium
			Actinobacteria	*Micromonosporaceae*	*Catellatospora*
			Actinobacteria	*Nocardioidaceae*	*Aeromicrobium*
176	411	35	*Actinobacteria*	*Geodermatophilaceae*	*Geodermatophilus*
174	411	156	*Actinobacteria*	*Corynebacteriaceae*	*Corynebacterium*
			Actinobacteria	*Brevibacteriaceae*	*Brevibacterium*
35	411	157	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*
176	411	158	*Actinobacteria*	*Nocardioidaceae*	*Nocardioides*
			Actinobacteria	*Kineosporiaceae*	*Kineococcus-like* bacterium
35	410	156	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*
174	410	156	*Actinobacteria*	*Cellulomonadaceae*	*Cellulomonas*
			Actinobacteria	*Micrococcaceae*	*Arthrobacter*
			Actinobacteria	*Intrasporangiaceae*	*Terrabacter*
			Actinobacteria	*Microbacteriaceae*	*Microbacterium*
			Actinobacteria	*Nocardioidaceae*	*Pimelobacter*
			Actinobacteria	*Micromonosporaceae*	*Pilimelia*
			Actinobacteria	*Frankiaceae*	*Frankia*
			Actinobacteria	*Nocardioidaceae*	*Nocardioides*
			Actinobacteria	*Brevibacteriaceae*	*Brevibacterium*
			Actinobacteria	*Actinosynnemataceae*	*Lechevalieria*
			Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium*
			Actinobacteria	*Nocardioidaceae*	*Kribbella*
			Actinobacteria	*Micrococcaceae*	*Micrococcus*
			Actinobacteria	*Streptomycetaceae*	*Streptomyces*
			Actinobacteria		*Sarraceniospora*
			Actinobacteria	*Pseudonocardiaceae*	*Actinoalloteichus*
			Actinobacteria	*Micromonosporaceae*	*Dactylosporangium*
			Actinobacteria	*Nocardioidaceae*	*Friedmanniella*
			Actinobacteria	*Micromonosporaceae*	*Micromonospora*
			Actinobacteria	*Cellulomonadaceae*	*Cellulomonas*
			Actinobacteria	*Micrococcaceae*	*Kocuria*
			Actinobacteria	*Nocardioidaceae*	*Hongia*
			Actinobacteria	*Intrasporangiaceae*	*Janibacter*
			Actinobacteria	*Micromonosporaceae*	*Catellatospora*
174	410	35	*Actinobacteria*	*Microbacteriaceae*	*Microbacterium*
			Actinobacteria	*Mycobacteriaceae*	*Mycobacterium*
			Actinobacteria	*Microbacteriaceae*	*Agromyces*
			Actinobacteria	*Gordoniaceae*	*Gordonia*
			Actinobacteria	*Geodermatophilaceae*	*Geodermatophilus*
			Actinobacteria	*Dietziaceae*	*Dietzia*
			Actinobacteria	*Corynebacteriaceae*	*Corynebacterium*
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*
			Actinobacteria	*Micromonosporaceae*	*Micromonosporaceae*
174	410	51	*Actinobacteria*	*Nocardioidaceae*	*Kribbella*
234	410	156	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
98	79	159	*Actinobacteria*	*Rubrobacteraceae*	*Rubrobacter*

Table A.2.8: Additional actinobacteria genera in the roots of wheat grown from EN2-inoculated seeds for six weeks in soil obtained from Haslam.

*Hinf*I	*Hha*I	*Mbo*I	Phylum	Family	Genus
241	420	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
			Actinobacteria	*Streptomycetaceae*	*Streptomyces*
241	420	162	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
241	420	163	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
177	421	159	*Actinobacteria*	*Streptosporangiaceae*	*Planomonospora*
177	420	159	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*
51	420	159	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
175	420	157	*Actinobacteria*	*Streptosporangiaceae*	genus unknown
236	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
236	419	788	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	419	517	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*
51	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
177	413	35	*Actinobacteria*	*Geodermatophilaceae*	*Geodermatophilus*
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*
236	412	158	*Actinobacteria*		Soil actinomycete (*Kitasatospora*)
175	412	35	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*
175	412	157	*Actinobacteria*	*Pseudonocardiaceae*	*Prauseria*
			Actinobacteria	*Nocardiopsaceae*	*Streptomonospora*
235	411	157	*Actinobacteria*		Soil actinomycete (*Kitasatospora*)
			Actinobacteria	*Streptomycetaceae*	*Kitasatospora*
51	411	157	*Actinobacteria*	*Actinomycineae*	genus unknown
176	411	158	*Actinobacteria*	*Nocardioidaceae*	*Nocardioides*
			Actinobacteria	*Kineosporiaceae*	*Kineococcus-like* bacterium
176	410	158	*Actinobacteria*	*Micromonosporaceae*	*Micromonosporaceae*
180	398	160	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*
176	387	158	*Actinobacteria*	*Micrococcaceae*	*Arthrobacter*
			Actinobacteria	*Nocardiaceae*	*Nocardia*
			Actinobacteria	*Kineosporiaceae*	*Kineococcus-like* bacterium
			Actinobacteria	*Pseudonocardiaceae*	*Streptoalloteichus*
			Actinobacteria	*Micromonosporaceae*	*Actinoplanes*
			Actinobacteria	*Micromonosporaceae*	*Spirilliplanes*
176	387	35	*Actinobacteria*	*Nocardiaceae*	*Nocardia*
175	387	157	*Actinobacteria*	*Micromonosporaceae*	*Actinoplanes*
			Actinobacteria	*Actinosynnemataceae*	*Actinosynnema*

Table A.2.9: Additional actinobacteria genera present in the roots of wheat grown from EN27-inoculated seeds for six weeks in soil obtained from Haslam.

*Hinf*I	*Hha*I	*Mbo*I	Phylum	Family	Genus
241	420	163			*Streptomyces*
241	420	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
			Actinobacteria	*Streptomycetaceae*	*Streptomyces*
241	420	162	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
51	423	160	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
177	421	159	*Actinobacteria*	*Streptosporangiaceae*	*Planomonospora*
177	420	159	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*
51	420	159	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
175	420	157	*Actinobacteria*	*Streptosporangiaceae*	
236	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	419	788	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	419	517	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*
51	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	418	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
235	418	157	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
236	418	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	418	758	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
174	418	156	*Actinobacteria*	*Streptosporangiaceae*	*Planobispora*
175	418	157	*Actinobacteria*		*Actinomyces* sp. Str. SR-25
178	414	160	*Actinobacteria*	*Pseudonocardiaceae*	*Thermocrispum*
			Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium*
177	413	35	*Actinobacteria*	*Geodermatophilaceae*	*Geodermatophilus*
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*
176	413	158	*Actinobacteria*	*Actinomycetaceae*	*Actinomyces*
236	412	158	*Actinobacteria*		Soil actinomycete (*Kitasatospora*)
175	412	35	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*
175	412	157	*Actinobacteria*	*Pseudonocardiaceae*	*Prauseria*
			Actinobacteria	*Nocardiopsaceae*	*Streptomonospora*
235	411	157	*Actinobacteria*		Soil actinomycete (*Kitasatospora*)
			Actinobacteria	*Streptomycetaceae*	*Kitasatospora*
51	411	157	*Actinobacteria*	*Actinomycineae*	Actinomycete (genus unknown) X87617
176	411	35	*Actinobacteria*	*Geodermatophilaceae*	*Geodermatophilus*
176	410	158	*Actinobacteria*	*Micromonosporaceae*	genus unknown
176	227	158	*Actinobacteria*	*Tsukamurellaceae*	*Tsukamurella*
176	227	35	*Actinobacteria*	*Gordoniaceae*	*Gordonia*

Table A.2.10: Additional actinobacteria genera present in the roots of wheat grown from EN46-inoculated seed for six weeks in soil obtained from Haslam.

*Hinf*I	*Hha*I	*Mbo*I	Phylum	Family	Genus
241	420	163	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
241	420	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
			Actinobacteria	*Streptomycetaceae*	*Streptomyces*
241	420	162	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
51	423	160	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
177	421	159	*Actinobacteria*	*Streptosporangiaceae*	*Planomonospora*
177	420	159	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*
51	420	159	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
175	420	157	*Actinobacteria*	*Streptosporangiaceae*	genus unknown
236	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
236	419	788	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	419	788	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	419	517	*Actinobacteria*	*Thermomonosporaceae*	*Thermomonospora*
51	419	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	418	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
235	418	157	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
236	418	158	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	418	758	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
174	418	156	*Actinobacteria*	*Streptosporangiaceae*	*Planobispora*
175	418	157	*Actinobacteria*		*Actinomyces* sp. Str. SR-25
178	416	160	*Actinobacteria*		Uncultured bacterium TAYNAYA-20
234	416	156	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
177	413	35	*Actinobacteria*	*Geodermatophilaceae*	*Geodermatophilus*
			Actinobacteria	*Nocardiaceae*	*Rhodococcus*
176	413	158	*Actinobacteria*	*Actinomycetaceae*	*Actinomyces*
178	413	160	*Actinobacteria*	*Streptomycetaceae*	*Streptomyces*
176	413	35	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*
236	412	158	*Actinobacteria*		Soil actinomycete (*Kitasatospora*)
175	412	35	*Actinobacteria*	*Mycobacteriaceae*	*Mycobacterium*
175	412	157	*Actinobacteria*	*Pseudonocardiaceae*	*Prauseria*
			Actinobacteria	*Nocardiopsaceae*	*Streptomonospora*
179	411	163	*Actinobacteria*	*Nocardioidaceae*	*Nocardioides*
235	411	157	*Actinobacteria*		Soil actinomycete (*Kitasatospora*)
			Actinobacteria	*Streptomycetaceae*	*Kitasatospora*
51	411	157	*Actinobacteria*	*Actinomycineae*	Genus Unknown
176	410	158	*Actinobacteria*	*Micromonosporaceae*	Genus Unknown

Appendix Three: Partial 16S rRNA Actinobacteria Sequencing

The three highest sequence matches for each 16S rRNA clone and the corresponding bit score and percentage identity for clones derived from the roots of wheat grown in Western Flat Swedes Flat soil with NutriLife 4/20™, Swedes Flat soil without NutriLife 4/20™ and Red Loam are shown in Table A.4.1, A.4.2, A.4.3 and A.4.4.

Table A.4.1: Three highest sequence matches of actinobacteria 16S rRNA clones from the roots of wheat grown in Western Flat soil and compared to the GenBank database.

CloneNo.	Match 1.	Bits	%	Match 2.	Bits	%	Match 3.	Bits	%
480	*Mycobacterium bohemicum*	1187	97	*Mycobacterium bohemicum*	1187	97	*Mycobacterium bohemicum*	1180	97
464	*Mycobacterium* sp. IMVS B76676	819	95	*Mycobacterium cookii*	809	96	*Mycobacterium* sp.	773	94
484	*Mycobacterium* sp. IMVS B76676	1166	97	*Mycobacterium cookii*	1138	97	*Mycobacterium* sp. 2333	1124	97
487	*Mycobacterium* sp. IMVS B76676	1166	97	*Mycobacterium cookii*	1138	97	*Mycobacterium* sp. 2333	1108	97
472	Uncultured maize root bacterium Zmrc174	1072	94	Uncultured eubacterium WD294	991	93	Uncultured bacterium	852	93
485	Uncultured eubacterium WD294	373	87	Uncultured eubacterium WR161	335	87	Uncultured division OP10 bacterium clone N41.110PG	303	86
499	*Mycobacterium* sp. 2333	876	95	*Mycobacterium* sp. IMVS B76676	876	95	*Mycobacterium cookii*	876	94
486	*Mycobacterium bohemicum*	557	91	*Mycobacterium bohemicum*	557	91	*Mycobacterium* sp. YM12	549	92
475	*Mycobacterium* sp. 2333	916	95	*Mycobacterium* sp. IMVS B76676	916	95	*Mycobacterium cookii*	906	94
476	*Mycobacterium* sp. IMVS B76676	1063	97	*Mycobacterium cookii*	1035	97	*Mycobacterium* sp. 2333	999	97
513	*Streptomyces* sp. EF-91	1227	98	*Streptomyces* sp. EF-93	1227	97	*Streptomyces* sp. VTT E-99-1335	1227	97
524	*Mycobacterium palustre*	1203	97	*Mycobacterium bohemicum*	1196	97	*Mycobacterium bohemicum*	1189	97

Table A.4.2: Three highest sequence matches of actinobacteria 16S rRNA clones from the roots of wheat grown in Swedes Flat soil with NutriLife 4/20™ and compared to the GenBank database.

Clone No.	Match 1.	Bits	%	Match 2.	Bits	%	Match 3.	Bits	%
304	*Mycobacterium aichiense* strain JS618	605	92	*Mycobacterium vaccae*	605	92	*Mycobacterium vaccae*	605	92
355	*Mycobacterium aichiense* strain JS618	1061	96	*Mycobacterium austroafricanum*	1061	96	*Mycobacterium austroafricanum*	1061	96
394	*Mycobacterium aichiense* strain JS618	1051	97	*Mycobacterium* sp. SM7.6.1	1051	97	*Mycobacterium* sp. T103	1051	97
319	*Mycobacterium austroafricanum*	831	93	*Mycobacterium austroafricanum*	829	93	*Mycobacterium aichiense*	825	93
313	*Mycobacterium chubuense*	821	96	*Mycobacterium* sp. DSM 44605	821	96	*Mycobacterium peregrinum*	821	96
328	*Mycobacterium cookii*	1253	95	*Mycobacterium* sp. 2333	1221	95	*Mycobacterium* sp. IMVS B76676	1197	94
327	*Mycobacterium interjectum*	618	87	*Mycobacterium interjectum*	618	87	*Mycobacterium* sp.	618	87
349	*Mycobacterium lacus*	500	88	*Mycobacterium asiaticum*	500	88	*Mycobacterium asiaticum*	500	88
359	*Mycobacterium scrofulaceum*	379	90	*Mycobacterium scrofulaceum*	379	90	*Mycobacterium palustre*	379	90
317	*Mycobacterium* sp.	1150	96	Mycobacterium sp. 'graecum DL049'	1142	95	*Mycobacterium intermedium*	1142	95
387	*Mycobacterium* sp. IMVS B76676	1086	96	*Mycobacterium cookii*	1059	96	*Mycobacterium* sp. 2333	1029	96
303	*Rhodococcus coprophilus*	1130	94	*Rhodococcus coprophilus*	1130	94	*Rhodococcus coprophilus*	1126	94
364	*Rhodococcus coprophilus*	886	98	*Rhodococcus coprophilus*	886	98	*Rhodococcus coprophilus*	886	98
352	*Streptomyces griseochromogenes*	1235	98	*Streptomyces galbus*	1227	98	*Streptomyces* sp. Z2	1227	98
383	*Streptomyces* sp. SE2	416	87	*Streptomyces* sp. EN35	416	87	*Streptomyces* sp. EN23	416	87
358	Uncultivated soil bacterium clone C019	878	93	Uncultured soil bacterium clone S1124	817	92	Uncultured bacterium clone KD6-84	775	91
371	*Nocardioides* sp. 4P1-A	688	91	Uncultured earthworm cast bacterium clone c224	682	93	*Nocardioides* sp. JS614	680	91
385	*Rhodococcus coprophilus*	946	98	*Rhodococcus coprophilus*	946	98	*Rhodococcus coprophilus*	946	98
322	*Mycobacterium* IWGMT 90174	1225	98	*Mycobacterium interjectum*	1205	98	*Mycobacterium heidelbergense*	1203	98
396	*Mycobacterium* IWGMT 90174	1249	98	*Mycobacterium interjectum*	1231	98	*Mycobacterium heidelbergense*	1229	98

Table A.4.3: Three highest sequence matches of actinobacteria 16S rRNA clones from the roots of wheat grown in Swedes Flat soil without NutriLife 4/20™ and compared to the GenBank database.

Clone No.	Match 1.	Bits	%	Match 2.	Bits	%	Match 3.	Bits %	
695	*Amycolatopsis* sp. GY152	884	98	*Amycolatopsis* sp. GY125	884	98	*Amycolatopsis lexingtonensis*	884	98
730	*Gordonia polyisoprenivorans*	1221	97	*Gordonia* sp. MN 110a	1217	97	*Gordonia polyisoprenivorans*	1203	97
713	*Mycobacterium aichiense* strain JS618	1187	94	*Mycobacterium austroafricanum*	1187	94	*Mycobacterium austroafricanum*	1187	94
696	*Mycobacterium cookii*	854	95	*Mycobacterium* sp. 2333	823	95	*Mycobacterium* sp. IMVS B76676	799	94
742	*Mycobacterium flavescens*	726	94	*Mycobacterium flavescens*	726	94	*Mycobacterium holsaticum*	726	94
705	*Mycobacterium* sp. 2333	1114	97	*Mycobacterium* sp. IMVS B76676	1114	97	*Mycobacterium cookii*	1104	97
709	*Mycobacterium* sp. 2333	1296	95	*Mycobacterium* sp. IMVS B76676	1296	95	*Mycobacterium cookii*	1263	94
680	*Mycobacterium* sp. IMVS B76676	1181	97	*Mycobacterium cookii*	1154	97	*Mycobacterium* sp. 2333	1116	97
708	*Mycobacterium* sp. IMVS B76676	1304	98	*Mycobacterium cookii*	1277	97	*Mycobacterium* sp. 2333	1239	97
737	*Mycobacterium* sp. IMVS B76676	1289	95	*Mycobacterium cookii*	1261	95	*Mycobacterium* sp. 2333	1233	95
658	*Mycobacterium* sp. IMVS B76676	1291	96	*Mycobacterium cookii*	1263	96	*Mycobacterium* sp. 2333	1225	96
733	*Mycobacterium* sp. IMVS B76676	938	94	*Mycobacterium cookii*	910	93	*Mycobacterium* sp. 2333	872	93
718	*Streptomyces* sp. SE2	504	90	*Streptomyces* sp. EN35	504	90	*Streptomyces* sp. EN23	504	90
739	*Amycolatopsis* sp. GY152	934	96	*Amycolatopsis* sp. GY125	934	96	*Amycolatopsis lexingtonensis*	934	96
666	*Mycobacterium* sp. 'MCRO 33'	1207	98	*Mycobacterium scrofulaceum*	1203	98	*Mycobacterium* IWGMT 90161	1203	98
736	Uncultured actinobacterium clone SMW4.128WL	694	95	*Mycobacterium cookii*	731	91	*Mycobacterium* sp. 2333	700	90

Table A.4.4: Three highest sequence matches of actinobacteria 16S rRNA clones from the roots of wheat grown in Red Loam soil and compared to the GenBank database.

Clone No.	Match 1.	Bits	%	Match 2.	Bits	%	Match 3.	Bits	%
230	*Micromonospora endolithica*	1128	95	*Micromonospora chersina*	1120	95	*Micromonospora citrea*	1120	95
221	*Micromonospora peucetica*	652	97	*Micromonospora* sp. IM-7020	652	97	*Catellatospora ishikariense*	644	97
241	*Mycobacterium cookii*	1051	95	*Mycobacterium* sp. 2333	1043	95	*Mycobacterium* sp. IMVS B76676	1011	94
238	*Mycobacterium heidelbergense*	1187	95	*Mycobacterium heidelbergense*	1181	95	*Mycobacterium* IWGMT 90174	1178	94
218	*Mycobacterium* sp.	630	90	*Mycobacterium* sp. JS621	599	89	*Mycobacterium rhodesiae*	599	89
277	*Mycobacterium* sp. 2333	888	93	*Mycobacterium* sp. IMVS B76676	888	93	*Mycobacterium cookie*	878	93
237	*Mycobacterium* sp. IMVS B76676	862	97	*Mycobacterium cookii*	858	98	*Mycobacterium palustre*	823	96
201	*Mycobacterium* sp. IMVS B76676	1160	97	*Mycobacterium cookii*	1150	97	*Mycobacterium* sp.	1104	95
251	*Mycobacterium* sp. IMVS B76676	1233	98	*Mycobacterium cookii*	1205	97	*Mycobacterium* sp. 2333	1162	98
227	*Mycobacterium* sp. IMVS B76676	1055	95	*Mycobacterium cookii*	1027	95	*Mycobacterium* sp. 2333	989	94
299	*Mycobacterium* sp. IMVS B76676	662	91	*Mycobacterium cookii*	660	91	*Mycobacterium* sp.	634	90
266	*Nocardia pseudobrasiliensis*	747	95	*Nocardia cyriacigeorgici*	739	94	*Nocardia ignorata*	739	94
216	*Rhodococcus coprophilus*	1316	97	*Rhodococcus coprophilus*	1312	97	*Rhodococcus coprophilus*	1308	97
203	*Streptomyces* sp. SE2	254	100	*Streptomyces* sp. EN35	254	100	*Streptomyces* sp. EN23	254	100
296	*Streptomyces thermolineatus*	858	97	*Streptomyces cattleya*	842	97	*Streptomyces cattleya*	841	97
271	*Streptomyces thermolineatus*	367	94	*Streptomyces rimosus*	351	93	*Amycolatopsis orientalis*	351	93
286	*Streptomyces thermolineatus*	535	88	*Amycolatopsis orientalis*	525	88	*Streptomyces rimosus subsp. Rimosus*	519	88

Appendix Four: Partial Fungal 18S rRNA Sequencing Results

The three highest sequence matche, bit score and percentage identity for each 18S rRNA clone derived from the roots of wheat grown in Swedes Flat soil with NutriLife 4/20™, Swedes Flat soil without NutriLife 4/20™, Red Loam and Western Flat are shown in Table A.5.1, A.5.2, A.5.3 and A.5.4.

Table A.5.1: Three highest sequence matches of fungal 18S rRNA clones from the roots of wheat grown in Swedes Flat soil with NutriLife 4/20™ and compared to the GenBank database.

Clone No.	Match 1.	Bits	%	Match 2.	Bits	%	Match 3.	Bits	%
13	*Westerdykella dispersa*	730	96	*Verruculina enalia*	730	96	Uncultured soil fungus clone Pent 3.2	722	96
55	*Westerdykella dispersa*	698	96	*Verruculina enalia*	698	96	*Trematosphaeria hydrela*	688	95
67		868	99		868	99		868	99
4	Uncultured soil ascomycete	684	96	*Pyrenophora tritici-repentis*	684	96	*Decorospora gaudefroyi*	976	95
46		591	92		591	92		593	92
63	*Talaromyces flavus*	692	96	*Spiromastix warcupii*	692	96	*Trichocomaceae sp.* G2521	686	96
52	Uncultured soil ascomycete	739	97	*Pyrenophora tritici-repentis*			*Ulocladium botrytis* strain UPSC 3539	731	97
15	*Clathrospora diplospora*	997	97	*Alternaria raphani*	997	97	*Alternaria brassicicola*	997	97
19		975	97		975	97		975	97
3		975	97		975	97		975	97
42		971	97		971	97		971	97
64		926	95		926	95		926	95
73		977	97		977	97		977	97
17		1009	97		1009	97		1009	97
45		997	97		997	97		997	97
39	*Cephaliophora muscicola*	991	97	*Rhizina undulata*	985	97	*Cephaliophora longispora*	985	97
27	*Trematosphaeria hydrela*	1037	98	*Verruculina enalia*	1007	98	*Delitschia winteri*	1005	97
30		1043	98		1013	97		1011 985	97
56		1011	97		981	97			
85		1017	97		987	97			97

Table A.5.2: Three highest sequence matches of fungal 18S rRNA clones from the roots of wheat grown in Swedes Flat soil without NutriLife 4/20™ and compared to the GenBank database.

Clone No.	Match 1.	Bits	%	Match 2.	Bits	%	Match 3.	Bits	%
101.	*Trematosphaeria hydrela*	979	98	*Verruculina enalia*	955	98	*Phoma herbarum* strain ATCC 22167	948	97
121.		979	98		955	97		948	97
181.		981	98		957	98		950	97
115.	Lasallia rossica	946	97	*Cephaliophora muscicola*	930	97	*Rhizina undulata*	914	96
116	*Trematosphaeria hydrela*	971	98	*Verruculina enalia*	948	98	*Phoma glomerate* strain ATCC 36804	940	97
118	*Endogone pisiformis*	777	94	*Scutellospora weresubiae*	704	97	*Scutellospora pellucida*	696	96
166.		809	95		712	97		704	97
122.	*Endogone pisiformis*	779	94	*Mortierella wolfii*	714	95	*Multiclavula mucida*	710	94
137.	*Endogone pisiformis*	795	94	*Scutellospora* sp. W2988	712	97	*Scutellospora pellucida*	704	97
176.	*Westerdykella dispersa*	561	91	*Verruculina enalia*	561	91	*Trematosphaeria hydrela*	559	91
143.	*Aleuria aurantia*	513	93	*Rhynchostoma minutum*	507	93	*Scutellinia scutellata*	490	92
148	*Aleuria aurantia*	811	95	*Rhynchostoma minutum*	799	94	*Pyronema domesticum*	793	94
158.	*Trematosphaeria hydrela*	1051	98	*Verruculina enalia*	1021	98	*Delitschia winteri*	1091	98
133.		1049	98		1019	98		1017	98
132.		1033	98		1003	98		1001	98
117.	*Endogone pisiformis*	769	94	*Mortierella wolfii*	702	95	*Scutellospora* sp. W2988	686	96
127.	*Pyronema domesticum*	940	96	*Rhynchostoma minutum*	938	97	*Aleuria aurantia*	936	97
142.	*Trematosphaeria hydrela*	892	96	*Verruculina enalia*	870	95	*Westerdykella dispersa*	862	95
178.	Uncultured Hypocreales	1003	99	*Nectria Lugdunensis* H4-2-4	1003	99	*Nectria Lugdunensis* strain CBE98	1003	99
185.	*Massaria platani*	1025	98	*Keissleriella cladophila*	1015	98	*Paraphaeosphaeria pilleata*	981	98
194.	*Pyronema domesticum*	975	97	*Rhynchostoma minutum*	973	97	*Scutellinia scutellata*	955	97

Table A.5.3: Three highest sequence matches of fungal 18S rRNA clones from the roots of wheat grown in Red Loam soil and compared to the GenBank database.

Clone No.	Match 1.	Bits	%	Match 2.	Bits	%	Match 3.	Bits	%
404	*Trematosphaeria hydrela*	1057	98	*Verruculina enalia*	1027	98	*Delitschia winteri*	1025	98
411		1057	98		1027	98		1025	98
417		1041	98		1011	98		1009	97
422		1043	98		1013	98		1011	97
435		1049	98		1035	98		1017	98
410	*Trematosphaeria hydrela*	995	98	*Verruculina enalia*	971	98	*Phoma herbarum* strain ATCC 22167	963	98
416	*Setosphaeria monoceras*	854	95	*Clathrospora diplospora*	854	95	*Pleospora herbarum*	854	95
423	*Phoma herbarum* strain ATCC 22167	605	89	*Westerdykella cylindrica*	605	89	*Sporomia lignicola*	605	89
428	*Westerdykella cylindrica*	482	85	*Sporormia lignicola*	482	85	*Westerdykella dispersa*	482	85
436		1027	99		1027	99		1011	99
429	*Ulocladium botrytis* strain UPSC 3539	997	99	*Ulocladium botrytis* strain CBS 173.82	997	99	*Clathrospora diplospora*	997	99
431	Fungal endophyte MUT 585	595	93	*Mycosphaerella mycopappi*	555	94	*Mycosphaerella mycopappi*	555	94
432	*Setosphaeria monoceras*	950	98	*Pleospora herbarum*	950	98	*Pleospora herbarum* DAOM 150679 nuclear	950	98
433	*Trematosphaeria hydrela*	995	98	*Verruculina enalia*	971	98	*Phoma herbarum* strain ATCC 22167	963	98
438	*Westerdykella cylindrica*	521	88	*Sporomia lignicola*	521	88	*Phoma herbarum* strain ATCC 22167	518	88
439	*Trematosphaeria hydrela*	981	98	*Verruculina enalia*	957	98	*Phoma herbarum* strain ATCC 22167	950	97
440	Uncultured ascomymete clone AT2-4	940	99	*Penicillium chrysogenum* strain KCTC6052	940	99	Uncultured ascomycete gene	940	99
442	*Clathrospora diplospora*	1027	99	*Alternaria raphani*	1027	99	*Alternaria brassicicola*	1027	99
443	*Termitomyces sp.* O1 gene	940	97	*Termitomyces sp.* Type A gene	940	97	*Lentinula edodes*	932	98
444	*Sordariomycete* sp. pgp-hsf	952	98	*Lecythophora sp.* HA1540	928	97	*Lecthophora mutabilis*	928	97
450	*Phoma herbarum* strain ATCC 22167	741	92	*Westerdykella dispersa*	741	92	*Westerdykella cylindrica*	726	92

Table A.5.4: Three highest sequence matches of fungal 18S rRNA clones from the roots of wheat grown in Western Flat soil and compared to the GenBank database.

Clone No.	Match 1.	Bits	%	Match 2.	Bits	%	Match 3.	Bits	%
552	*Pleospora rudis*	1076	99	*Dothideomycete* sp. G9-S53	1068	99	*Dothideomycete* sp. G9-S53	1068	99
553		1037	98		1029	98		1029	98
577		1092	99		1084	99		1084	99
578		1082	99		1074	99		1074	99
559	*Uncultured ascomycete clone AT2-4*	989	98	*Penicillium chrysogenum strain KCTC6052*	989	98	*Uncultured ascomycete gene*	989	98
561	*Uncultured Hypocreales*	1011	99	*Nectria Lugdunensis H4-2-4*	1011	99	*Nectria Lugdunensis strain CBE98*	1011	99
566	*Raciborskiomyces longisetosum*	789	94	Uncultured soil ascomycete	789	94	Uncultured ascomycete	789	94
567		789	94		789	94	gene	789	94
572	*Phoma sp.201*	1043	100	*Pleospora rudis*	1043	100	*Phoma glomerate strain ATCC 36804*	1035	99
576	*Phoma sp.201*	1019	99	*Pleospora sp. Fh4*	1019	99	*Pleospora rudis*	1019	99
579		1017	99		1017	99		1017	99
584	*Dothideomycete* sp. G9-S53	914	98	*Dothideomycete* sp. G9-S53	914	98	*Dothideomycete* sp. G6-S57	914	98
632		523	89		523	89		523	89
587	*Pseudoplectania nigrella*	908	96	*Rhizina undulata*	908	96	*Monacrosorium doedycoides*	908	96
605		912	96		912	96		912	96
581	Uncultured soil fungus clone Pent 3.5	955	97	*Pseudoplectania nigrella*	955	97	*Rhizina undulata*	955	97
588		955	97		955	97		955	97
639		948	96		936			936	96
585	*Engyodontium* album NRRL 28022	1059	99	*Tritirachium* sp. IAM14522	1779	98	*Tritirachium* sp. IAM14522	1043	98
589	*Raciborskiomyces longisetosum*	844	93	Uncultured ascomycete	844	93	*Cladosporium cladosporioides*	839	93
590	*Pseudoplectania nigrella*	420	88	*Wolfina aurantiopsis*	420	88	*Verpa conica*	420	88
592	*Leotiomycete* sp. G2-5-S70	1049	*98*	*Leotiomycete sp.* G2-5-RU342	1049	98	*Leotiomycete* sp. G2-5-PU349	1049	98
601	*Raciborskiomyces longisetosum*	829	95	Uncultured ascomycete	829	95	*Cladosporium cladosporioides* UPSC 1657	829	95
607	*Cladophialophora boppii nuclear*	977	99	*Cladophialophora devriessi nuclear*	934	98	*Graphium calicioides*	932	97

Appendix Five: Real Time RT-PCR Data

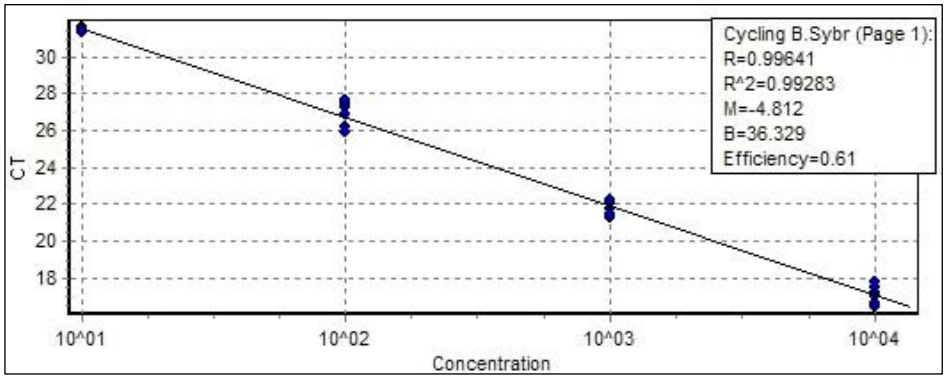


Figure A.5.1: Standard curve of *Act2* and *Act8* Real-time RT-PCR primer amplification efficiency.

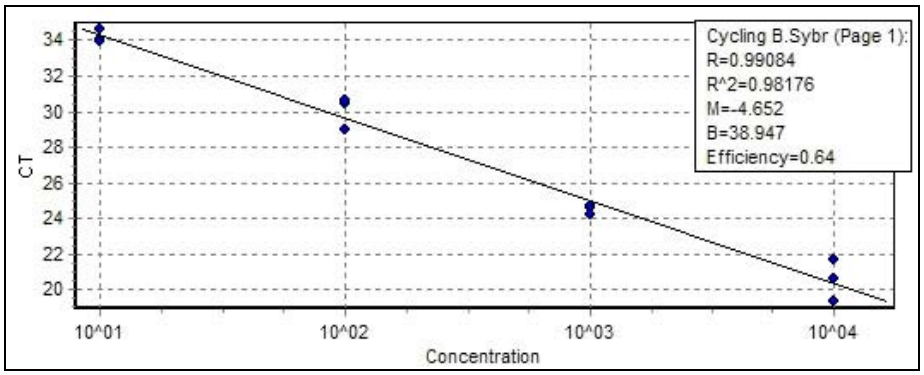


Figure A.5.2: Standard curve of *PR-1* Real-time RT-PCR primer amplification efficiency.

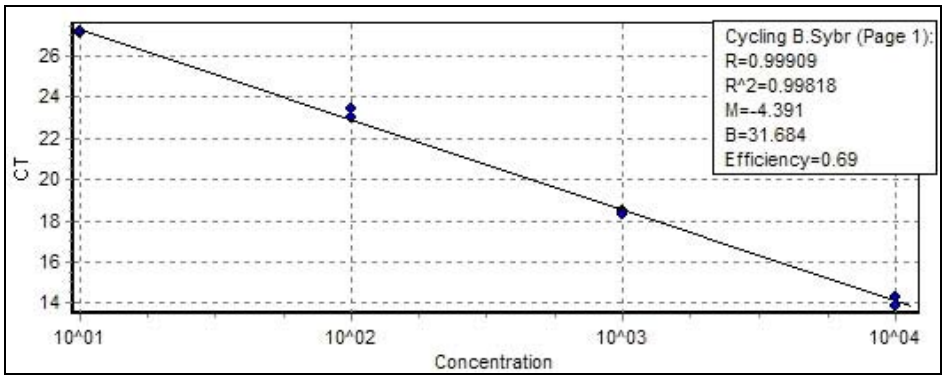


Figure A.5.3: Standard curve of *PR-5* Real-time RT-PCR primer amplification efficiency.

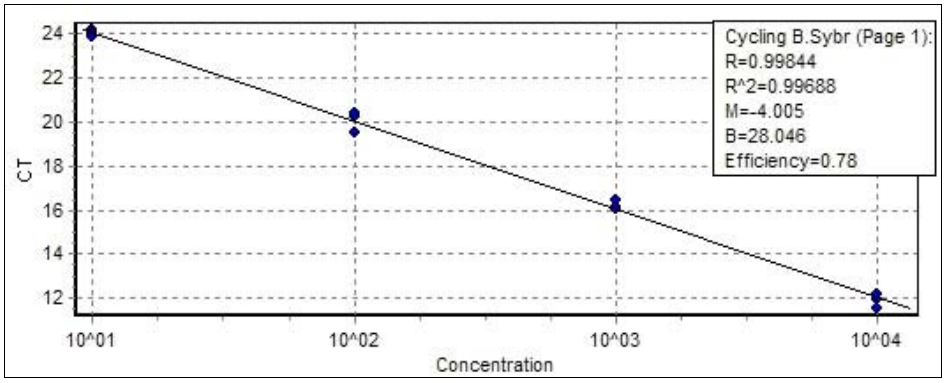


Figure A.5.4: Standard curve of *PDF1.2* Real-time RT-PCR primer amplification efficiency.

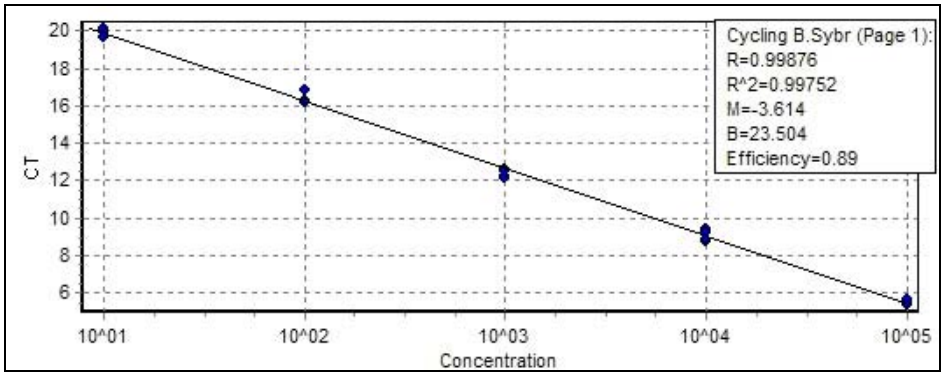


Figure A.5.5: Standard curve of *Hel* Real-time RT-PCR primer amplification efficiency.

Appendix Six: Publications, Conference Presentations and Awards

A.6.1 Publications

Conn, V.M., and Franco, C.M.M. (2004). Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by Terminal Restriction Fragment Length Polymorphism and sequencing of 16S rRNA clones. Applied and Environmental Microbiology **70,** 1787-1794.

Abstract: The endophytic actinobacterial population in the roots of wheat grown in three different soils obtained from the southeast part of South Australia was investigated by terminal restriction fragment length polymorphism (T-RFLP) analysis of the amplified 16S rRNA genes. A new, validated approach was applied to the T-RFLP analysis in order to estimate, to the genus level, the actinobacterial population that was identified. Actinobacterium-biased primers were used together with three restriction enzymes to obtain terminal restriction fragments (TRFs). The TRFs were matched to bacterial genera by the T-RFLP Analysis Program, and the data were analyzed to validate and semiquantify the genera present within the plant roots. The highest diversity and level of endophytic colonisation were found in the roots of wheat grown in a dark loam from Swedes Flat, and the lowest were found in water-repellent sand from Western Flat. This molecular approach detected a greater diversity of actinobacteria than did previous culture-dependent methods, with the predominant genera being *Mycobacterium* (21.02%) in Swedes Flat, *Streptomyces* (14.35%) in Red Loam, and *Kitasatospora* (15.02%) in Western Flat. This study indicates that the soil that supported a higher number of indigenous organisms resulted in wheat roots with higher actinobacterial diversity and levels of colonisation within the plant tissue. Sequencing of 16S rRNA clones, obtained using the same actinobacterium-biased PCR primers that were used in the T-RFLP analysis, confirmed the presence of the actinobacterial diversity and identified a number of *Mycobacterium* and *Streptomyces* species.

Conn, V.M., and Franco, C.M.M. (2004). Effect of Microbial Inoculants on the Indigenous Actinobacterial Endophyte Population in the Roots of Wheat as Determined by Terminal Restriction Fragment Length Polymorphism. Applied and Environmental Microbiology **70,** 6407-6413.

Abstract: The effect of single actinobacterial endophyte seed inoculants and a mixed microbial soil inoculant on the indigenous endophytic actinobacterial population in wheat roots was investigated by using the molecular technique terminal restriction

fragment length polymorphism (T-RFLP). Wheat was cultivated either from seeds coated with the spores of single pure actinobacterial endophytes of *Microbispora* sp. strain EN2, *Streptomyces* sp. strain EN27, and *Nocardioides albus* EN46 or from untreated seeds sown in soil with and without a commercial mixed microbial soil inoculant. The endophytic actinobacterial population within the roots of 6-week-old wheat plants was assessed by T-RFLP. Colonisation of the wheat roots by the inoculated actinobacterial endophytes was detected by T-RFLP, as were 28 to 42 indigenous actinobacterial genera present in the inoculated and uninoculated plants. The presence of the commercial mixed inoculant in the soil reduced the endophytic actinobacterial diversity from 40 genera to 21 genera and reduced the detectable root colonisation by approximately half. The results indicate that the addition of a nonadapted microbial inoculum to the soil disrupted the natural actinobacterial endophyte population, reducing diversity and colonisation levels. This was in contrast to the addition of a single actinobacterial endophyte to the wheat plant, where the increase in colonisation level could be confirmed even though the indigenous endophyte population was not adversely affected.

A.6.2 Conference Presentations

13th International Symposium on Biology of Actinomycetes

2003, 1-5 December, Melbourne, Australia

Poster Title: Analysis of the Endophytic Actinobacteria Population in the Roots of Wheat (*Triticum aestivum*) in response to different field soils with and without the addition of microbial inoculants by Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Partial Sequencing of 16S rDNA.

Abstract

Introduction: Endophytes are microorganisms that live within healthy plant tissue causing no apparent disease symptoms. A number of the biologically active endophytes have been isolated and belong to the actinobacteria phylum (Coombs, 2002). Actinobacteria are high G+C, gram-positive, filamentous bacteria capable of secondary metabolite production such as antibiotics and anti-fungal compounds. It is well known that soil type, host plant, cropping practices, growth stage and other environmental factors are capable of affecting the microbial population present in the rhizosphere, however, the effect of soil type and microbial inocula on the endophyte population is not known (Smalla *et al.*, 2001). Endophytic populations have been isolated and characterised primarily by cultivation-based methods, but as not all microorganisms can be grown *in vitro*, molecular techniques need to be used for microbial community analysis.

Aim: The aim of this study was to analyse how the endophytic actinobacteria

population in wheat roots change in response to cultivation in different field soils with and without microbial inocula using the terminal restriction fragment length polymorphism (T-RFLP) technique.

Methods: Cultivation of wheat (cv. Krichauff) in three field soils, a soil with NutriLife 4/20 microbial inoculum, and in a field soil with added actinobacteria endophyte inoculants. The endophytic bacterial DNA was extracted from wheat roots and the endophytic actinobacteria population determined by T-RFLP analysis of 16S rRNA.

Results: The endophytic actinobacteria population was significantly affected by the soil type. The highest diversity and level of endophytic colonisation was found in wheat grown in a dark loam (Swedes Flat) and the lowest in a non-wetting sand (Western Flat). Over 40 different endophytic actinobacteria genera were identified in three field soils, of these 10 genera were common in all soils. The addition of NutriLife 4/20 reduced the endophytic actinobacteria diversity from 40 genera to 21, and the endophytic colonisation by approximately half. In contrast the addition of a single endophyte inoculum by seed application has a very small effect on endophytic colonisation and diversity. Of 28 genera identified in the roots there was a change of colonisation levels in only six genera.

Conclusions: The endophytic actinobacteria population of wheat is affected by the soil type. Soils that support a higher, indigenous microbial population result in higher endophytic actinobacteria diversity and level of colonisation. The addition of a mixed microbial inoculum to the soil reduces the natural endophytic actinobacteria population. It would appear microbes present in the NutriLife 4/20 inoculum out compete the indigenous actinobacteria microflora, preventing their access to the seed and ultimately endophytic colonisation. This is in contrast to the addition of a single actinobacteria endophyte to the wheat plant, where the population remained relatively stable.

International Joint Workshop on PR-Proteins and Induced Resistance

Denmark, Helsingor, May 5-9, 2004

Poster Title: Induction of Key Genes in the Systemic Acquired Resistance snd Jasmonic Acid/Ethylene Pathways of *Arabidopsis Thaliana* by Endophytic Actinobacteria.

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Abstract: Endophytic actinobacteria, isolated from healthy wheat roots in our laboratory, have been shown to enhance disease resistance to multiple pathogens in wheat when coated onto the seed before sowing. *Arabidopsis thaliana* was used as a model system to investigate the mechanism of resistance. Real Time RT-PCR was used to determine if key genes in the Systemic Acquired Resistance (*PR-1* and *PR-5*) and Jasmonic Acid/Ethylene (*Pdf1.2* and *HEL*) pathways were induced. Coating the *A. thaliana* (Col-0) seeds with the endophytic actinobacteria, *Streptomyces* sp. (EN27 and EN28), *Micromonospora* sp. (EN43) and *Nocardioides albus* (EN46) induced a low level of *PR-1*, *Pdf1.2* and *HEL* gene expression. The level of gene induction was significantly lower compared with plants infected with a pathogen. Infection with the bacterial pathogen *Erwinia carotovora* triggers the JA/Ethylene pathway and all endophytic actinobacteria tested were able to induce *Pdf1.2* gene expression above the level detected in the wild-type infected plant, with EN43 and EN46 inducing the highest *Pdf1.2* expression (6.5-fold increase over the wild-type infected plant). In comparison, challenge with the fungal pathogen *Fusarium oxypsorum* strongly induced the SAR pathway, with EN28 inducing *PR-1* and *PR-5* genes 86 and 21-fold over the wild-type infected plant, respectively. The endophytic actinobacteria appear to be able to prime both the SAR and JA/Ethylene pathways, upregulating genes in either pathway depending on the infecting pathogen. Application of the culture filtrate of the endophytic actinobacteria EN43 induced *PR-1* and *PR-5* in FL026 media, and *Pdf1.2* and *HEL* in FL031 media suggesting that a secreted metabolite(s) or a cell factor(s) produced under different culture conditions are capable of priming the plant for pathogen infection. These results indicate the endophytic actinobacteria are capable of priming the plant when the spores are coated onto the seed before sowing, triggering induced systemic resistance. Inoculation with individual, or combinations of endophytic actinobacteria may provide a greater level of disease resistance in crops when used as biocontrol agents.

A.6.3 Awards

2004: GRDC Eureka Prize for Research to Improve the Environmental Sustainability of Graingrowing.

References

Adams, P.D., and Kloepper, J.W. (2002). Effect of host genotype on inidigenous bacterial endophytes of cotton (*Gossypium hirsutum* L.). Plant and Soil **240,** 181-189.

Aguilar, I., Alamillo, J.M., Garcia-Olmedo, F., and Rodriguez-Palenzuela, P. (2002). Natural variability in the Arabidopsis response to infection with *Erwinia carotovora* subsp. *carotovora*. Planta **215,** 205-209.

Akinsanmi, O.A., Mitter, V., Simpfendorfer, S., Backhouse, D., and Chakraborty, S. (2004). Identity and pathogenicity of *Fusarium* spp. isolated from wheat fields in Queensland and northern New South Wales. Australian Journal of Agricultural Research **55,** 97-107.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25,** 3389-3402.

An, Y., McDowell, J.M., Huang, S., McKinney, E.C., Chambliss, S., and Meagher, R.B. (1996). Strong, constitutive expression of the *Arabidopsis ACT2/ACT8* actin subclass in vegetative tissues. The Plant Journal **10,** 107-121.

Araujo, W.L., Maccheroni, W., Jr., Aguilar-Vildoso, C.I., Barroso, P.A., Saridakis, H.O., and Azevedo, J.L. (2001). Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Canadian Journal of Microbiology **47,** 229-236.

Araujo, W.L., Marcon, J., Maccheroni, W., Jr., Van Elsas, J.D., Van Vuurde, J.W., and Azevedo, J.L. (2002). Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Applied and Environmental Microbiology **68,** 4906-4914.

Arenskotter, M., Baumeister, D., Berekaa, M.M., Potter, G., Kroppenstedt, R.M., Linos, A., and Steinbuchel, A. (2001). Taxonomic characterization of two rubber degrading bacteria belonging to the species *Gordonia polyisoprenivorans* and analysis of hyper variable regions of 16S rDNA sequences. FEMS Microbiology Letters **205,** 277-282.

Arnold, E.A., Mejia, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N., and Herre, E.A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences USA **100,** 15649-15654.

Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K.e. (1990). Current protocols in molecular biology.

Axelrood, P.E., Chow, M.L., Radomski, C.C., McDermott, J.M., and Davies, J. (2002). Molecular characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. Canadian Journal of Microbiology **48,** 655-674.

Bacilio-Jimenez, M., Aguilar-Flores, S., Ventura-Zapata, E., Perez-Campos, E., Bouquelet, S., and Zenteno, E. (2003). Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria. Plant and Soil **249,** 271-277.

Baldani, J.I., Caruso, l., Baldani, V.L.D., Goi, S., and Dobereiner, J. (1997). Recent advances in BNF with non-legume plants. Soil Biology and Biochemistry **29,** 911-922.

Barron, G. (1990). New *Cephaliophora* species capturing rotifers and tarigrades. Canadian Journal of Botany **68,** 685-690.

Basilio, A., Gonzalez, I., Vicente, M.F., Gorrochategui, J., Cabello, A., Gonzalez, A., and Genilloud, O. (2003). Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. Journal of Applied Microbiology **95,** 814-823.

Behrend, C., and Heesche-Wagner, K. (1999). Formation of hydride-Meisenheimer complexes of picric acid (2,4, 6-trinitrophenol) and 2,4-dinitrophenol during mineralization of picric acid by *Nocardioides* sp. strain CB 22-2. Applied and Environmental Microbiology **65,** 1372-1377.

Benedetti, C.E., Xie, D., and Turner, J.G. (1995). COI1-dependent expression of an Arabidopsis vegetative storage protein in flowers and siliques and in response to coronatine or methyl jasmonate. Plant Physiology **109,** 567-572.

Benhamou, N., Kloepper, J.W., Quadt-Hallman, A., and Tuzun, S. (1996). Induction of defense-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. Plant Physiol **112,** 919-929.

Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C.W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C.H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M.A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B.G., Parkhill, J., and Hopwood, D.A. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature **417,** 141-147.

Berbee, M.L. (1996). Loculoascomycete origins and evolution of filamentous ascomycete morphology based on 18S rRNA gene sequence data. Molecular Biology and Evolution **13,** 462-470.

Berbee, M.L., and Taylor, J.W. (1992). Two ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. Molecular Biology and Evolution **9,** 278-284.

Berch, S.M., and Fortin, J.A. (1983). Germination of zygospores of *Endogone pisiformis*. Mycologia **75,** 328-332.

Berdy, J. (1989). The discovery of new bioactive microbial metabolites: screening and indentification. Progress in Industrial Microbiology **27,** 3-5.

Berrocal-Lobo, M., and Molina, A. (2004). Ethylene Response Factor 1 mediates Arabidopsis resistance to the soilborne fungus *Fusarium oxysporum.* Molecular Plant Microbe Interactions **17,** 763-770.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. The Plant Journal **29,** 23-32.

Blackwood, C.B., Marsh, T., Kim, S.H., and Paul, E.A. (2003). Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Applied and Environmental Microbiology **69,** 926-932.

Bloemberg, G.V., and Lugtenberg, B.J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Current Opinion in Plant Biology **4,** 343-350.

Bockus, W.W., and Shroyer, J.P. (1998). The impact of reduced tillage on soilborne plant pathogens. Annual Review of Phytopathology **36,** 485-500.

Boosalis, M.G. (1956). Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. Phytopathology **46,** 473-478.

Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F., and Dongag, X. (1997). The CPR5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. The Plant Cell **9,** 1573-1584.

Briones, A.M., Okabe, S., Umemiya, Y., Ramsing, N.B., Reichardt, W., and Okuyama, H. (2002). Influence of different cultivars on populations of ammonia-oxidizing bacteria in the root environment of rice. Applied and Environmental Microbiology **68,** 3067-3075.

Brooks, D.S., Gonzalez, C.F.A., D. N., and Filer, T.H. (1994). Evaluation of endophytic bacteria as potential biological control agents for oak wilt. Biological Control **4,** 373-381.

Burg, R.W., Miller, B.M., Baker, E.E., Birbaum, J., Currie, S.A., Harman, R., Kong, Y.-L., Monaghan, R.L., Olsen, G., Putter, I., Tunae, J.B., Wallick, H., Stapley, E.O., Oiura, R., and Omura, S. (1979). Avermectins, a new family of potent antihelminthic agents. Producing organism and fermentation. Antimicrobial Agents and Chemotherapy **15,** 361-378.

Burgess, L.W., and Swan, L.J. (2001). Crown rot of wheat in the northern grain belt of NSW: 2000 in review. Proceedings of the Second Australasain soilborne disease symposium.

Buttner, M., and Singh, K.B. (1997). *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. Proceedings of the National Academy of Sciences USA **94,** 5961-5966.

Cameron, R.K.a., Paiva, N.L., Lamb, C.J., and Dixon, R.A. (1999). Accumulation of salicylic acid and *PR-1* gene transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. *tomato* in Arabidopsis. Physiological and Molecular Plant Pathology **55,** 121-130.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583-1592.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X. (1997). The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88,** 57-63.

CDC. (2003). Center For Disease Control and Protection. http://www. cdc.gov/ ncidod/dbmd/diseaseinfo/nocardiosis_t.htm.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., and Prasher, D. (1994). Green fluorescent protein as a marker for gene expression. Science **263,** 802-805.

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. (1993). Arabidopsis ethylene response gene ETR1: similarity of product to two-component regulators. Science **262,** 539-544.

Charrier, B., Champion, A., Henry, Y., and Kreis, M. (2002). Expression profiling of the whole Arabidopsis shaggy-like kinase multigene family by real-time reverse transcriptase-polymerase chain reaction. Plant Physiology **130,** 577-590.

Chelius, M.K., and Triplett, E.W. (2001). The diversity of archaea and bacteria in association with the roots of *Zea mays* L. Microbial Ecology **41,** 252-263.

Chen, Y., Mei, R., Lu, S., Liu, L., and Kloepper, J.W. (1996). The use of yield increasing bacteria (YIB) as plant growth-promoting rhizobacteria in Chinese agriculture. Utkhede RS and Gupta VK (eds) Management of Soil Borne Diseases**,** 165-176.

Chernin, L., Ismailov, Z., Haran, S., and Chet, I. (1995). Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Applied and Environmental Microbiology **61,** 1720-1726.

Cheung, P.Y., and Kinkle, B.K. (2001). Mycobacterium diversity and pyrene mineralization in petroleum-contaminated soils. Applied and Environmental Microbiology **67,** 2222-2229.

Ciuffetti, L.M., Tuori, R.P., and Gaventa, J.M. (1997). A single gene encodes a selective toxin causal to the development of tan spot of wheat. The Plant Cell **9,** 135-144.

Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X. (1998). Uncoupling PR gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis cpr6-1 mutant. Plant Cell **10,** 557-569.

Clay, K., Marks, S., and Cheplick, G.P. (1993). Effects of insect herbivory and fungal endophyte infection on competitive interactions among grasses. Ecology **74,** 1767-1777.

Clement, S.L., Elberson, L.R., Youssef, N.N., Davitt, C.M., and Doss, R.P. (2001). Incidence and diversity of Neotyphodium fungal endophytes in tall fescue from Morocco, Tunisia and Sardinia. Crop Science **41,** 570-576.

Coleman, N.V., Mattes, T.E., Gossett, J.M., and Spain, J.C. (2002). Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites. Applied and Environmental Microbiology **68,** 6162-6171.

Conn, V.M., and Franco, C.M.M. (2004a). Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by Terminal Restriction Fragment Length Polymorphism and sequencing of 16S rRNA clones. Applied and Environmental Microbiology **70,** 1787-1794.

Conn, V.M., and Franco, C.M.M. (2004b). Effect of microbial inoculants on the indigenous actinobacterial endophyte population in the roots of wheat as determined by Terminal Restriction Fragment Length Polymorphism. Applied and Environmental Microbiology **70,** 6407-6413.

Conrath, U., Pieterse, C.M., and Mauch-Mani, B. (2002). Priming in plant-pathogen interactions. Trends Plant Science **7,** 210-216.

Cook, R.J. (2001). Management of wheat and barley root diseases in modern farming systems. Australasian Plant Pathology **30,** 119-126.

Cook, R.J. (2003). Take-all of wheat. Physiological and Molecular Plant Pathology **62,** 73-86.

Cook, R.J., Thomashow, L.S., Weller, D.M., Fujimoto, D., Mazzola, M., Bangera, G., and Kim, D.S. (1995). Molecular mechanisms of defense by rhizobacteria against root disease. Proceedings of the National Academy of Sciences USA **92,** 4197-4201.

Coombs, J.T. (2002). The isolation and characterisation of endophytic actinomycetes from wheat (*Triticum Aestivum*). In Department of Medical Biotechnology (Adelaide: Flinders University), pp. 273.

Coombs, J.T., and Franco, C.M. (2003a). Isolation and identification of actinobacteria from surface-sterilized wheat roots. Applied and Environmental Microbiology **69,** 5603-5608.

Coombs, J.T., and Franco, C.M. (2003b). Visualization of an endophytic *Streptomyces* species in wheat seed. Applied and Environmental Microbiology **69,** 4260-4262.

Coombs, J.T., Franco, C.M., and Loria, R. (2003). Complete sequencing and analysis of pEN2701, a novel 13-kb plasmid from an endophytic *Streptomyces* sp. Plasmid **49,** 86-92.

Coombs, J.T., Michelsen, P.P., and Franco, C.M.M. (2004). Evaluation of endophytic actinobacteria as antagonists of *Gaeumannomyces graminis* var. *tritici* in wheat. Biological Control **29,** 359-366.

Covert, T.C., Rodgers, M.R., Reyes, A.L., and Stelma, G.N., Jr. (1999). Occurrence of nontuberculous mycobacteria in environmental samples. Applied and Environmental Microbiology **65,** 2492-2496.

Crawford, D.L., Lynch, J.M., Whipps, J.M., and Ousley, M.A. (1993). Isolation and characterization of actinomycete antagonists of a fungal root pathogen. Applied and Environmental Microbiology **59,** 3899-3905.

CRCTP, C.R.C.f.T.P.P. (2002). Wheat. http://www.tpp.uq.edu.au/commo/wheat.htm.

Creelman, R.A., and Mullet, J.E. (1997). Biosynthesis and action of jasmonates in plants. Annual Review of Plant Physiology and Plant Molecular Biology **48,** 355-381.

Deacon, J. (2001). The microbial world: biology and control of take-all disease. http://helios.bto.ed.ac.uk/bto/microbes/takeall.htm#Top.

Dean, R.A. (1997). Signal pathways and appressorium morphogenesis. Annual Review of Phytopathology **35,** 211-234.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science **266,** 1247-1250.

Devadas, S.K., Enyedi, A., and Raina, R. (2002). The Arabidopsis hrl1 mutation reveals novel overlapping roles for salicylic acid, jasmonic acid and ethylene signalling in cell death and defence against pathogens. The Plant Journal **30,** 467-480.

Dickson, J.G. (1956). Diseases of field crops. (New York: McGraw Hill).

Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A., and Dangl, J.L. (1994). Arabidopsis mutants simulating disease resistance response. Cell **77,** 565-577.

Dong, X., and Durrant, W.E. (2004). Systemic acquired resistance. Annual Review of Phytopathology **42,** 185-209.

Dong, Y., Iniguez, A.L., Ahmer, B.M., and Triplett, E.W. (2003). Kinetics and strain specificity of rhizosphere and endophytic colonization by enteric bacteria on seedlings of *Medicago sativa* and *Medicago truncatula.* Applied and Environmental Microbiology **69,** 1783-1790.

Dong, Z., McCully, M.E., and Canny, M.J. (1997). Does *Acetobacter diazotrophicus* live and move in the xylem of sugarcane stems? Anatomical and physiological data. Annals of Botany **80,** 147-158.

Dong, Z., Heydrich, M., Bernard, K., and McCully, M.E. (1995). Further Evidence that the N2-Fixing Endophytic Bacterium from the Intercellular Spaces of Sugarcane Stems Is Acetobacter diazotrophicus. Applied and Environmental Microbiology **61,** 1843-1846.

Dong, Z., Canny, M.J., McCully, M.E., Roboredo, M., Cabadilla, C.F., Ortega, E., and Rodés, R. (1994). A nitrogen-fixing endophyte of sugarcane stems. Plant Physiology **105,** 1139-1147.

Doumbou, C., Akimov, V., Cote, M., Charest, P., Beaulieu, C. (2001). Taxonomic study on nonpathogenic streptomycetes isolated from common scab lesions on potato tubers. Systematic Applied Microbiolgoy **24,** 451-456.

Dufour, R. (2001). Biointensive integrated pest management (IPM). http://www.attra.ncat.org/attra-pub/PDF/ipm.pdf.

Duijff, B.J., Alabouvette, C., and Lemanceau, P. (1996). Involvement of induced systemic resistance in the control of fusarium wilt of tomato by *Fusarium oxysporum* strain FO47 and *Pseudomonas fluorescens* strain WCS417r. Int. Organ. Biol. Integrated Control Noxious Anim. Plants/West Palaearctic Reg. Sec. Bull. **19,** 120-124.

Duineveld, B.M., Kowalchuk, G.A., Keijzer, A., van Elsas, J.D., and van Veen, J.A. (2001). Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. Applied and Environmental Microbiology **67,** 172-178.

Dunbar, J., Ticknor, L.O., and Kuske, C.R. (2000). Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Applied and Environmental Microbiology **66,** 2943-2950.

Dunfield, K.E., and Germida, J.J. (2001). Diversity of bacterial communities inteh rhizosphere and root interior of field-grown genetically modified *Brassica napus*. FEMS Microbiology Ecology **38,** 1-9.

Ecker, J.R. (1995). The ethylene signal transduction pathway. Science **268,** 667-675.

Egert, M., and Friedrich, M.W. (2003). Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. Applied and Environmental Microbiology **69,** 2555-2562.

El-Tarabily, K.A. (2003). An endophytic chitinase-producing isolate of *Actinoplanes missouriensis*, with potential for biological control of root rot of lupin caused by *Plectosporium tabacinum*. Australian Journal of Botany **51,** 257-266.

El-Tarabily, K.A. (2004). Suppression of *Rhizoctonia solani* diseases of sugar beet by antagonistic and plant growth-promoting yeasts. Journal of Applied Microbiology **96,** 69-75.

El-Tarabily, K.A., Hardy, G.E.J., Sivasithamparam, K., Hussein, A.M., and Kurtboke, D.I. (1997). The potential for the biological control of cavity-spot disease of carrots, caused by *Pythium coloratum*, by streptomycete and non-streptomycete actinomycetes. New Phytologist **137,** 495-507.

El-Tarabily, K.A., Soliman, M.H., Nassar, A.H., Al-Hassani, H.A., Sivasithamparam, K., McKenna, F., and St. J. Hardy, G.E. (2000). Biological control of *Sclerotinia minor* using chinolytic bacterium and actinomycetes. Plant Pathology **49,** 573-583.

Elbeltagy, A., Nishioka, K., Sato, T., Suzuki, H., Ye, B., Hamada, T., Isawa, T., Mitsui, H., and Minamisawa, K. (2001). Endophytic colonization and in

planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Applied and Environmental Microbiology **67,** 5285-5293.

Ellis, C., and Turner, J.G. (2001). The Arabidopsis mutant cev1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. Plant Cell **13,** 1025-1033.

Embley, T.M., and Stackebrandt, E. (1994). The molecular phylogency and systematics of the actinomycetes. Annual Review of Microbiology **48,** 257-289.

Englund, S., Bolske, G., and Johansson, K.E. (2002). An IS900-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. FEMS Microbiology Letters **209,** 267-271.

Epple, P., Apel, K., and Bohlmann, H. (1995). An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol **109,** 813-820.

Epple, P., Apel, K., and Bohlmannl, H. (1997). Overexpression of an endogenous thionin enhances resistance of Arabidopsis against *Fusarium oxysporum.* The Plant Cell **9,** 509-520.

Ernst, M., Mendgen, K.W., and Wirsel, S.G.R. (2003). Endophytic fungal mutualists: seed-borne *Stagonospora* spp. enhance reed biomass production in axenic microcosms. Molecular Plant Microbe Interactions **16,** 580.587.

Feys, B.J.F., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. The Plant Cell **6,** 751-759.

Franco, C.M., and Coutinho, L.E. (1991). Detection of novel secondary metabolites. Critical Reviews in Biotechnology **11,** 193-276.

Fravel, D.R., Kim, K.K., and Papavizas, G.C. (1987). Viability of microsclerotia of *Verticillium dahliae* reduced by a metabolite produced by *Talaromyces flavus*. Phytopathology **77,** 616-619.

Gadkar, V., David-Schwartz, R., Kunik, T., and Kapulnik, Y. (2001). Arbuscular mycorrhizal fungal colonization. Factors involved in host recognition. Plant Physiology **127,** 1493-1499.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261,** 754-756.

Garbeva, P., van Overbeek, L.S., van Vuurde, J.W.L., and van Elsas, J.D. (2001). Analysis of endophytic bacterial communities of potato by plating and Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rDNA based PCR fragments. Microbial Ecology **41,** 369-383.

Garcia-Garrido, J.M., Tribak, M., Rejon-Palomares, A., Ocampo, J.A., and Garcia-Romera, I. (2000). Hydrolytic enzymes and ability of arbuscular mycorrhizal fungi to colonize roots. Journal of Experimental Botany **51,** 1443-1448.

Garcia-Olmedo, F., Rodriguez-Palenzuela, P., Hernandez-Lucas, C., Ponz, F., Marana, C., Carmona, M.J., Lopez-Fando, J., Fernandez, J.A., and Carbonero, P. (1989). The thionins: A protein family that includes purothionins, viscotoxins and crambins. Oxford Surveys of Plant Molecular and Cell Biology **6,** 31-60.

Gardner, P.A., Angus, J.F., Pitson, G.D., and Wong, P.T.W. (1998). A comparison of six methods to control take-all in wheat. Australian Journal of Agricultural Research **49,** 1225-1240.

Geraats, B.P., Bakker, P.A., and van Loon, L.C. (2002). Ethylene insensitivity impairs resistance to soilborne pathogens in tobacco and *Arabidopsis thaliana*. Molecular Plant Microbe Interactions **15,** 1078-1085.

Germida, J.J., Sicilliano, S.D., de Freitas, J.R., and Seib, A.M. (1998). Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). FEMS Microbiology Ecology **26,** 43-49.

Girlanda, M., Ghignone, S., and Luppi, A.M. (2002). Diversity of sterile root-associated fungi of two Mediterranean plants. New Phytologist **155,** 481-498.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual Review of Phytopathology**,** 9.1-9.23.

Glazebrook, J., and Ausubel, F.M. (1994). Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proceedings of the National Academy of Sciences USA **91,** 8955-8959.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1996). Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics **143,** 973-982.

Glick, B.R., Penrose, D.M., and Ma, W. (2001). Bacterial promotion of plant growth. Biotechnology Advances **19,** 135-138.

Goetz, M.A., McCormick, P.A., Monaghan, R.L., Ostlind, D.A., Hensens, O.D., and Liesch, J.M.A.-S., G. (1985). L-155175: A new antiparasitic macrolide: Fermentation, isolation and structure. Journal of Antibiotics **38,** 161-168.

Goodwin, A., Lumb, R., Patkin, M., and Bastian, I. (1998). Isolation of a novel mycobacterium from an adolescent with cervical lymphadenitis. Eur J Clin Microbiol Infect Dis **17,** 516-518.

Gorlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H., and Ryals, J. (1996). Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell **8,** 629-643.

Greenberg, J.T., Guo, A., Klessig, D.F., and Ausubel, F.M. (1994). Programmed cell death in plants: a pathogen-triggered response activated with multiple defense functions. Cell **77,** 551-563.

Guzman, P.E., J. R. (1990). Exploiting the triple response of Arabídopsís to identify ethylene-related mutants. The Plant Cell **2,** 513-523.

Gyaneshwar, P., James, E.K., Mathan, N., Reddy, P.M., Reinhold-Hurek, B., and Ladha, J.K. (2001). Endophytic colonization of rice by a diazotrophic strain of *Serratia marcescens*. Journal of Bacteriology **183,** 2634-2645.

Haas, W.H., Kirschner, P., Ziesing, S., Bremer, H.J., and Bottger, E.C. (1993). Cervical lymphadenitis in a child caused by a previously unknown mycobacterium. The Journal of Infectious Diseases**,** 237-240.

Hallmann, J. (2001). Plant interactions with endophytic bacteria. Biotic Interactions in Plant-Pathogen Associations.**,** 87-119.

Hallmann, J., Rodriguez-Kabana, R., Kloepper, J. W. (1999). Chitin-mediated changes in bacterial communities of the soil, rhizosphere and within roots of cotton in relation to nematode control. Soil Biology and Biochemistry **31,** 551-W.

Hallmann, J., Rodriguez-Kabana, R., Kloepper, J. W., Quadt-Hallmann, A., Mahaffee, W.F., and Kloepper, J.W. (1997). Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology **43,** 895-914.

Hammerschmidt, R. (1999). Induced disease resistance: how do induced plants stop pathogens? Physiological and Molecular Plant Pathology **55**.

Harrier, L.A. (2001). The arbuscular mycorrhizal symbiosis: a molecular review of the fungal dimension. Journal of Experimental Botany **52,** 469-478.

Harrington, F.A., Pfister, D.H., Potter, D., and Donoghue, M. (1999). 18S rRNA sequence data and classification. Mycologia **91,** 41-50.

Harrison, M.J. (1999). Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annual Review of Plant Physiology and Plant Molecular Biology **50,** 361-389.

Harvey, P., and Hawke, B.G. (2002). Little-known Pythium disease stunts crop growth. Farming Ahead **125,** 42-43.

Heil, M., and Bostock, R.M. (2002). Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. Ann Bot (Lond) **89,** 503-512.

Heuer, H., Krsek, M., Baker, P., Smalla, K., and Wellington, E.M. (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Applied and Environmental Microbiology **63,** 3233-3241.

Heuer, H., Hartung, K., Wieland, G., Kramer, I., and Smalla, K. (1999). Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. Applied and Environmental Microbiology **65,** 1045-1049.

Hirsch, A.M., and Kapulnik, Y. (1998). Signal transduction pathways in mycorrhizal associations: comparisons with the rhizobium-legume symbiosis. Fungal Genet Biol **23,** 205-212.

Hirsch, A.M., Lum, M.R., and Downie, J.A. (2001). What makes the rhizobia-legume symbiosis so special? Plant Physiology **127,** 1484-1492.

Hirsch, P., Mevs, U., Kroppenstedt, R.M., Schumann, P., and Stackebrandt, E. (2004). Cryptoendolithic actinomycetes from antarctic sandstone rock samples: *Micromonospora endolithica* sp. nov. and two isolates related to *Micromonospora coerulea* Jensen 1932. Systematic Applied Microbiolgoy **27,** 166-174.

Hogan, J., Barrett, D., Berry, P., Lawrance, L., Connell, P., and Mollard, W. (2004). Australian crop report (Canberra: Australian Bureau of Agricultural and Resource Economics).

Hoisington, D., Khairallah, M., Reeves, T., Ribaut, J.M., Skovmand, B., Taba, S., and Warburton, M. (1999). Plant genetic resources: what can they contribute toward increased crop productivity? Proceedings of the National Academy of Sciences USA **96,** 5937-5943.

Hoitink, H., and Boehm, M. (1999). Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. Annual Review of Phytopathology **37,** 427-446.

Hopwood, D.A. (2003). The *Streptomyces* genome-be prepared! Nature Biotechnology **21,** 505-506.

Horowitz, S., Freeman, S., and A., S. (2002). Use of green fluorescent protein-transgenic strains to study pathogenic and non-pathogenic lifestyles in *Colletotrichum acutatum*. Phytopathology **92,** 743-749.

Iniguez, L.A., Dong, Y., Carter, H.D., Ahmer, B.M.M., Stone, J.M., and Triplett, E.W. (2005). Regulation of enteric endophytic bacterial colonization by plant defenses. Molecular Plant Microbe Interactions **18,** 169-178.

James, E.K., Reis, V.N., Olivares, F.L.B., J. L., and Dobereiner, J. (1994). Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus.* Journal of Experimental Botany **45,** 757-766.

Kachroo, P., Kachroo, A., Lapchyk, L., Hildebrand, D., and Klessig, D.F. (2003). Restoration of defective cross talk in ssi2 mutants: role of salicylic acid, jasmonic acid, and fatty acids in SSI2-mediated signaling. Molecular Plant Microbe Interactions **16,** 1022-1029.

Kahmann, R., and Basse, C. (2001). Fungal gene expression during pathogenesis-related development and host plant colonization. Current Opinion in Microbiology **4,** 374-380.

Kent, A.D., and Triplett, E.W. (2002). Microbial communities and their interactions in soil and rhizosphere. Annual Review of Microbiology**,** 012302.161120.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical *Streptomyces* genetics. (The John Innes Foundation, Norwich).

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. Plant Cell **12,** 2339-2350.

Klamer, M., Roberts, M.S., Levine, L.H., Drake, B.G., and Garland, J.L. (2002). Influence of elevated CO(2) on the fungal community in a coastal scrub oak forest soil investigated with terminal-restriction fragment length polymorphism analysis. Applied and Environmental Microbiology **68,** 4370-4376.

Kloepper, J.W., Rodriguez-Kabana, R., Zehnder, G.W., Murphy, J.F., Sikora, E., and Fernandez, C. (1999). Plant root-bacterial interactions in biological control of soilborne diseases and potential extension to systemic and foliar diseases. Australasian Plant Pathology **28,** 21-26.

Kmcel, A., Mauch, F., Winzeler, M., Winzeler, H., and Dudler, R. (1995). Quantative field resistance of wheat to powdery mildew and defense reactions at the seedling stage: identification of a potential marker. Physiological and Molecular Plant Pathology **47,** 185-199.

Koch, C., Kroppenstedt, R.M., and Stackebrandt, E. (1996). Intragenetic relationships of the Actinomycete genus *Micromonospora*. International Journal of Systematic Bacteriology **46,** 383-387.

Kuske, C.R., Barns, S.M., and Busch, J.D. (1997). Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. Applied and Environmental Microbiology **63,** 3614-3621.

Lamb, T.G., Tonkyn, D.W., and Kluepfel, D.A. (1996). Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. Canadian Journal of Microbiology **42,** 1112-1120.

Landvik, S., Egger, K.N., and Schumacher, T. (1997). Towards a subordinal classification of the Pezizales (Ascomycota): phylogenetic analyses of SSU rRNA sequences. Nordic Journal of Botany **17,** 403-418.

Larran, S., Perello, A.S., M.R., and Moreno, V. (2002). Isolation and analysis of endophytic microorganisms in wheat (*Triticum aestivum* L.) leaves. World Journal of Microbiology and Biotechnology **18,** 683-686.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. Molecular Plant Microbe Interactions **8,** 863-870.

Lawton, K.A., Potter, S.L., Uknes, S., and Ryals, J. (1994). Acquired resistance signal transduction in Arabidopsis is ethylene independent. The Plant Cell **6,** 581-588.

Le Dantec, C., Duguet, J.P., Montiel, A., Dumoutier, N., Dubrou, S., and Vincent, V. (2002). Occurrence of mycobacteria in water treatment lines and in water distribution systems. Applied and Environmental Microbiology **68,** 5318-5325.

Lechevalier, M.P. (1989). Actinomycetes in Agriculture and Forestry. In Actinomycetes in Biotechnology. Goodfellow, M., Williams, S. T., and Modarshki, M. (eds)**,** 327--358.

Leeman, M., van Pelt, J.A., den Ouden, F.M., Heinsbroek, M., Bakker, P.A.H.M., and Schippers, B. (1995). Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. European Journal of Plant Pathology **101,** 655-664.

Liew, E.C., Aptroot, A., and Hyde, K.D. (2000). Phylogenetic significance of the pseudoparaphyses in *Loculoascomycete* taxonomy. Molecular Phylogenetics and Evolution **16,** 392-402.

Lipps, P.E. (1996). Take-all disease of wheat. http://ohioline.osu.edu/ac-fact/0001.html.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25,** 402-408.

Lopez-Garcia, P., Philippe, H., Gail, F., and Moreira, D. (2003). Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge. Proceedings of the National Academy of Sciences USA **100,** 697-702.

Lorenzo, O., Piqueras, R., Sánchez-Serrano, J.J., and Solano, R. (2003). Ethylene Response Factor 1 integrates signals from ethylene and jasmonate pathways in plant defense. The Plant Cell **15,** 165-178.

Loria, R., Bukhalid, R.A., Creath, R.A., Leiner, R.J., Olivier, M., and Steffens, J.C. (1995). Differential production of thaxtomins by pathogenic *Streptomyces* species *In vitro*. Phytopathology **85,** 537-541.

Lukow, T., Dunfield, P.F., and Liesack, W. (2000). Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiology Ecology **32,** 241-247.

Lumb, R., Goodwin, A., Ratcliff, R., Stapledon, R., Holland, A., and Bastian, I. (1997). Phenotypic and molecular characterization of three clinical isolates of *Mycobacterium interjectum*. J Clin Microbiol **35,** 2782-2785.

Lumbsch, T.H., and Lindemuth, R. (2001). Major lineages of *Dothideomycetes (Ascomycota)* inferred from SSU and LSU rDNA sequences. Mycological Research **105,** 901-908.

Maidak, B.L., Cole, J.R., Parker, C.T., Garrity, G.M.J., Larsen, N., Li, B., T. G. Lilburn, McCaughey, M.J., Olsen, G.J., Overbeek, R., Pramanik, S., Schmidt, T.M., Tiedje, J.M., and Woese, C.R. (1999). A new version of the RDP [Ribosomal Database Project]. Nucleic Acids Research **27,** 171-173.

Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J., and Cameron, R.K. (2002). A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. Nature **419,** 399-403.

Maldonado, L.A., Stainsby, F.M., Ward, A.C., and Goodfellow, M. (2003). *Gordonia sinesedis* sp. nov., a novel soil isolate. Antonie Van Leeuwenhoek **83,** 75-80.

Manners, J.M., Penninckx, I.A., K., V., Kazan, K., Brown, R.L., Morgan, A., Maclean, D.J., Curtis, M.D., Cammue, B.P.A., and Broekaert, W.F. (1998). The promoter of the plant defensin gene *PDF1.2* from Arabidopsis is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. Plant Molecular Biology **38,** 1071-1080.

Manning, B., Southwell, R., Hayman, P., and Moore, K. (2000). Fusarium head blight in northern NSW. NSW Agriculture Research Update.

Marsh, T.L., Saxman, P., Cole, J., and Tiedje, J. (2000). Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Applied and Environmental Microbiology **66,** 3616-3620.

Mayfield, C.I., Williams, S.T., Ruddick, S.M., and Hatfield, H.L. (1972). Studies on the ecology of Actinomycetes in soil IV. Observations on the form and growth of *Streptomyces* in soil. Soil Biology and Biochemistry **4,** 79-91.

McCully, M.E. (2001). Niches for bacterial endophytes in crop plants: a plant biologist's view. Australian Journal of Plant Physiology **28,** 983-900.

McCully, M.E., Shane, M.W., Baker, A.N., Huangs, C.X., Lings, L.E.C., and Canny, M.J. (2000). The reliability of cryoSEM for the observation and quantification of xylem embolisms and quantitative analysis of xylem sap *in situ*. Journal of Microscopy. **198,** 24-33.

McInroy, J.A., and Kloepper, J.W. (1995). Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. Can. J. Microbiol. **41**.

McLaren, D.L., Huang, H.C., Kozub, G.C., and Rimmer, S.R. (1994). Biological control of Sclerotinia wilt of sunflower with *Talaromyces flavus* and *Coniothyrium minitans*. Plant Disease **78,** 231-235.

McSpadden Gardener, B.B., and Weller, D.M. (2001). Changes in populations of rhizosphere bacteria associated with take-all disease of wheat. Applied and Environmental Microbiology **67,** 4414-4425.

Mendgen, K., Hahn, M., and Deising, H. (1996). Morphogenesis and mechanisms of penetration by plant pathogenic fungi. Annual Review of Phytopathology **34,** 367-386.

Miche, L., and Balandreau, J. (2001). Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. Applied and Environmental Microbiology **67,** 3046-3052.

Miles, A.A., and Misra, S.S. (1938). The estimation of the bactericidal activity of blood. Journal of Hygiene **38,** 732-749.

Miles, C.O., di Menna, M.E., Jacobs, S.W., Garthwaite, I., Lane, G.A., Prestidge, R.A., Marshall, S.L., Wilkinson, H.H., Schardl, C.L., Ball, O.J., and Latch, G.C. (1998). Endophytic fungi in indigenous Australasian grasses associated with toxicity to livestock. Applied and Environmental Microbiology **64,** 601-606.

Miller, H.J., Henken, G., and van Veen, J.A. (1989). Variation and composition of bacterial populations in the rhizosphere of maize, wheat, and grass cultivars. Can. J. Microbiol. **35**.

Miyadoh, S.e. (1997). Atlas of Actinomycetes.

Molina, A., Gorlach, J., Volrath, S., and Ryals, J. (1999). Wheat genes encoding two types of PR-1 proteins are pathogen inducible, but do not respond to activators of systemic aquired resistance. Molecular Plant Microbe Interactions **12,** 53-58.

Moller, S.G., and Chua, N.H. (1999). Interactions and intersections of plant signaling pathways. Journal of Molecular Biology **293,** 219-234.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113,** 935-944.

Mucciarelli, M., Scannerini, S., Bertea, C., and Maffei, M. (2002). An ascomycetous endophyte isolated from *Mentha piperita* L.: biological features and molecular studies. Mycologia **94,** 28-39.

Murray, F., Llewellyn, D., McFadden, H., Last, D., Dennis, E.S., and Peacock, J.W. (1999). Expression of the *Talaromyces flavus* glucose oxidase gene in cotton and tobacco reduces fungal infection, but is also phytotoxic. Molecular Breeding **5,** 219-232.

Muyzer, G., de Waal, E.C., and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology **59,** 695-700.

Nandi, A., Kachroo, P., Fukushige, H., Hildebrand, D.F., Klessig, D.F., and Shah, J. (2003). Ethylene and jasmonic acid signaling affect the NPR1-independent expression of defense genes without impacting resistance to *Pseudomonas syringae* and *Peronospora parasitica* in the Arabidopsis ssi1 mutant. Molecular Plant Microbe Interactions **16,** 588-599.

Nawrath, C., and Metraux, J.P. (1999). Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11,** 1393-1404.

Neeno-Eckwall, E.C., and Schottel, J.L. (1999). Occurance of antibiotic resistance in the biological control of potato scab disease. Biological Control **16,** 199-208.

Nejad, P., and Johnson, P.A. (2000). Endophytic bacteria induce growth promotion and wilt disease suppression in oilseed rape and tomato. Biological Control **18,** 208-215.

Nogales, B., Moore, E.R., Llobet-Brossa, E., Rossello-Mora, R., Amann, R., and Timmis, K.N. (2001). Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Applied and Environmental Microbiology **67,** 1874-1884.

Nolan, R.D., and Cross, T. (1988). Isolation and screening of actinomycetes. In Actinomycetes in Biotechnology. Goodfellow, M. Williams, S. T., Modarski, M. (eds).

Norman-Setterblad, C., Vidal, S., and Palva, E.T. (2000). Interacting signal pathways control defense gene expression in Arabidopsis in response to cell wall-degrading enzymes from *Erwinia carotovora*. Molecular Plant Microbe Interactions **13,** 430-438.

Norman, C., Vidal, S., and Palva, E.T. (1999). Oligogalacturonide-mediated induction of a gene involved in jasmonic acid synthesis in response to the cell-wall-degrading enzymes of the plant pathogen *Erwinia carotovora*. Molecular Plant Microbe Interactions **12,** 640-644.

Onate-Sanchez, L., and Singh, K.B. (2002). Identification of Arabidopsis ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection. Plant Physiology **128,** 1313-1322.

Osborn, A.M., Moore, E.R., and Timmis, K.N. (2000). An evaluation of terminal-restriction fragment length polymorphism (T- RFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology **2,** 39-50.

Park, K.S., and Kloepper, J.W. (2000). Activation of PR-1a promoter by rhizobacteria that induce systemic resistance in tobacco against *Pseudomonas syringae* pv. *tabaci*. Biological Control. **18,** 2-9.

Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J. (1996). Characterization of eds1, a mutation in Arabidopsis suppressing resistance to to *Peronospora parasitica* specified by several different RPP genes. Plant Cell **8,** 2033-2046.

Patten, C.L., and Glick, B.R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Applied and Environmental Microbiology **68,** 3795-3801.

Paulitz, T.C., and Belanger, R.R. (2001). Biological control in greenhouse systems. Annual Review of Phytopathology **39,** 103-133.

Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M., and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. Plant Cell **8,** 2309-2323.

Penrose, D.M., Moffat, B.A., and Glick, B.R. (2001). Determination of 1-aminocyclopropane-1-carboxylic acid (ACC) to assess the effects of ACC deaminase-containing bacteria on roots of canola seedlings. Canadian Journal of Microbiology **47,** 77-80.

Perret, X., Staehelin, C., and Broughton, W.J. (2000). Molecular basis of symbiotic promiscuity. Microbiol Mol Biol Rev **64,** 180-201.

Peterson, R.R. (1965). Wheat. Botany, cultivation, and utilization. (New York, U.S.A: Interscience Publishers Inc.).

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research **29,** e45.

Pierson, E.A., and Weller, D.M. (1994). Use of mixtures of fluorescent pseudomonads to suppress take-all and improve the growth of wheat. Phytopathology **84,** 940-947.

Pierson, L.S., and Pierson, E.A. (2000). Microbial gossiping: signalling in the rhizosphere. Proceedings of 5th PGPR Workshop.

Pieterse, C.M., van Wees, S.C., Hoffland, E., van Pelt, J.A., and van Loon, L.C. (1996). Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell **8,** 1225-1237.

Pieterse, C.M., van Wees, S.C., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., and van Loon, L.C. (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell **10,** 1571-1580.

Pieterse, C.M.J., van Wees, S. C. M., Ton, J., van Pelt, J. A. and van Loon, L. C. (2002). Signalling in rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. Plant Biology **4**.

Piret, J.M., and Demain, A.L. (1989). Actinomycetes in Biotechnology.

Provorov, N.A., Borisov, A.Y., and Tikhonovich, I.A. (2002). Developmental genetics and evolution of symbiotic structures in nitrogen-fixing nodules and arbuscular mycorrhiza. Journal of Theoretical Biology **214,** 215-232.

Raajimakers, J.S., and Weller, D.M. (1998). Natural plant protection by 2,4-diacetylphloroglucinol-producing *Psuedomonas* spp.in take-all decline soils. Molecular Plant-Microbe Interactions **11,** 144-152.

Rainey, F.A., Klatte, S., Kroppenstedt, R.M., and Stackebrandt, E. (1995). *Dietzia*, a new genus including *Dietzia maris* comb. nov., formerly *Rhodococcus maris*. Int J Syst Bacteriol **45,** 32-36.

Rairdan, G.J., and Delaney, T.P. (2002). Role of salicylic acid and NIM1/NPR1 in race-specific resistance in Arabidopsis. Genetics **161,** 803-811.

Rajeevan, M.S., Ranamukhaarachchi, D.G., Vernon, S.D., and Unger, E.R. (2001). Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. Methods-Orlando **45,** 443-451.

Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S., and Greenberg, J.T. (1999). The gain-of-function Arabidopsis acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. The Plant Cell**,** 1695-1708.

Rathmell, B. (2001). QWCRC Media Release Press Kit. http://www.wheat-research.com.au/media/media_releases/QWCRC_Press_Kit.pdf. Quality Wheat Cooperative Research Centre Website.

Reis, V.W., Olivares, F.L., and Dobereiner, J. (1994). Improved methodology for isolation of *Acetobacter diazotrophicus* and conformation of its endophytic habitat. World Journal of Microbiology and Biochemistry **10,** 401-405.

Reiter, B., Pfeifer, U., Schwab, H., and Sessitsch, A. (2002). Response of endophytic bacterial communities in potato plants to infection with *Erwinia carotovora* subsp. *atroseptica*. Applied and Environmental Microbiology **68,** 2261-2268.

Rogers, E.E., and Ausubel, F.M. (1997). Arabidopsis enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. Plant Cell **9,** 305-316.

Roget, D.K., and Rovira, A.D. (1991). The relationship between incidence of infection by the take-all fungus (*Gaeumannomyces graminis* var. *tritici*), rainfall and yield of wheat in South Australia. Australian Journal of Experimental Agriculture **31,** 509-513.

Ross, A.F. (1961). Systemic acquired resistance induced by localized virus infections in plants. Virology **14,** 340-358.

Rossello-Mora, R., and Amann, R. (2001). The species concept for prokaryotes. FEMS Microbiology Reviews **25,** 39-67.

Roth, A., Reischl, U., Streubel, A., Naumann, L., Kroppenstedt, R.M., Habicht, M., Fischer, M., and Mauch, H. (2000). Novel diagnostic algorithm for identification of mycobacteria using genus-specific amplification of the 16S-23S rRNA gene spacer and restriction endonucleases. Journal of Clinical Microbiology **38,** 1094-1104.

Rovira, A.D. (1986). Influence of crop rotation and tillage on Rhizoctonia bare patch of wheat. Phytopathology **76,** 669-673.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.-Y., and Hunt, M.D. (1996). Systemic Acquired Resistance. The Plant Cell **8,** 1809-1819.

Ryu, C., Farag, M.A., Hu, C., Reddy, M.S., Kloepper, J.W., and Pare, P.W. (2004). Bacterial volatiles induce systemic resistance in Arabidopsis. Plant Physiology **134,** 1017-1026.

Saikkonen, K., Faeth, S.H., Helander, M., and Sullivan, T.J. (1998). Fungal endophytes: a continuum of interactions with host plants. Annual Review of Ecology and Syst. **29,** 319-343.

Sardi, P., Saracchi, M., Quaroni, S., Petrolini, B., Borgonovi, E., and Merli, S. (1992). Isolation of endophytic *Streptromyces* strains from surface-sterilized roots. Applied and Environmental Microbiology **58,** 2691-2693.

Schaffrath, U., Freydl, E., and Dudler, R. (1997). Evidence for different signalling pathways activated by inducers of acquired resistance in wheat. Molecular Plant Microbe Interactions **10,** 779-783.

Schardl, C.L., Leuchtmann, A., and Spiering, M.J. (2004). Symbiosis of grasses with seedborne fungal endophytes. Annual Review of Phytopathology **55,** 315-340.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M. (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proceedings of the National Academy of Sciences USA **97,** 11655-11660.

Schippers, B., Bakker, A., and Bakker, P. (1987). Interactions Of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annual Review of Phytopathology **25,** 339.

Scholthof, H.B. (2001). Molecular plant-microbe interactions that cut the mustard. Plant Physiology **127,** 1476-1483.

Schottel, J.L., Shimizu, K., and Kinkel, L.L. (2001). Relationships of *in vitro* pathogen inhibition and soil colonization to potato scab biocontrol antagonistic *Streptomyces* spp. Biological Control **20,** 102-112.

Schultze, M., and Kondorosi, A. (1998). Regulation of symbiotic root nodule development. Annu Rev Genet **32,** 33-57.

Sessitsch, A., Reiter, B., and Berg, G. (2004). Endophytic bacterial communities of field-grown potato plants and their plant-growth -promoting and antagonistic abilities. Canadian Journal of Microbiology **50,** 239-249.

Sessitsch, A., Reiter, B., Pfeifer, U., and Wilhelm, E. (2001). Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and Actinomycetes-specific PCR of 16S rRNA genes. FEMS Microbiology Ecology **1305,** 1-10.

Sevilla, M., Burris, R.H., Gunapala, N., and Kennedy, C. (2001). Comparison of benefit to sugarcane plant growth and 15N2 incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and Nif- mutants strains. Molecular Plant Microbe Interactions **14,** 358-366.

Shah, J., Kachroo, P., and Klessig, D.F. (1999). The Arabidopsis SSI1 mutation restores pathogenesis-related gene expression in NPR1 plants and renders defensin gene expression salicylic acid dependent. Plant Cell **11,** 191-206.

Shimizu, M., Nakagawa, Y., Sato, Y., Furumai, T., Igarashi, Y., Onaka, H., Yoshida, R., and Kunoh, H. (2000). Studies on endophytic actinomycetes (I) *Streptomyces* sp. isolated from rhododendron and its antifungal activity. J. Gen. Plant Pathol. **66,** 360-366.

Siciliano, S.D., Theoret, C.M., de Freitas, J.R., Huci, P.J., and Germida, J.J. (1998). Differences in the microbial communities associated with the roots of different cultivars of canola and wheat. Can. J. Microbiol. **44,** 844-851.

Siciliano, S.D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., Ouellette, D., Roy, R., Whyte, L.G., Banks, M.K., Schwab, P., Lee, K., and Greer, C.W. (2001). Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Applied and Environmental Microbiology **67,** 2469-2475.

Sieber, T., Riesen, T.K., Muller, E., and Fried, P.M. (1988). Endophytic fungi in four winter wheat cultivars (*Triticum aestivum* L.) differing in resistance against *Stagonospora nodorum* (Berk.) Cast. and germ.= *Septoria nodorum* (Berk.) Berk. Journal of Phytopathology **122,** 289-306.

Simon, L., Lalonde, M., and Bruns, T.D. (1992). Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Applied and Environmental Microbiology **58,** 291-295.

Simpfendorfer, S., Kirkegaard, J.A., and Heenan, D.P. (1999). Soil microorganisms limit the early growth of direct drilled wheat in south-eastern Australia. Proceedings of the First Australasian Soilborne Disease Symposium, Magarey, R.C. (ed) Gold Coast, Australia.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., and Berg, G. (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Applied and Environmental Microbiology **67,** 4742-4751.

Smit, E., Leeflang, P., Glandorf, B., van Elsas, J.D., and Wernars, K. (1999). Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Applied and Environmental Microbiology **65,** 2614-2621.

Smit, E., Leeflang, P., Gommans, S., van den Broek, J., van Mil, S., and Wernars, K. (2001). Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Applied and Environmental Microbiology **67,** 2284-2291.

Smith, K.P., and Goodman, R.M. (1999). Host variation for interations with beneficial plant-associated microbes. Annual Reveiw of Phytopathology **37,** 473-491.

Song, J., Weon, H.Y., Yoon, S.H., Park, D.S., Go, S.J., and Suh, J.W. (2001). Phylogenetic diversity of thermophilic actinomycetes and *Thermoactinomyces* spp. isolated from mushroom composts in Korea based on 16S rRNA gene sequence analysis. FEMS Microbiology Letters **202,** 97-102.

Spaink, H.P. (2000). Root nodulation and infection factors produced by rhizobial bacteria. Annual Review of Microbiology **54,** 257-288.

Spoel, S.H., Koornneef, A., Claessens, S.M., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., and Pieterse, C.M. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15,** 760-770.

Stackebrandt, E., Rainey, F.A., and Ward-Rainey, N.L. (1997). Proposal for a new hierarchic classification system, actinobacteria classis nov. International Journal of Systematic Bacteriology **47,** 479-491.

Staswick, P.E., and Tiryaki, I. (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell **16,** 2117-2127.

Staswick, P.E., Tiryaki, I., and Rowe, M.L. (2002). Jasmonate response locus JAR1 and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. The Plant Cell **14,** 1405-1415.

Stenroos, S.K., and DePriest, P.T. (1998). SSU rRNA phylogeny of cladoniiform lichens. American Journal of Botany **85,** 1548-1559.

Stosz, S.K., Fravel, D.R., and Roberts, D.P. (1996). *In vitro* analysis of the role of glucose oxidase from *Talaromyces flavus* in biocontrol of the plant pathogen *Verticillium dahliae*. Applied and Environmental Microbiology **62,** 3183-3186.

Strobel, G.A. (2003). Endophytes as sources of bioactive products. Microbes and Infections **5,** 535-544.

Sturz, A.V. (1995). The role of endophytic bacteria during seed piece decay and tomato tuberization. Plant Soil **175,** 257-263.

Sturz, A.V., and Nowak, J. (2000). Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. Applied Soil Ecology **15,** 183-190.

Sturz, A.V., Christie, B.R., and Matheson, B.G. (1998). Associations of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. Canadian Journal of Microbiology **44,** 162-167.

Sturz, A.V., Christie, B.R., Matheson, B.G., Arsenault, W.J., and Buchanan, N.A. (1999). Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil-borne plant pathogens. Plant Pathology **48,** 360-369.

Surette, M.A., Sturz, A.V., Lada, R.R., and Nowak, J. (2003). Bacterial endophytes in processing carrots (*Daucus carota* L. var. *sativus*): their localization, population density, biodiversity and their effects on plant growth. Plant and Soil **253,** 381-390.

Taechowisan, T., Peberdy, J.F., and Lumyong, S. (2003). Isolation of endophytic actinomycetes from selected plants and their antifungal activity. World Journal of Microbiology and Biotechnology **19,** 381-385.

Tanabe, Y., Nagahama, T., Saikawa, M., and Sugiyama, J. (1999). Phylogenetic relationship of *Cephaliophora* to nematophagous hyphomycetes including

taxonomic and nomenclatural emendations of the genus *Lecophagus*. Mycologia **91,** 830-835.

Thaler, J.S., Owen, B., and Higgins, V.J. (2004). The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. Plant Physiology **135,** 530-538.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A., and Heinen, E. (1999). Housekeeping genes as internal standards: use and limits. J Biotechnol **75,** 291-295.

Thevissen, K., Ghazi, A., De Samblanx, G.W., Brownlee, C., Osborn, R.W., and Broekaert, W.F. (1996). Fungal membrane responses induced by plant defensins and thionins. Journal of Biological Chemistry **271,** 15018-15025.

Thomma, B., Tierens, KFM., Pennickx, IAMA., Mauch-Mani, B., Broekaert, WF., Cammue, BPA. (2001). Different microorganisms differentially induce Arabidopsis disease response pathways. Plant Physiology and Biochemisty **39,** 673-680.

Tokala, R.K., Strap, J.L., Jung, C.M., Crawford, D.L., Hamby Salove, M., Deobald, L.A., Bailey, F.J., and Morra, M.J. (2001). Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). Applied and Environmental Microbiology **68,** 2161-2171.

Torkko, P., Suomalainen, S., Iivanainen, E., Tortoli, E., Suutari, M., Seppanen, J., Paulin, L., and Katila, M.L. (2002). *Mycobacterium palustre* sp. nov., a potentially pathogenic, slowly growing mycobacterium isolated from clinical and veterinary specimens and from Finnish stream waters. International Journal of Systematic and Evolutionary Microbiology **52,** 1519-1525.

Torkko, P., Suomalainen, S., Iivanainen, E., Suutari, M., Paulin, L., Rudback, E., Tortoli, E., Vincent, V., Mattila, R., and Katila, M.L. (2001). Characterization of *Mycobacterium bohemicum* isolated from human, veterinary, and environmental sources. Journal of Clinical Microbiology **39,** 207-211.

Tucker, S.L., and Talbot, N.J. (2001). Surface attachment and pre-penetration stage development by plant pathogenic fungi. Annu. Rev. Phytopathol. **39,** 385-417.

Turenne, C., Chedore, P., Wolfe, J., Jamieson, F., Broukhanski, G., May, K., and Kabani, A. (2002). *Mycobacterium lacus* sp. nov., a novel slowly growing, non-chromogenic clinical isolate. International Journal of Systematic and Evolutionary Microbiology **52,** 2135-2140.

Turenne, C.Y., Tschetter, L., Wolfe, J., and Kabani, A. (2001). Necessity of quality-controlled 16S rRNA gene sequence databases: identifying

nontuberculous *Mycobacterium* species. Journal of Clinical Microbiology **39,** 3637-3648.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired resistance in Arabidopsis. Plant Cell **4,** 645-656.

Valois, D., Fayad, K., Barbasubiye, T., Garon, M., Dery, C., Brzezinski, R., and Beaulieu, C. (1996). Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi*, the casaul agent of raspberry root rot. Applied and Environmental Microbiology **62,** 1630-1635.

van Loon, L.C., and van Stein, E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparitive analysis of PR-1 type proteins. Physiological and Molecular Plant Pathology **55,** 85-97.

van Peer, R., Niemann, G.J., and Schippers, B. (1991). Induced resistance and phytoalexin accumulation in biological control of fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. Phytopathology **81,** 728-734.

van Vuurde, J.W.L., and Elivra-Recuenco, M. (2000). Endophyte management as a tool to optimize plant quality. Proceedings of 5th PGPR Workshop.

van Wees, S.C., Luijendijk, M., Smoorenburg, I., van Loon, L.C., and Pieterse, C.M. (1999). Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. Plant Mol Biol **41,** 537-549.

van Wees, S.C., de Swart, E.A., van Pelt, J.A., van Loon, L.C., and Pieterse, C.M. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences USA **97,** 8711-8716.

Verhagen, B.W.M., Glazebrook, J., Zhu, T., Chang, H.S., van Loon, L.C., and Pieterse, C.M. (2004). The transcriptome of rhizobacteria-induced systemic resistance in Arabidopsis. Molecular Plant Microbe Interactions **17,** 895-908.

Vidal, S., Eriksson, A.R.B., Montesano, M., Denecke, J., and Palva, E.T. (1998). Cell wall-degrading enzymes from *Erwinia carotovora* cooperate in the salicylic acid-independent induction of a plant defense response. Molecular Plant Microbe Interactions **11,** 23-32.

Wang, K.L.C., Li, H., and Ecker, J.R. (2002). Ethylene Biosynthesis and Signaling Networks. The Plant Cell **Supplement 2002,** S131-S151.

Watkins, J. (2003). Wheat root and crown rot. http://pdc.unl.edu/wheat/CrownRootRot/ (http://pdc.unl.edu/wheat/CrownRootRot/: Plant Disease Central).

Wayne, L.G., Good, R.C., Bottger, E.C., Butler, R., Dorsch, M., Ezaki, T., Gross, W., Jonas, V., Kilburn, J., Kirschner, P., Krichevsky, M.I., Ridell, M., Shinnick, T.M., Springer, B., Stackebrandt, E., Tarnok, I., Tarnok, Z., Tasaka, H., Vincent, V., Warren, N.G., Knott, C.A., and Johnson, R. (1996). Semantide- and chemotaxonomy-based analyses of some problematic phenotypic clusters of slowly growing mycobacteria, a cooperative study of the International Working Group on Mycobacterial Taxonomy. Int J Syst Bacteriol **46,** 280-297.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol **173,** 697-703.

Weller, D.M., Raaijmakers, J.M., Gardener, B.B., and Thomashow, L.S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annual Review of Phytopathology **40,** 309-348.

Whipps, J.M. (2001). Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany **52,** 487-511.

Wiese, M.V. (1977). Compendium of wheat diseases. (Minnesota, U.S.A: The American Phytopathological Society).

Wilson, D. (1995). Endophyte - the evolution of a term, and clarification of its use and definition. Oikos **73,** 274-276.

Wintzingerode, F.V., Gobel, U.B., and Satackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews **21,** 213-229.

Wood, D.W., Gong, F., Daykin, M.M., Williams, P., and Pierson, L.S.r. (1997). N-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. Journal of Bacteriology **179,** 7663-7670.

Wood, M., and Stevens, F. (2004). The fungi of California. http://www.mykoweb.com/CAF/species/Aleuria_aurantia.html.

Wu, Z., Tsumura, Y., Blomquist, G., and Wang, X.R. (2003). 18S rRNA gene variation among common airborne fungi, and development of specific oligonucleotide probes for the detection fungal isolates. Applied and Environmental Microbiology **69,** 5389-5397.

Zehnder, G.W., Murphy, E.J.S., and Kloepper, J.W. (2001). Application of rhizobacteria for induced resistance. European Journal of Plant Pathology **107,** 39-50.

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. Proceedings of the National Academy of Sciences USA **96,** 6523-6528.

Zhou, J., Bruns, M.A., and Tiedje, J.M. (1996). DNA recovery from soils of diverse composition. Applied and Environmental Microbiology **62,** 316-322.

Zhou, J.M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., and Klessig, D.F. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the *PR-1* gene required for induction by salicylic acid. Molecular Plant Microbe Interactions **13,** 191-202.

Zinniel, D.K., Lambrecht, P., Harris, N.B., Feng, Z., Kuczmarski, D., Higley, P., Ishimaru, C.A., Arunakumari, A., Barletta, R.G., and Vidaver, A.K. (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Applied and Environmental Microbiology **68,** 2198-2208.