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THE CULTURE-INDEPENDENT ANALYSIS OF FUNGAL ENDOPHYTES OF WHEAT GROWN IN KWAZULU-NATAL, SOUTH AFRICA

By

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Thesis summary

Fungal endophytes are of interest due to their diverse taxonomy and biological functions. A range of definitions exists based on their identity, morphology, location and relationship with their host. Fungal endophytes belong to a wide range of taxa and they are categorized by a variety of characteristics. The detection and identification of these fungal endophytes can be performed using culture-dependent and culture-independent methods. These organisms have a range of application in pharmaceutical discovery and agriculture. Agricultural applications include the exploitation of the growth promoting and protective properties of fungal endophytes in crops such as wheat. This important crop is grown in South Africa where biotic and environmental stresses pose a challenge to its cultivation. Fungal endophytes have demonstrated potential to ameliorate these challenges. Future research will reveal how they can be harnessed to fight food insecurity brought about by stress factors such as climate change.

Extraneous DNA interferes with PCR studies of endophytic fungi. A procedure was developed with which to evaluate the removal of extraneous DNA. Wheat (*Triticum aestivum*) leaves were sprayed with *Saccharomyces cerevisiae* and then subjected to physical and chemical surface treatments. The fungal ITS1 products were amplified from whole tissue DNA extractions. ANOVA was performed on the DNA bands representing *S. cerevisiae* on the agarose gel. Band profile comparisons using permutational multivariate ANOVA (PERMANOVA) and non-metric multidimensional scaling (NMDS) were performed on DGGE gel data, and band numbers were compared between treatments. Leaf surfaces were viewed under Variable Pressure Scanning Electron Microscopy (VPSEM). Yeast band analysis of the agarose gel showed that there was no significant difference in the mean band DNA quantity after physical and chemical treatments, but they both differed significantly ($p < 0.05$) from the untreated control. PERMANOVA revealed a significant difference between all treatments ($p < 0.05$). The mean similarity matrix showed that the physical treatment results were more reproducible than those from the chemical treatment results. The NMDS showed that the physical treatment was the most consistent. VPSEM indicated that the physical treatment was the most effective treatment to remove surface microbes and debris. The use of

molecular and microscopy methods for the post-treatment detection of yeast inoculated onto wheat leaf surfaces demonstrated the effectiveness of the surface treatment employed, and this can assist researchers in optimizing their surface sterilization techniques in DNA-based fungal endophyte studies.

Denaturing gel electrophoresis (DGE) can be used in culture-independent studies of microbial community composition and the technique has several variants. This work compared two of these variants, namely denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient electrophoresis (TTGE), to establish their relative performance in terms of resolution and detection, as well as cost and preparation time. Per gel reagent and material costs and preparation times were recorded for comparison. Conversion formulae were developed to standardize denaturing conditions for comparison of DGGE and TTGE gels. For all gel samples, band numbers, positions, peak height and base width were recorded. Samples run on DGGE gels tended to be clearer and more distinct from each other and DGGE tended to provide higher band numbers and better resolution. However, TTGE was quicker and cheaper to prepare. The TTGE and DGGE gel data were strongly correlated but DGGE provided more accurate dendrograms for comparisons of pure fungal isolates. Non-metric multidimensional scaling showed that TTGE data profiles were more heterogeneous, while DGGE produced tighter clustering of replicate samples. Although TTGE could be an acceptable technique for resolving DNA sequences in certain applications, DGGE is preferable for fungal wheat endophyte studies.

Fungal endophyte community composition can be affected by various factors, such as the host genome. Research into the host genome effects on fungal endophyte composition can assist in harnessing the potential benefits of such relationships in agro-ecosystems. Several culture-based studies have investigated the presence of a cultivar effect on endophyte composition. However, a culture-based approach can only detect organisms that can be isolated and grown. Culture-independent methods can detect both culturable and non-culturable fungal endophytes for comparisons of fungal endophyte community composition (ECC) between wheat cultivars. Denaturing gradient gel electrophoresis (DGGE), high-resolution melt (HRM) analysis of community profiles, quantitative PCR, and sequence

analysis were used to analyse and compare the fungal ECC of four wheat cultivars grown under field conditions. A significant organ and cultivar x organ interaction effects on fungal biomass were observed. A chytrid, namely *Olpidium brassicae* formed a significant component of the fungal endophyte community across all tissues in wheat. This finding highlighted the utility of the culture-independent in revealing cryptic interactions and endophytes, and raised questions about the factors that influence the organisms that reside within field-grown wheat.

Systemic fungicides used in wheat production are pathogenic to many plant-inhabiting fungi such as fungal endophytes. The aim of the study was to reveal the effect of tebuconazole on the eukaryotic endophytes of wheat flag leaves using next generation sequencing (NGS). Treated and untreated leaves were surface sterilized prior to metagenomic DNA (mDNA) extraction. NGS was performed on DNA amplified using universal ITS primers. SCATA analysis was used for operational taxonomic unit (OTU) assignment of sequences, which were identified against CBS, UNITE and Genbank databases. A maximum likelihood (ML) tree was developed for taxonomic assignment of key genera. OTU mean read numbers and OTU richness were compared. The treatment effects were analysed using Principal Component Analysis (PCA), permutational multivariate ANOVA (PERMANOVA), distance-based test for homogeneity of multivariate dispersions (PERMDISP) and similarity percentage analysis (SIMPER). With one exception, non-wheat OTUs belonged to the Dikarya. *Puccinia* read numbers differed significantly ($p = 0.01$) between treatments and fungicide treatment tended to reduce total OTU read numbers and OTU richness. The variability of most key OTUs correlated positively with unsprayed samples. Treatment influenced OTU composition. Treated samples had the greatest homogeneity in endophyte composition and *Puccinia* made the greatest contribution to variation, with low contribution from the other OTUs. Dikarya were the dominant wheat flag leaf endophytes, and while the fungicide suppressed *Puccinia* and reduced fungal endophyte abundance, it did not significantly alter the community assemblage.

PCR-based studies of plant pathogen and endophyte community composition are constrained by primer problems such as variable amplification efficiency and non-target sequence amplification. This study developed non-extendable blocking primers (NEBPs) for use with universal eukaryotic ITS-PCR primers, to enhance target *Puccinia* Pers. and endophyte sequence amplification while suppressing host wheat DNA amplification. These NEBPs were 100% complementary to the priming site and flanking regions for wheat. ITS-PCR products under increasing concentrations of NEBPs were compared on agarose gels. Diluted ITS-PCR products were used as template in qPCR assays of wheat host and *Puccinia* amplicon production under increasing NEBP concentrations. Gel analysis showed the suppression of wheat DNA amplification, while non-host target sequence amplification was enhanced as NEBP concentrations increased. The qPCR assay of wheat amplicons from ITS-PCR products showed a linear decrease in wheat amplicons as NEBP concentration increased. *Puccinia*-specific qPCR of ITS-PCR products showed a non-linear association between *Puccinia* sequence quantities and increasing NEBP concentrations. *Puccinia* sequence amplification increased up to a specific NEBP concentration after which amplification was suppressed. It was shown that universal primers used with optimal NEBPs concentrations successfully suppressed host wheat DNA amplification with enhanced *Puccinia* and eukaryotic endophyte DNA amplification.

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Declaration 1: Plagiarism

I, Richard Jörn Burgdorf, declare that:

1) The research reported in this thesis, except where otherwise indicated or acknowledged, is my original work.

2) This thesis has not been submitted in full or in part for any degree or examination to any other university.

3) This thesis does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

4) This thesis does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, their words have been re-written but the general information attributed to them has been referenced; where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

5) This thesis does not contain text, graphics or tables copied and pasted from the Internet.

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Declaration 2: Publications

The following work has been published in a peer-reviewed ISI-accredited scientific journal:

Chapter 2-A procedure to evaluate the efficiency of surface sterilization methods in culture-independent fungal endophyte studies. 2014. R.J. Burgdorf, M.D. Laing, C.D. Morris, S.F. Jamal-Ally, *Brazilian Journal of Microbiology* 45 (3)**:** 977-983.

The following work has been submitted to peer-reviewed ISI-accredited scientific journals and is currently under review:

Chapter 6- Enhanced PCR detection of *Puccinia* and fungal endophytes of wheat using non-extendable blocking primers. 2016. R.J. Burgdorf, M.D. Laing, C.D. Morris, S.F. Jamal-Ally. *Canadian Journal of Microbiology* (First submitted June 2016. A revised version was re-submitted in November 2016 and is currently under review).

My role in all of the published or submitted work was that I conceived the hypotheses and identified the research problems, decided on the experimental approach, performed all the experimental work, collected the data, performed statistical analyses and wrote the text.

The co-authors assisted with experimental design, statistical analyses, reviewed the work and provided funding for the research materials.

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List of abbreviations and acronyms

18s	ribosomal small subunit
A	adenine
AM fungi	arbuscular mycorrhizal fungi
ANOVA	analysis of variance
APS	ammonium persulfate
ARC	Agricultural Research Council
BFAP	Bureau for Food and Agricultural Policy
BLAST	basic local alignment search tool
bp	base pair/s
C	cytosine
C-endophytes	clavicipitaceous endophytes
cfu	colony forming unit
CTAB	cetyltrimethylammonium bromide
CV	coefficient of variation
DGE	denaturing gradient electrophoresis
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSE	dark septate endophytes
ECC	endophyte community composition
EDTA	ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
FISH	fluorescent in situ hybridization
G	guanidine
g	earth's gravitational acceleration
GC	guanidine and cytosine
GFP	green fluorescent protein
ISR	induced systemic resistance
ITS	internal transcribed spacer
kb	kilo bases

MEB	malt extract broth
NaOCl	sodium hypochlorite
NGS	next generation sequencing
NEBP	non-extendable blocking primer
NC-endophytes	non-clavicipitaceous endophytes
NJ	neighbour-joining
ML	maximum likelihood
NMDS	non-metric multidimensional scaling
NCBI	National Centre for Biotechnology Information
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PERMANOVA	permutational multivariate analysis of variance
PERMDISP	distance-based test for homogeneity of multivariate dispersions
PVP	polyvinylpyrrolidone
qPCR	quantitative PCR
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
mDNA	metagenomic DNA
rDNA	ribosomal DNA
rRNA	ribosomal RNA
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
SIMPER	similarity percentage analysis
SGI	Small Grain Institute
sp.	species (singular)
spp.	species (plural)
T	thymine
TAE	Tris-acetic acid-EDTA
TBE	Tris-boric acid-EDTA
TEM	transmission electron microscopy

TEMED	N,N,N',N'-tetramethylethylenediamine
TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism
Tris	tris (hydroxymethyl) aminomethane
TTGE	temporal temperature gradient electrophoresis
UPGAMA	unweighted pair-group arithmetic average
VOC	volatile organic compound
VPSEM	variable pressure scanning electron microscopy
w/v	weight per volume
Y	cytosine or thymine

General introduction

Global food security is under threat due to the increasing human population and climate change (Wheeler and von Braun, 2013). It is projected that after 2030 yields from staple crops are expected to decrease (Challinor *et al.*, 2014). New strategies are being explored to increase yields and lower inputs for food production. Plant breeding efforts, as well as GMO strategies, are achieving some gains (Eisenstein, 2013, Hawkesford *et al.*, 2013). However, plant-associated microbes also offer the potential for increased plant growth and for crop protection (Sessitsch and Mitter, 2015).

Plant-associated microbes can include organisms known as endophytes, which live within the tissues of the host plants but do not cause disease symptoms. Their ecological significance is increasingly recognized since all plants in natural ecosystems probably harbor them (Rodriguez *et al*., 2009a). As initial model organisms, endophytes of forage grasses have been extensively studied for their role in grass biology, producing compounds that repel herbivores and because they affect the reproductive cycle of infected plants (Clay, 1988). Subsequent interest in endophytes also increased due to the identification of new species, the discovery of novel pharmacological compounds, and the harnessing of endophytes for crop protection and plant growth promotion. Many of these endophytes have been shown to increase yields under challenging conditions such as drought or low nutrient availability. In addition, endophytes have also been shown to protect against pathogens where conventional pesticides are ineffective (Zhang *et al*., 2006). Various endophytes have been reported in association with important crop plants, including wheat (*Triticum aestivum* L.) (Larran *et al.*, 2016).

Problem statement

Wheat is regarded as one of the worlds' most important food crops used for human consumption, providing much of the global human dietary protein and carbohydrates (Gustafson *et al*., 2009). According to the Food and Agricultural Organization of the United Nations (http://www.fao.org), wheat was ranked third in tons of cereal grains produced (675M tonnes), after maize (875M tonnes) and rice (718M tonnes). In South Africa, it is the second most important cereal (1.8M tonnes) produced after maize (12.5M tonnes). South Africa is the largest producer of wheat in Southern Africa. In a 2009 estimate, wheat accounted for approximately 17% of the total per capita calorific intake for South Africans. Additionally, due to urbanization, wheat consumption is increasing in southern Africa (Byerlee and Morris, 1990). The South African Bureau for Food and Agricultural Policy's 10th BFAP Baseline report indicated that South Africa will be importing more wheat than it produces by 2022. Any factors that could affect wheat production are of major importance in South Africa and globally. Therefore, the role of endophytes in local wheat cultivation requires investigation.

Purpose of the study

The identification of the wheat endophytes that are present in locally grown wheat is a necessary step in developing the potential of endophytes to enhance wheat production in South Africa. Wheat cultivation occurs under irrigation in the western and southern central parts of KwaZulu-Natal, South Africa (Burger and Kilian, 2009). This was an important practical consideration that allowed sampling of wheat material grown under typical local field conditions and transport of the samples to our laboratories for further processing, which took place within a few hours.

Research has suggested that there may be a cultivar-based effect on the selection of the types of endophytes associating with wheat (Larran *et al.*, 2007). There is theoretically the potential to incorporate endophyte association as a beneficial trait that could be selectively bred in wheat to bequeath desirable benefits on the host plant, since endophytes have been shown to enhance wheat yields and disease resistance (Marshall and Tunali, 2000). Research into the epigenetic effects of such endophytes on plants could also be explored in the context of wheat production because epigenetic regulation of plants by fungal endophytes has been established (Rodriguez *et al.*, 2009b).

Several culture-based studies have been performed to determine whether the genetic differences between wheat cultivars affect the diversity of fungal endophytes in them (Sieber *et al*., 1988; Crous *et al*., 1995; Larran *et al*., 2002; Larran *et al*., 2007). These studies only evaluated the microorganisms that could be cultured. In this study, a DNA-based approach was used. This was performed to see if the detection of the culturable and non-culturable fungal endophytes could reveal a cultivar effect on fungal endophyte community composition and species richness in the roots, stems, and leaves of different wheat cultivars. Bacterial endophytes were also understood to be important but they were beyond the scope of this work which focussed on the fungal endophytes.

While many fungi can be isolated from wheat with relative ease (Crous *et al.*, 1995), this does not provide a complete picture of the endophytic fungal community composition. A culture-independent approach can detect those organisms which cannot be isolated and

identified by axenic culture. A culture-independent approach can employ techniques such as the polymerase chain reaction (PCR) to amplify regions that can be used to identify fungal species. This PCR is then coupled with techniques such as denaturing gradient electrophoresis (DGE) to resolve DNA from different species, high-resolution melt (HRM) analysis for profile discrimination, quantitative PCR (qPCR) to quantify relative fungal endophyte presence, clone libraries with Sanger sequencing to identify the fungal species present, and next generation sequencing to provide high sequence depth with operational taxonomic unit (OTU) analysis and identification. These are more recent approaches in microbial research that can be optimized and applied to the exploration of wheat fungal endophyte community composition.

Aim of the study

The broad aim of this study is to develop and evaluate appropriate methods of sample preparation and DNA analysis for fungal endophytes of wheat, followed by comparisons of fungal endophyte community composition in different cultivars of wheat, as well as in fungicide-treated wheat. More detailed descriptions of aims are outlined in the research questions below.

Research Questions:

1. The first question that was raised was how the sample processing in terms of surface DNA decontamination could affect the detection of fungal endophytes. Microbial sampling procedures can greatly impact on the description of fungal community composition (Unterseher *et al.*, 2011). In culture-based studies, a chemical surface sterilization procedure is typically used for isolation of endophytes (Bacon, 1988). The methods of surface sterilization in the traditional culture of endophytes on agar media is predicated on the destruction of surface microbial cells and spores, to ensure that they are not capable of growth, thus only allowing for the growth of those microbes left alive within the plant tissues (Schulz *et al*., 1993). As such, evaluating the efficiency of surface sterilization has been considered an important first step in culture-based endophyte studies (Schulz *et al*., 1993; Reissinger *et al*., 2001; Paulus *et al*., 2003). In culture-independent fungal endophyte studies, it is necessary to remove DNA originating from fungal epiphytes and other incidental surface organisms. Establishing a way to determine and compare the efficiency of techniques used to destroy the DNA of epiphytes is thus of similar importance in DNA-based endophyte diversity studies (Guo, 2010). It was decided to investigate this and develop an effective surface decontamination procedure to use when comparing the cultivar-specific communities.

2. The next question which arose was on which variant of denaturing gel electrophoresis (DGE) would be most suitable for the comparison of wheat fungal endophyte community profiles. A PCR-based approach combined with a form of DGE such as denaturing gradient gel electrophoresis (DGGE) was to be employed since this approach had been successfully

employed in another study of endophytes in different host cultivars (Sessitsch *et al.*, 2002). The production of large numbers of DGE gels for the screening of fungal community profiles of the roots, stems, and leaves of several wheat cultivars is a laborious process. DGE is based on a principle that can be implemented in several different ways either by chemical or thermal denaturing of DNA as it moves through a gel (Muyzer *et al*., 1993; Muyzer and Smalla, 1998; Cornejo *et al*., 2004; Manzano *et al*., 2005). Both TTGE and DGGE could be performed on the available equipment. Both methods needed to be evaluated for their various attributes and performance, aiming to reduce the cost and effort of performing this type of fungal community profile analysis without losing resolution. In this context, it was hypothesized that TTGE could be a desirable alternative to DGGE. Once it was established which technique was most suitable for fungal community profile analysis, that method would be employed in the subsequent research to determine whether there was a selective effect by wheat cultivars on the endophytic populations associated with wheat.

3. After addressing the technical considerations for determining the fungal endophyte composition, the research could investigate the principle focus of the work, namely the effect of wheat cultivar on fungal endophyte community composition. The genetic composition of the host plant has been shown to influence the composition of endophytes within it (Saikkonen *et al*., 2010), so it was anticipated that such an effect could be observed for different cultivars of wheat. Field grown wheat was to be sampled, surface sterilized and analyzed by PCR, followed by the appropriate form of DGE. Quantitative PCR and high-resolution melt profiles would also be performed to explore the nature of fungal endophyte colonization amongst different wheat cultivars and to reveal the utility of these techniques in arriving at conclusions that might not be apparent from PCR-DGE alone.

4. The samples analysed for the investigation on the effect of cultivar on fungal endophyte community composition were grown under commercial cultivation conditions where fungicides were applied. The question that arose from this was how such fungicides affected the composition of eukaryotic endophytes, i.e., including non-fungal organisms such as oomycetes. This would necessitate the use of universal eukaryotic primers with the deep sampling power of next generation sequencing (NGS). It was expected that fungicides could

significantly affect fungal community composition (Karlsson *et al.*, 2014) and that non-fungal eukaryotes may opportunistically occupy the niche vacated by fungi. It was also expected that there would be useful data on the extent of primer bias and interference by host DNA, as seen in PCR-base endophyte studies (Arenz *et al.*, 2015).

5. The data from the use of the universal eukaryotic primers would undoubtedly result in the detection of host DNA sequences. The final question was whether it was possible to inhibit this amplification without using taxon-specific primers that introduce bias. The data acquired from the previous experiment could be used to develop a solution to this problem that affects culture-independent PCR-based fungal endophyte and pathogen studies, i.e., the presence and interference of host plant DNA and amplification bias from primer mismatches. By aligning the detected wheat, fungal endophyte, and pathogen sequences, it would be possible to develop non-extendable blocking primers to solve this issue.

The research questions would be answered and the aims of the research achieved by working towards a series of study objectives.

Objectives of the study:

1-Evaluate surface sterilization methods using DNA-based techniques to develop a sample treatment protocol for wheat tissue samples for surface decontamination of any non-endophytic DNA. (Chapter 2)

2-Evaluate and compare the temporal temperature gradient gel electrophoresis (TTGE) and denaturing gradient gel electrophoresis (DGGE) variants of denaturation gradient electrophoresis in terms of cost, time and performances on the Bio-Rad Universal Mutation Detection System. This would determine the most appropriate method for comparing fungal wheat endophyte community profiles. (Chapter 3)

3-Compare DGE, qPCR and HRM profile data from the organs of different wheat cultivars to reveal any cultivar-based influence on fungal endophyte community composition; and to

identify the dominant fungal endophytic taxa by DNA sequencing of cloned ITS1 to ITS4 region sequences. (Chapter 4)

4-Perform NGS analysis of the effect of a triazole fungicide on eukaryotic wheat endophytes, as detected by universal eukaryotic primers targeting the ITS1 to ITS4 regions. (Chapter 5)

5-Develop a non-extendable blocking primer system to allow for the use of universal primers for unbiased PCR amplification and to suppress host DNA amplification in future PCR-based fungal wheat endophyte and pathogen studies. (Chapter 6)

Thesis format

This thesis follows an official University of KwaZulu-Natal format whereby all experimental chapters are written in the form of discreet research papers. Some duplication is unavoidable, such as some references and materials and methods, although it was attempted to keep repetition to a minimum in the Introductions and Discussions of the chapters. There are six chapters. The first chapter is a review of relevant literature focused on fungal endophytes, their diversity, evolution, and how they can and have been utilized in agriculture, particularly wheat, touching briefly upon wheat and its cultivation. This is followed by five experimental chapters. A final Thesis Overview discusses the findings of each experimental chapter. It allows for a more speculative and philosophical review of the research, its implications, as well as a starting point for future research.

As mentioned, the experimental chapters are presented in a stand-alone research paper format, consisting of an Introduction, Materials and Methods, Results and Discussion (with a Conclusion where this has been required by a journal) and references sections. This document has been produced in Microsoft Word (2013). For all the unpublished work in this thesis, the citations and references are in the author-date format, in a modified form of the author-date referencing style used in the journal FEMS Microbiology Ecology. This was implemented using Endnote X7. Where published work is included, it is in the format of the journal that the work was published and this is indicated in a footnote.

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Chapter 1

A review of fungal endophytes and their relation to wheat cultivation

1.1 Abstract

Fungal endophytes are of interest due to their diverse taxonomy and biological functions. A range of definitions exists based on their identity, morphology, location and relationship with their host. Fungal endophytes belong to a wide range of taxa and they are categorized by a variety of characteristics. The detection and identification of these fungal endophytes can be performed using culture-dependent and culture-independent methods. These organisms have a range of application in pharmaceutical discovery and agriculture. Agricultural applications include the exploitation of the growth promoting and protective properties of fungal endophytes in crops such as wheat. This important crop is grown in South Africa where biotic and environmental stresses pose a challenge to its cultivation. Fungal endophytes have demonstrated potential to ameliorate these challenges. Future research will reveal how they can be harnessed to fight food insecurity brought about by stress factors such as climate change.

1.2 Introduction

Endophytes are members of the plant microbiome that colonize the internal tissues of host plants (Bacon and White, 2016), i.e., the bacteria, fungi, and any other microbes that live inside plants. The study of these organisms encompasses a wide area of research. Aside from the taxonomy of the diverse array of endophytes found within a host plant, research on endophytic organisms includes the observation of the relationships with the host, host pathogens, and other endophytic co-inhabitants. Such investigations can broadly involve aspects of plant physiology and pathology, in particular, with relation to plant signaling and defence mechanisms; microbial ecology, in terms of interaction and succession; and microbial diversity, an evolving body of knowledge driven by developments in taxonomy, systematics, and phylogenetics. All of these areas have a wide scope. However, this review

will briefly discuss the concept of 'endophytism', with a primary focus on fungal endophytes; describe fungal endophyte research methodologies in terms of how endophytes are detected and identified; and review the applications of fungal endophytes in crop production, particularly in relation to the global and local cultivation of wheat.

Endophyte research is partly characterized by the allure of developing beneficial applications in agricultural systems and the discovery of novel metabolites (Gillespie, 1988; Hallmann *et al.*, 1997; Vallad and Goodman, 2004; Strobel, 2006; Johnson, 2008; Sessitsch and Mitter, 2015). An important aspect of these discoveries and developments is the identification of endophytic organisms that have been isolated from or detected within the host plant. This inadvertently develops our knowledge of the ecological functions of all microbes, and their diversity.

Even though most, if not all, plants are involved in symbiotic relationships with endophytes (Rodriguez *et al.*, 2009), until relatively recently little was known about endophyte diversity, function, and ecology (Sturz and Nowak, 2000); but this knowledge is steadily growing. This is evident in the growth in endophyte research. Research on endophytes produced just over one research paper a year for nineteen years since 1971. This has expanded to more than fifteen papers a year for about six years following 2001, in English-language journals; and research on endophyte-linked issues including novel metabolites has increased more than two hundredfold (Arnold, 2007). This increased interest requires a scrutiny of what is meant by the term 'endophyte'.

1.3 Defining endophytes

Several definitions have been applied to the plethora of organisms that have in common the trait of residing within a host plant. The term 'endophyte' was derived from the Greek meaning 'in the plant' (Siegel *et al*., 1987). It was apparently the famed mycologist, Thomas de Bary that was reported to have first suggested the term 'endophyte' (Kusari *et al.*, 2012). There have subsequently been varying opinions on the criteria for membership in this group.

Wilson (1995) discussed the definition, pointing out that fundamentally the term referred to the location of the organism; that it most commonly referred to bacteria and fungi; but that

referring to any organism living in a plant, as an endophyte, was not useful. It was proposed that these organisms must live entirely within the plant (thereby excluding mycorrhizae) and not produce disease symptoms during at least part of their life cycle (thereby including many plant pathogens). It was further suggested that endophytes are not only mutualists because the same mutualistic endophyte within a plant can present antagonistic properties, as observed for some clavicipitaceous endophytes. A noteworthy characteristic that Wilson (1995) raised was that endophytes do not trigger host defenses while they colonize the tissues. This could serve as a primary trait to qualify as an endophyte. Wilson (1995) was of the view that pathogens could be regarded as endophytes unless they immediately produced disease symptoms. This contrasted with Wennström (1994) who emphasized that pathogens such as rusts and smuts should not be classed as endophytes. Wilson (1995) also argued that simply because an organism was isolated from what appeared to be healthy tissue, did not mean it was not a pathogen, e.g., when a biotrophic pathogen is isolated at an early stage of infection, or when the pathogenic effects of the infection are not obvious.

Further to the location of endophytes being a qualifying trait, Saikkonen *et al*. (1998) considered endophytes to be predominantly leaf inhabitants and only occasionally residing in roots. They went further to propose, as did Wilson (1995), that mycorrhizal fungi, such as arbuscular mycorrhizal (AM) fungi or ectomycorrhizae, could not be regarded as endophytes since their hyphae extended outside of the roots. Dark septate endophytes (DSE) are fungal root inhabitants (Addy *et al*., 2005), and many other inhabitants of roots conform to the definition of an endophyte, e.g., the clavicipitaceous fungus *Metarhizium robertsii* J.F. Bisch., Rehner and Humber (Sasan and Bidochka, 2012). Busby *et al.* (2015) even mentioned that some epiphytes also occur as endophytes. Therefore, aside from the spatial location, i.e., whether the organism can or does reside within the plant internal tissues or not, the location of the fungus does not always assist in defining endophytes.

The taxonomic domains represented by endophytes have also varied according to their definition. Some definitions have limited the term ‘endophytes’ to fungi (Carroll, 1988), while others regarded endophytes as only bacteria that reside in the plant with or without any symbiotic interaction (Hallmann *et al*., 1997). This was probably in the context of the

research being performed. Brundrett (2006) suggested that any organism could be regarded as an endophyte when it occupies a plant without causing disease symptoms. Presumably, this would exclude insects, for example, which consume plant tissue, thereby harming the host while residing within it.

The manner by which endophytes are detected also has implications for their definition. Describing endophytes as 'bacteria and fungi that can be detected at a particular moment within tissues of apparently healthy plant hosts' (Schulz and Boyle, 2006), excludes non-culturable microorganisms. Archaea, for example, have been reported as endophytes using DNA-based detection methods (Chelius and Triplett, 2001; Ma *et al*., 2013). Microbes such as members of the Archaea and fungal obligate biotrophs cannot be cultured by axenic methods (Epstein, 2013), therefore, it is proposed here that this definition is inadequate .

The shift from the location of the microorganism and its detection to the relationship with the host has played a significant part in trying to define endophytes (Wennström, 1994; Wilson, 1995). Initially, endophytes were regarded as microbial contaminants from unsuccessful surface sterilization, or as disease organisms, but it was later realized that these microorganisms could, in fact, have beneficial or neutral relationships with host plants. Therefore, authors have used the term 'endophyte' to refer to any organism living within plants, or algae, in a range of commensal and symbiotic relationships (Schulz and Boyle, 2006). Schulz *et al*. (1999) considered endophytes as existing in a state of 'balanced antagonism' with the host, whereby an endophyte is distinguished from a pathogen in that the former fails to overcome the host defenses. Kogel *et al*. (2006) maintained a similar view but specified that the 'balance' was related to the response to, and production of, reactive oxygen species (ROS) by the endophyte or the plant. Photita *et al.* (2004) reported that some endophytes are latent plant pathogens, i.e., they eventually overcome the plant host defenses, supporting the argument by Wilson (1995) that even pathogens can be regarded as endophytes at some stage in their life-cycle. Kusari *et al*. (2012) also argued that the function of the organism was relevant to its qualification as an endophyte. Therefore, the relationship between host and microorganism does not seem to offer unambiguous support for membership to the endophyte category either.

The broadest definitions of endophytes categorize them as organisms that actively colonize the inside of a plant, spread throughout it and reproduce within it (van Overbeek, 2006). Sikora *et al.* (2007) described endophytes as 'any organisms that live in plant tissue whether neutral, beneficial or detrimental'. These last two definitions effectively classify all organisms that spend any part of their life cycle within living plant tissue as endophytes. Since there is nothing in the etymology of the word 'endophyte' that prescribes a specific functional attribute to classification as such, these are acceptable definitions. However, the use of the term 'endophyte' has been implicitly used to imply a mutualism (Backman and Sikora, 2008), or at worst, commensalism. However, it is suggested that the term 'endophyte' should include members of the plant microbiome that are not immediately pathogens, as proposed by Wilson (1995). More recently, in a review of endophytes, they were described as microbes that inhabit plant tissues without causing disease symptoms (Porras-Alfaro and Bayman, 2011). Maybe the term endophyte should be seen from a temporal perspective; in that, once a microbe becomes pathogenic to the host it is no longer an endophyte. This suggests that endophytism can be a transitory property of various microbes.

It becomes apparent that the definition of an endophyte may be difficult to restrict to a narrow set of traits. It may be simpler to consider that endophytes are not an exclusive group of organisms, but rather that endophytism is a trait that many organisms may possess. In this light, *sensu lato*, the definition used for this review is that an endophyte is a microorganism that partially or entirely resides within a plant (to include various mycorrhizal associations); spending a part of its life cycle inside a plant (to include facultatively biotrophic organisms); and which is not immediately pathogenic (to include latent pathogens).

1.4 What are fungal endophytes?

There term 'fungal endophyte' can refer to fungi that belong to a range of taxa. Certain highly adapted grass symbionts are members of the Clavicipitaceae, as described in the detailed review by Clay (1990); these endophytes have been regarded as the most frequently studied of all microbial endophytes that display a mutualistic interaction with the host plant (Anand, 2006). Another group of fungi, the dark septate endophytes (DSE), are

considered to be the most plentiful (Sieber and Grünig, 2006). The DSE are often members of the Ascomycota, they are taxonomically diverse and may include ectendomycorrhizal species (Jumpponen, 2001; Addy *et al*., 2005). As stated previously, for the purpose of this review, mycorrhizae are included as endophytes. The AM fungi are members of the Glomeromycota (Krüger *et al*., 2009) and ectomycorrhizal fungi can be members of the Basidiomycota, Ascomycota, and Zygomycota (Tedersoo *et al*., 2010). Even the Chytridiomycota have been represented as endophytes (Barrow *et al*., 1997), although this is uncommon. It appears then, that 'endophytism' is not limited to specific taxonomic groups and that endophytes can differ greatly in terms of their biology.

Fungal endophytes are considered to occur in almost all land and aquatic plants (Stone *et al*., 2000), and even seaweeds (Suryanarayanan, 2012). Stone *et al.* (2000) discussed their presence in grasses, dicotyledonous plants, lichens, mosses and ferns, tree bark, xylem vessels, plant roots, galls, and cysts. It would seem that they are pervasive in the tissues of plants and their relatives. In some cases, the host type has been used to categorize fungal endophytes.

Several systems have been devised to categorize different types or classes of endophytes. The nomenclature for classifying fungal endophytes devised by Carrol (1988) defined Type I and Type II endophytes as constitutive mutualists and inducible mutualists, respectively (Yuan, 2009). Rodriguez *et al.* (2009a) described Type I endophytes as systemic endophytes, usually belonging to the family Clavicipitaceae, transmitted via seeds and Type II endophytes as belonging to a wide variety of organisms that are horizontally transmitted (Yuan, 2009). Horizontal transmission occurs when endophytes are transmitted from the environment, including from other plants, to the host plant (Chung and Schardl, 1997); whereas transmission from parent to seed is referred to as vertical transmission (Afkhami and Rudgers, 2008). The way in which a fungal endophyte reaches its host was one of the several factors used by Rodriguez *et al.* (2009a) to develop a system that categorizes fungal endophytes. They reasoned that clavicipitaceous (C) endophytes and non-clavicipitaceous (NC) endophytes could be grouped into four classes based on a set of criteria that included host range, tissues colonized, the degree of colonization and diversity, manner of

transmission and the fitness benefits conferred on the host. Class 1 endophytes include clavicipitaceous endophytes that have a narrow host range; colonize shoots and rhizomes extensively; have a low *in planta* biodiversity; are vertically and horizontally transmitted; and confer non-habitat-adapted benefits to the host. These benefits include enhanced plant growth or tolerance of dry conditions, traits which are frequently found to be independent of where the plants grow. Habitat-adapted benefits are derived due to the evolution encouraged by conditions in specific habitats, e.g. acidity and temperature. Class 2, 3 and 4 endophytes are all non-clavicipitaceous endophytes and have broad host ranges. Class 2 endophytes colonize shoots, roots and rhizomes extensively, have low *in planta* biodiversity, are vertically and horizontally transmitted and confer both non-habitat-adapted and habitat-adapted benefits to the host plant. Class 3 endophytes only colonize shoots to a limited extent, with high *in planta* biodiversity. They are horizontally transmitted while conferring non-habitat-adaptive benefits to the host. Class 4 endophytes colonize the roots extensively and have undetermined degrees of *in planta* biodiversity. They are horizontally transmitted and confer non-habitat-adapted benefits to the host. Clay and Schardl (2002) further categorize C-endophytes into three types (Table 1.1).

Table 1.1 Categorization of C-endophytes, according to Clay and Schardl (2002).

Type	Characteristic
I	Symptomatic/pathogenic
II	Mixed-interaction
III	Asymptomatic

According to Rodriguez *et al.* (2009a) fungal endophytes belonging to the same genus can vary in their categorization, e.g., *Epichloë* species can be either Type I or II. These endophytes can control how they are transmitted, and in some cases, they can control plant development via the production of plant hormones, including auxins. Backman and Sikora (2008) categorized endophytes in general as belonging to three groups: pathogens of other host plants that are harmless as endophytes; non-pathogenic microbes; or modified pathogens that have lost their pathogenicity but not their ability to colonize the host.

All these systems of classification are difficult to apply with any degree of certainty. It also raises the question as to why a system of classification needs to exist at all, considering the range of organisms; the highly variable nature of their interactions with the host plant; and the highly variable range of hosts that some endophytes can be found in. Like some aspects of taxonomy, the categorization of endophytes is artificial. It is suggested here that endophytism is a strain trait, as opposed to endophytes being a distinct group of organisms.

There is some dispute whether mycorrhizae should be regarded as endophytes (Schulz and Boyle, 2006); and authors such as Kusari *et al.* (2012) who suggested that endophytes must reside entirely within the plant. Brundrett (2006) was more specific, stating that endophytes differ from mycorrhizae since they lack specialized hyphae that interface with the plant cell, their development is not synchronized with that of the plant and they do not transfer nutrients to the host. The last point is problematic in that the root endophyte *Piriformospora indica* Sav. Verma, Aj. Varma, Rexer, G. Kost and P. Franken has been shown to transfer phosphates to the host plant from the soil (Kumar *et al*., 2011). Mycorrhizae are usually beneficial to plants, possessing specialized hyphae which interface with host cells and provide nutrients to the plant from the hyphae extended beyond the root into the soil (Brundrett, 2006); however, some can also affect the host negatively (Jumpponen, 2001; Schulz and Boyle, 2006). In the same way that Wilson (1995) regarded rusts (which have specialized hyphal haustoria, are latent pathogens and are obligate biotrophs) as endophytes, it seems difficult not to regard mycorrhizae as endophytes because they largely meet the definition of endophytes as living within the host plant tissue and that they are 'microorganisms that establish neutral or beneficial interactions with their host plants' (Anand *et al*., 2006). Considering that Jumpponen (2001) suggested that dark septate endophytes (DSE) could be considered as having a mycorrhizal relationship with plants, conversely, should mycorrhizae not be classed as endophytes if they colonize internal plant tissues? It would simplify matters if mycorrhizae were considered as a type of endophyte but which is distinct from the DSE and other endophytes. Since mycorrhizae are unable to exist unless they colonize the internal tissues of the host plant roots, it could even be suggested that they are obligate endophytes. It should at least be acknowledged that mycorrhizae possess the property of endophytism.

Carroll and Petrini (1983) suggested that the ability for a wide variety of fungal endophytes to occupy a single host was due to the division of resources amongst these endophytes with differing substrate requirements. In studies on both fungal endophytes and the fungal epiphytes in coffee bean plants, endophytes were found to be more numerous than epiphytes (Santamaria and Bayman, 2005). This suggests that the *in planta* environment provides a more stable and hospitable environment than the plant surface, and that it is an environment in which many organisms can co-exist.

It is clear that the host range, taxonomy and ecological roles of fungal endophytes are extremely wide and varied. However, they share the ability to live within the host without hostility from the plant. New technologies are revealing not only the identities of these organisms but also how they interact and reside inside the plant without triggering host defence mechanisms (Kaul *et al*., 2016). Such studies of fungal endophytes and their interactions with other organisms at a molecular level will help to reveal how they occupy this niche, what they do there and how they evolved to do so.

1.5 The evolution of fungal endophytes

Studies of the ancient mycoflora in the Rhynie Chert Ecosystem, a 400-million-year-old fossilized ecosystem belonging to the early Devonian period, indicated that some endophytes may have begun as endomycorrhizae. These organisms are hypothesized to have transitioned from a parasitic to mutualistic relationship with the host plant, offering protection and improved phosphate uptake in the host (Taylor and Taylor, 2000). White *et al.* (2000) hypothesized that the clavicipitaceous endophytes probably evolved from an epiphytic grass symbiont; from there, groups developed separate phylogenies as they co-evolved with their hosts. White *et al.* (2000) reasoned that the switch from epiphyte to endophyte was due to higher nutrient availability and escape from desiccation and predation. In exchange, the host received herbivore-deterring secondary metabolites, growth stimulating hormones and protection from other fungal pathogens, giving the host a competitive advantage over other plants. However, even though endophytes conferred a competitive advantage, Schardl and Wilkinson (2000) noted that dependence of a host on endophyte is rare.

The great number of non-clavicipitaceous endophytes that have been identified indicates that they also evolved to provide an advantage to the host. Freeman and Rodriguez (1993) proposed that the loss of a virulence factor by mutation could change a pathogen into an endophyte. There is further evidence supporting this, for example, when apparently harmless endophytes cause disease symptoms in stressed hosts; or the use of a limited number of substrates, typical of parasitic organisms, by certain endophytes; as well as the close taxonomic relationship between many endophytes and pathogens. Carroll (1988) suggested that endophytes evolved from plant pathogens and that the precondition for the formation of fungal endophytes was latency and mycotoxin production. Carroll (1988) also argued that plant-endophyte associations are due to the plant acquiring a form of chemical protection from the fungal endophyte; while fungal traits can evolve relatively quickly, plant defences do not, thus the hosting of a protective endophyte is highly desirable.

An interesting proposal by Rodriguez *et al*. (2009a) was that some endophytes evolved from insect pathogens, migrating along the 'nutrient stream' from insect to plant, forming endophytes since they did not possess plant pathogenic genes. The fact that *Beauveria bassiana* (Bals.-Criv.) Vuill. is an entomopathogen and readily forms an endophytic association with various plants (Wagner and Lewis, 2000; Ownley *et al*., 2004; Quesada-Moraga *et al*., 2006; Akello *et al*., 2007; Akello *et al*., 2008; Ownley *et al*., 2008; Tefera and Vidal, 2009) reinforces this suggestion. The presence of an entomopathogenic resident would certainly be advantageous for a plant that was vulnerable to attack by insects; therefore, a plant that actively promoted such an association would have an evolutionary advantage over competitor plants in an ecosystem. Further support for this hypothesis is most likely to be found in the understanding of the genetic composition of endophytes relative to their non-endophytic counterparts.

Although fossil records of fungi do exist (Taylor and Taylor, 2000), the information they provide is limited relative to that of, for example, fossils of ancient reptiles and mammals. However, our ability to understand the genetic differences between organisms and subsequently their phylogenetic relationships as inferred by evolutionary models is

improving. These emerging methods form part of a range of methodological approaches to understanding the origins, diversity, interactions and functions of fungal endophytes.

1.6 Fungal endophyte diversity research methods

Fungal endophytes research methods continue to evolve with new technological developments, as is the case for biological research in general. Just as in other areas of biological research, work on fungal endophytes can be observational, where the diversity, quantity, and effect of fungal endophytes are deduced from naturally occurring populations; or experimental, where the fungal endophyte composition or biology is manipulated. For either of these experimental processes to occur, fungal endophytes need to be detected and distinguished by their unique features, i.e., their morphology or molecular composition. Schultz and Boyle (2006) outlined four general approaches for doing so, i.e., by microscopy; by isolation on growth media after surface sterilization of plant tissue; by specific biochemistry, such as ELISA; and finally, by molecular techniques involving PCR detection.

However, endophyte research methods are not restricted to a limited number of techniques, although they are often described as belonging to two general approaches that are used to identify and characterize fungal endophytes. These two approaches are regarded as, either dependent on isolating and growing the endophytes (culture-dependent), or observing and analysing the endophytes without doing so (culture-independent) (Arnold *et al*., 2007). Combinations of the two methods can occur, for example, by the introduction of a fungal endophyte and its subsequent detection by PCR to establish how successfully it has colonized the host (Landa *et al*., 2013). Host colonization is also often the focus of *in planta* studies (Vági *et al*., 2014).

In planta studies can be considered as a combinational approach because the endophytes are not necessarily studied while cultured on growth media, but that does not mean that they cannot be grown. In fact, the endophyte being studied may first have been isolated, grown and characterized before being applied to and colonizing a host plant. Once they have colonized the host, knowledge derived from DNA-based techniques may be used to study them. An example of this is with the use of confocal laser scanning microscopy that produces images such as those shown in Figure 1.1. Using this form of microscopy in conjunction with

FISH probes (Kutter *et al.*, 2006; Vági *et al*., 2014) or with modified organisms that produce green fluorescent protein (GFP) (Coombs and Franco, 2003; Bolwerk *et al.*, 2005) can reveal the location of fungal endophytes of interest relative to other co-inhabitants, and the interactions between them.

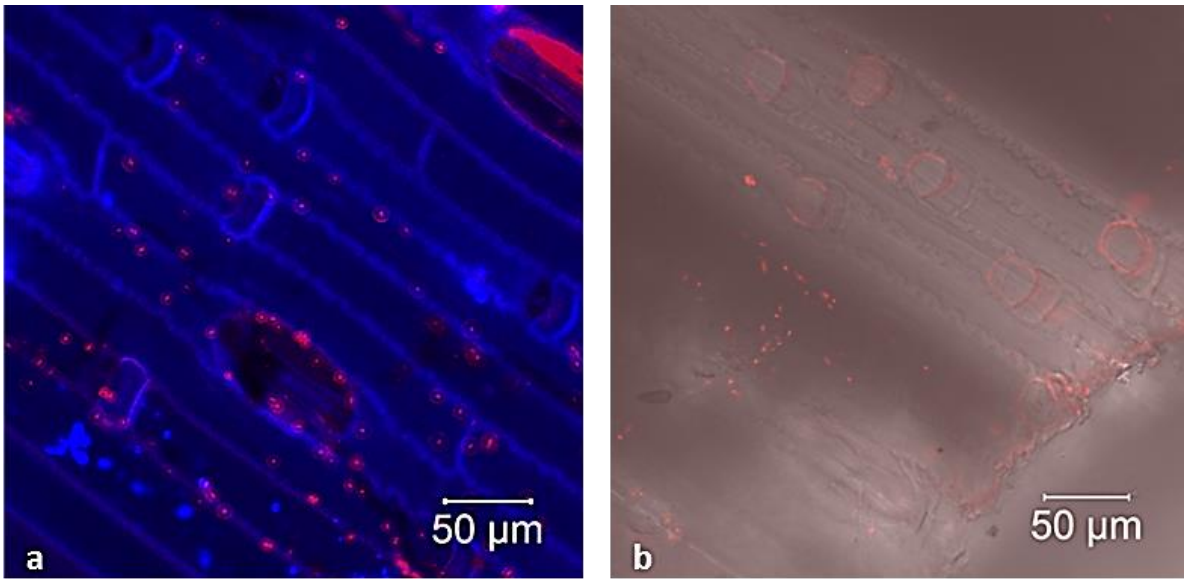

Figure 1.1 Confocal Laser Scanning Microscopy images showing (a) blue auto-fluorescence of wheat leaf epidermal cell walls and (b) Differential Interference Contrast (DIC) image of the wheat leaf surface. Both show pink fluorescence of epiphytic microbes stained with a fluorescent nucleic acid dye (Images taken by Richard Burgdorf).

The development of such techniques, therefore, can depend on the data produced from both culture-dependent and culture-independent methods; however, these methods are typically regarded as distinct approaches to understanding the identity and nature of fungal endophytes.

1.6.1 Culture-dependent methods

Hallmann *et al.* (2006) extensively reviewed the various approaches to isolating and culturing endophytes. Their review explained that culture-dependent endophyte research, by definition, requires the endophyte to be grown as an axenic culture, i.e., a pure fungal isolate that is free from contaminants. Aside from incidental contaminants that may be present from incomplete sterilization of media or equipment, the main consideration for the isolation of fungal endophytes is the removal or destruction of non-endophytic microbes

that exist on plant surfaces. This is referred to as 'surface sterilization' and is the first step in fungal endophyte isolation from host plant material. Surface sterilization methods can vary, but generally involve the immersion of plant tissue in disinfectants such as hydrogen peroxide, formaldehyde, sodium hypochlorite (bleach) and ethanol, or combinations thereof, followed by rinsing with sterile water (Schulz *et al*., 1993; Allen *et al*., 2003; Arnold *et al*., 2007) under aseptic conditions.

Schulz *et al*. (1993) evaluated the most common methods of surface sterilization that used bleach, ethanol, and formaldehyde at various concentrations and durations, finding that with the exception of immersion in 50% ethanol for 5 minutes, all of the other methods reliably sterilized the tested plant surfaces. Reissinger *et al*. (2001) compared the efficacy of bleach, ethanol, and peracetic acid in surface sterilization and found that while peracetic acid was the most effective in destroying certain ascospores, it also resulted in damage to internal plant tissues and endophytes residing there. Meyer and Hoy (2008) found that immersion in a 6% bleach solution for 1 minute was sufficient to kill all surface fungi on insects. Surface sterilization, using a combination of ethanol, bleach and then rinsing with water, as described by Arnold *et al.* (2007), seems to be adequate as a method of killing epiphytic fungi and bacteria, although variations can be experimented with, followed by surface sterility checks performed by applying treated plant surfaces to nutrient media (Hallmann *et al*., 2006).

After surface sterilization, standard culture procedures for selecting fungi can be applied, such as the use of bactericidal or bacteriostatic antibiotics or the alteration of factors such as pH and temperature (Hallmann *et al*., 2006). There is not a great deal of literature on selective media for fungal endophytes; however, an example of selective media being used to isolate a specific fungal endophyte is the work by Meyling and Eilenberg (2006) who used media containing dodine to isolate *B. bassiana* from plant leaves. Media such as *Trichoderma* Selective Media (Askew and Laing, 1993) could also be adapted to selectively isolate these fungi when they occur as endophytes as well. On such selective media, pieces of whole plant tissue can be placed, as shown in Figure 1.2. Other methods that extract

tissue fluids can also be used, e.g., centrifugation or vacuum extraction (Hallmann *et al.*, 2006).

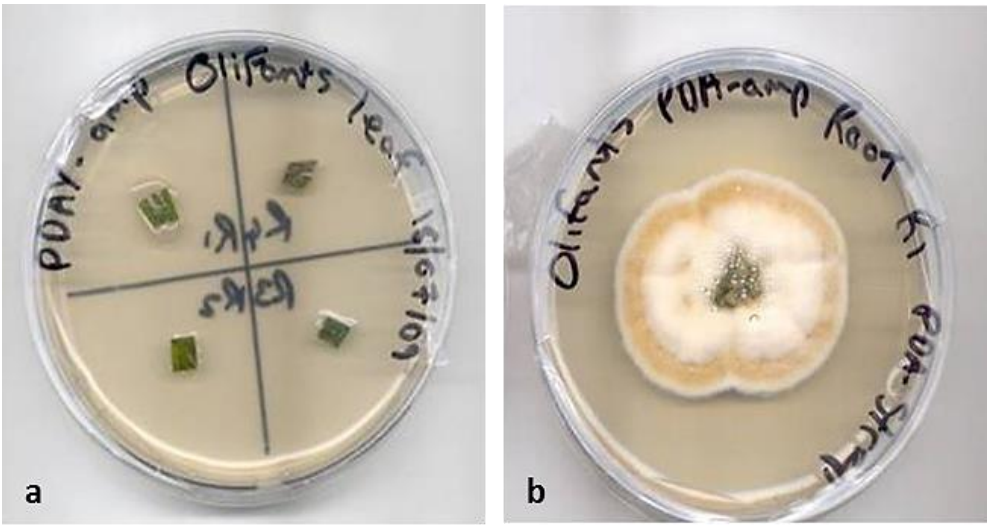

Figure 1.2 (a) Plating of surface sterilized plant material from which cultures were later isolated (b). (Images were taken by Richard Burgdorf).

After their isolation, the identification of fungi follows standard methods that include microscopic observations of the morphology of hyphae, conidia, conidiophores, sclerotia, chlamydospores and, where possible, sexual fruiting bodies. Images taken under light microscopy are used to record morphological structures that are used in taxonomical assignations (Dugan, 2006). Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) are also used to distinguish between taxa (Guarro *et al.*, 1999). Guarro *et al*. (1999) further discussed various physiological and biochemical techniques to distinguish between fungal organisms, such as growth rates under varying conditions or on different media; analysis of secondary metabolites; ubiquinones; fatty acids; cell wall and protein composition.

More recently, molecular approaches utilizing extracted nucleic acids to identify fungal isolates have become popular because of the relative ease and speed with which they can be performed. This was proposed by Bruns *et al.* (1991) who provided an extensive review of fungal systematics by nucleic acid analysis. They discussed DNA-DNA hybridization, restriction fragment analysis, electrophoretic karyotyping, and sequence analysis, in

particular with regard to sequences that code for ribosomal RNA (rRNA) within the fungal genome. These rRNA sequences, amplified from genomic DNA by polymerase chain reaction (PCR) using universal primers, have been regarded as good determinants of fungal phylogeny. Beyond phylogenetic associations, sequencing of the internal transcribed spacer (ITS) regions, flanking the 5.8S ribosomal unit sequence between the small and large subunit rRNA sequences, are often used to identify species by comparing sequences to those in the Genbank database (Tooley *et al*., 1997; Guarro *et al*., 1999; Allen *et al*., 2003; Martin and Rygiewicz, 2005; Arnold *et al*., 2007; Xin *et al*., 2009; Blaalid *et al*., 2013). Consequently, the ITS region has become known as the 'barcode' for fungal identification (Schoch *et al*., 2012).

Identifying fungal isolates usually forms part of a larger study on the diversity or composition (or function) of fungal endophytes within the host. In such studies, culture-dependent methods have been observed to exert a selective bias by enriching for certain microbial groups, revealing that this approach can be inadequate for accurately describing microbial communities (Wagner *et al.*, 1993; Allen *et al.*, 2003). Most microbial cells from environmental samples belong either to known organisms which are in a non-culturable state; or cannot be cultured with the method used; or are unknown organisms hitherto not cultured by axenic means (Amann *et al.*, 1995). Subsequently, there has been skepticism that culture-dependent methods reflect the true number of endophytes in host plant tissue (Hallmann, 2006). Another limitation of the culture-dependent approach to endophyte studies is that it is not possible to guarantee the complete sterilization of plant surfaces (Anand *et al*. 2006). In addition, it has been proposed that the concentrations of chemicals required to completely sterilize plant tissue surfaces may also damage endophytic microbes within the tissues (Hallman, 1997). However, the need to isolate and grow fungal endophytes persists and the use of multiple culture-dependent approaches can increase the quantity and diversity of isolated organisms (Unterseher and Schnittler, 2009); and utilizing dilution-to-extinction plating as described by Collado *et al.* (2007) has proven to isolate a greater number of endophytes with greater ease than traditional plating methods. These techniques have increased the number of culturable organisms that can be detected within host plant tissues.

While culture-dependent studies of fungal endophyte diversity may fall out of favour relative to modern methods that are less laborious in detecting fungal endophytes, the need to culture fungal endophytes is unlikely to disappear. Fungal endophytes are often of interest due to the metabolites they produce (Kusari *et al*., 2012). The production of these chemicals for pharmaceutical and agricultural applications will require the organism to be studied in isolation to optimize growth and productivity conditions in much the same way as, for example, penicillin production from *Penicillium* spp. Therefore, the culture of fungal endophytes will continue to develop and new techniques and media will emerge as we learn more about the types of fungi that exist as endophytes, particularly through the use of culture-independent techniques.

1.6.2 Culture-independent methods

The term 'culture-independent' commonly refers to microbial research which does not require the axenic culture of the organism being studied. Stating that most microorganisms cannot be cultured on growth media, Su *et al*. (2012) comprehensively reviewed the various methods used to study environmental microorganisms *in situ*, including spectrometric detection, fluorescent *in situ* hybridization and microarrays, as well as DNA-based studies by PCR and the derivatives thereof. Considering that Hawksworth (1991) estimated that 83% of known fungi cannot be readily cultured, the methodologies that were discussed by Su *et al*. (2012) are readily applicable to fungal endophyte studies as well. These methods can be employed to study functional aspects of endophyte colonization, but often the aim of such work is to identify the range of fungi that have colonized the host plant.

The use of molecular methods to identify endophytes is a relatively recent approach, and although there are limitations, it is likely to shed more light on endophytic populations than currently exists on the various taxonomic and functional aspects of plant colonization by microbes (Brundrett, 2006), especially since it can simultaneously detect those organisms which can be cultured and those which cannot (van Overbeek *et al*., 2006). As discussed previously, the culture of plant-associated microbes is understood to provide a limited representation of the total population of microbes that inhabit plant tissues (Bayman and Otero, 2006; Hallmann *et al*., 2006), while culture-independent methods are considered to

be better at revealing the true composition of fungal communities within plant tissues (Yang *et al*., 2001; Ma *et al*., 2005; Unterseher *et al*., 2016). A good example of this is in research on arbuscular mycorrhizal composition (where axenic culture is not possible), where Ma *et al.* (2005) found that culture-independent analysis of root material showed greater diversity than spore inspections.

Culture-independent fungal endophyte research methods tend to refer largely to those which can detect, quantify and characterize fungal endophytes by the PCR-based detection of targeted DNA sequences within the host after the extraction of total host and endophyte DNA (Hallmann *et al*, 2006). This is a highly sensitive technique that requires the removal of contaminating non-endophytic extraneous DNA. Burgdorf *et al.* (2014) demonstrated that surface decontamination methods can influence the detection of fungal endophytes and the apparent composition of the population within the host. Therefore, PCR-based fungal endophyte analyses also have factors that can bias results.

Primer choice can also introduce bias to PCR-based studies of fungal endophyte composition. This choice is governed by the region of interest, although many primer combinations can exist for individual regions, as evidenced by the various primers that amplify the fungal ITS region, as discussed by Martin and Rygiewicz (2005). While several regions have been used for phylogenetic analyses of fungal endophytes, e.g., elongation factor and tubulin genes (Zhu *et al*., 2013), those that amplify the ITS regions are most commonly used to elucidate fungal species composition (Schoch *et al*., 2012). Primers that amplify this region in eukaryotic organism were first developed by White *et al*. (1990) from ribosomal RNA sequences. Subsequently, the suitability of the ITS region for fungal diversity studies, and the strengths and weaknesses thereof have been discussed in great depth (Nilsson *et al*., 2008; Nilsson *et al*., 2009; Lindner and Banik, 2011; Schoch *et al*., 2012; Blaalid *et al*., 2013). This region continues to be used in fungal endophyte composition studies (Christian *et al*., 2016, Ofek-Lalzar *et al*., 2016, Unterseher *et al*., 2016); however, scrutiny has revealed bias from priming sequences used in the amplification of ITS sequences (Bellemain *et al*., 2010). This bias is caused by mismatches to the priming sequences, which result in some target sequences being amplified more efficiently than others. New primers

are continually developed to address this problem (Ihrmark *et al*., 2012; Toju *et al*., 2012). However, a better option may be the use of blocking primers, as shown by Burgdorf *et al.* (2016a), where universal eukaryotic primers are used to anneal to highly conserved flanking ribosomal DNA sequences, but host sequences are prevented from being amplified by prior annealing of non-extendable oligonucleotide sequences.

After PCR of the target fungal sequences (in many cases, the ITS region) from the mixed host and endophyte template DNA, several downstream procedures can be employed to compare endophyte population composition between host plants. Muyzer (1999) suggested a protocol for culture-independent microbial community studies, starting with total DNA extraction and ending either with sequence analysis or FISH probe development for microscopy, as shown in Figure 1.3.

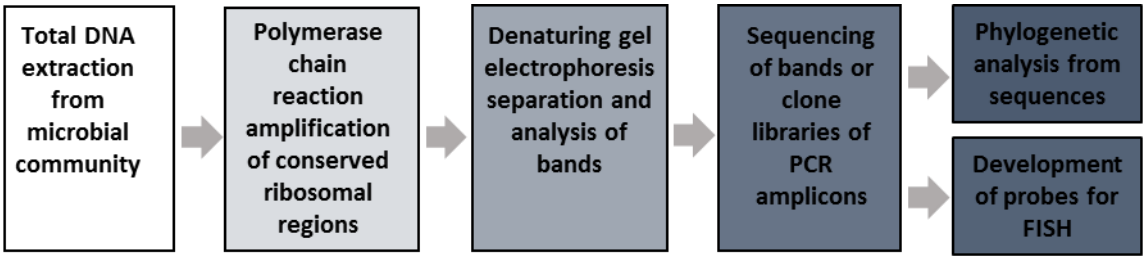

Figure 1.3 Common culture-independent approach to environmental diversity studies adapted from Muyzer (1999).

Many studies on microbial community composition have utilized techniques such as terminal restriction fragment length polymorphisms (T-RFLP's) analysis and the production of clone libraries (Hallmann *et al*., 2006); however denaturing gel electrophoresis analysis of PCR amplified ITS fragments has been a popular choice for such research (Muyzer, 1999; Garbeva *et al.*, 2001; Yang *et al.*, 2001).

Denaturing gel electrophoresis (DGE) was described by Fromin *et al.* (2002) as having three main forms, i.e., denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and temporal temperature gel electrophoresis (TTGE). The principle of all DGE is the same, whereby the migration of PCR amplification products, or amplicons, that have different DNA sequences will migrate to a different position in a denaturing gel

because of the varying rate at which they denature stops or slows their migration in the gel, as illustrated in Figure 1.4 (Muyzer, 1999; Ercolini, 2004).

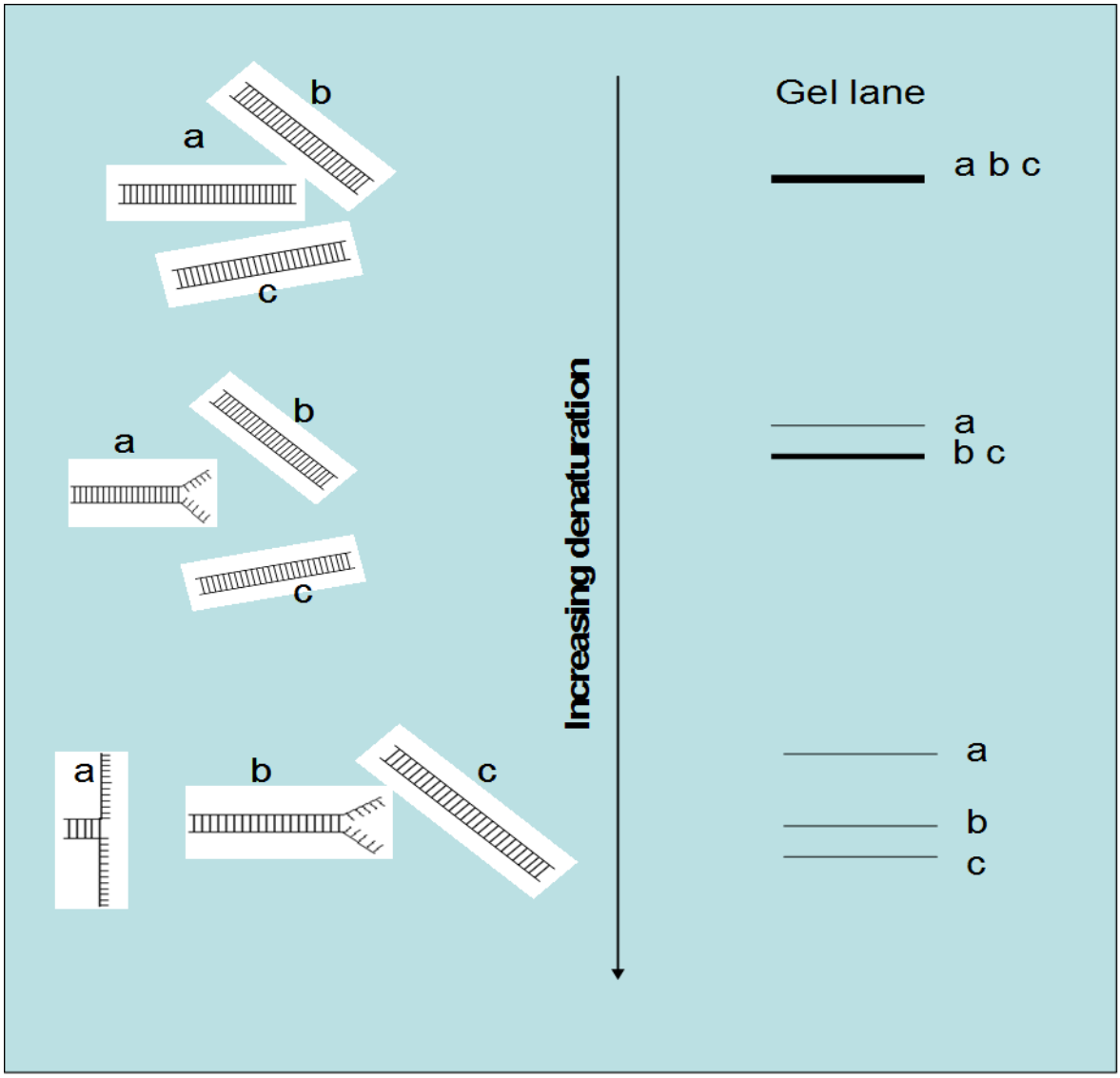

Figure 1.4 An illustration of the effect of a denaturation gradient gel on mixed amplicons 'a', 'b' and 'c' which separate as they move along a denaturing gradient. As amplicon 'a' denatures due to its lower denaturing properties, its movement is slowed and it separates from 'b' and 'c'. All amplicons eventually separate according to their sequence differences.

The various forms of DGE have been developed as standard tools in microbial diversity studies (Muyzer and Smalla, 1998; Muyzer, 1999; Ercolini, 2004; Mühling *et al.*, 2008) and DGGE has been used in a variety of endophyte studies (Garbeva *et al.*, 2001; Ma *et al.*, 2005; Duong *et al.*, 2006; van Overbeek *et al.*, 2006; Andreote *et al.*, 2010; Fernandes *et al.*, 2015).

In 2006 it was noted that, until then, TGGE had not yet been applied to endophyte studies (van Overbeek *et al*., 2006). However, TTGE has been used in microbial diversity studies (Ogier *et al.*, 2002) and Likar and Regvar (2009) used this method in a study of the fungal endophyte diversity in the roots of plants grown in heavy metal polluted soils based on their belief that it was more reproducible than DGGE. This assertion was ascribed to the work by Yoshino *et al.* (1991), although they did not evaluate DGGE as a comparative method, stating that their TTGE results were supported by TGGE and that the resulting gels were of good quality. Aside from that paper, there do not appear to be any published comparisons between methods to establish their suitability for fungal endophyte studies. These would be useful to select the variant of DGE to be used in endophyte studies.

Muyzer *et al.* (1993) and Murray *et al.*(1996) suggested that the gel-banding profiles produced by DGE may not represent all the organisms present in a habitat because multiple taxa could be represented by a single band. On the other hand, Wakelin *et al.* (2007) regarded distinct bands as operational taxonomic units (OTUs), with their intensities reflecting their relative abundance. Adding to this, a study on ericoid mycorrhizal fungi revealed that multiple sequences represented a single fungal organism, due to heterogeneous repeats of ribosomal genes or differences in alleles with dikaryotic nuclei (Allen *et al.*, 2003). This intragenomic variation of ITS sequences could result in inflated estimates of OTU diversity (Lindner and Banik, 2011). From a DGE perspective, it means that multiple bands could represent a single organism. However, this may not be an issue when comparing community profiles, where specific bands matter less than the general community changes that occur over time or between hosts. Therefore, despite some limitations, PCR-DGGE is a technique that will probably continue to be used for some time, even with the current and future advances in DNA sequencing technology (Izard, 2015).

DNA sequencing has been used extensively in research on fungal endophyte diversity. Clone libraries have been produced from PCR amplification of mixed-template genomic DNA targeting the ITS region (Gao *et al*., 2005), followed by Sanger sequencing (Sanger *et al*., 1977). This approach identified the most frequent fungal endophyte sequences that were detected within the host tissue. The procedures in these studies were laborious and costly,

using ‘high-throughput’ multi-capillary sequencing systems to produce clone libraries of a few hundred sequences that were distributed amongst multiple samples, resulting in low sequence depth (Vandenkoornhuyse et al., 2002; O'Brien et al., 2005; Parfitt et al., 2010). By contrast, the development of next-generation sequencing (NGS) in the last decade has revolutionized the tools available for research into fungal endophyte composition within the host plant. The process for species composition analysis commonly follows pre-sequencing PCR-enrichment of taxonomically informative regions, such as the ITS1 or ITS2 region, as used by Bullington and Larkin (2015) to investigate foliar pine fungal endophytes. Several million sequence reads and over a thousand OTUs were generated from less than 10 samples, indicating that depth of sampling is several orders of magnitude greater than for the approach of clone library development. This sequence depth has allowed for reduced sample replicates numbers in culture-independent studies exploring microbial diversity from metagenomic DNA samples (Smith and Peay, 2014).

A variety of technologies exist that are classified as NGS have been emerged since 2005. These technologies use approaches that can be separated into two major categories, i.e., sequencing by synthesis and single-molecule sequencing. The main sequencing by synthesis platforms are the Roche, Illumina and Life Technologies systems. These platforms all make use of PCR during library preparation and sequence detection, but differ in the detection methods. The Roche 454 system uses an emulsion PCR enrichment step, followed by sequence detection via the production of light from the release of a pyrophosphate molecule during PCR synthesis. The Illumina MiSeq® and HiSeq® systems also use an enrichment step producing immobilized sequence clusters on a flow cell. These clusters then undergo PCR synthesis with fluorescently labelled nucleotides and the light released during PCR nucleotide incorporation reveals the cluster sequences. The Life Technologies Ion Torrent™ detects the sequences of DNA within a DNA library, during PCR synthesis, by measuring the hydrogen ion concentration as synthesis proceeds. The single molecule sequence platforms, namely the Helicos Bisociences HeliScope™ and the Pacific Biosciences SMRT Sequencer differ from the previously mentioned systems in that they do not utilize a pre-sequencing enrichment PCR step. However, the sequencing process also makes use of PCR synthesis for nucleotide detection. These NGS platforms and technologies have varying

capabilities in terms of read length and run time. Each system has advantages and disadvantages; however, in general the sequence depth of all of these systems enables the detection of small community composition differences that were undetectable with the prior approach of clone libraries and Sanger sequencing (Shokralla *et al.,* 2012).

However, an issue that emerges from NGS studies is the large quantity of data produced. The analysis of such data presents new challenges that were not present with the relatively small numbers of samples and sequences involved in clone library sequencing projects. Lindahl *et al*. (2013) provided a comprehensive guide to the various NGS software pipelines, such as QIIME, MOTHUR, and SCATA, which assign OTUs to sequences, interpret the data and, in some cases, present the data for biological interpretation. They also detailed the various procedures and platforms used for designing and preparing studies on fungal community composition. This provides an invaluable guide for researchers engaging in such work.

Increasingly, NGS is being applied to fungal endophyte studies. These studies are revealing interesting aspects of fungal endophyte and plant pathogen diversity within host plants; however, it is being found that data handling can also affect the conclusions made from such analyses (Kemler *et al*., 2013). Undoubtedly, the methods of handling the information derived from NGS will evolve as quickly as the technology itself, and this will be to the benefit of endophyte research and its applications.

1.7 Applications of fungal endophytes

The symbioses between plants and fungal endophytes are a major motivation for fungal endophyte research because of their ecological role in natural systems and their potential impact on agricultural production systems. Endophytes are regarded as having significant potential in the commercial application of biological control and plant growth promotion (Compant *et al*., 2005) and they are used in commercial agriculture (Kloepper and Ryu, 2006). The regulation of the composition of endophytes in commercially important plants, such as cereal crops is starting to be regarded as being a vital aspect in plant breeding since the presence and variety of endophytes within a host can be more influential on plant properties than the genetic differences between host cultivars (Easton, 2007). Some plant

breeders even screen for 'endophyte enhanced' hosts (Rodriguez *et al*., 2009b). Within the host, fungal endophytes receive nutrients and protection. In return, they provide plant growth promoting substances, as well as compounds that help protect the plant from herbivores (Choudhary, 2012), pathogens (Busby *et al*., 2015) and abiotic stress (Azad and Kaminskyj, 2016). Some endophytes produce insecticidal secondary metabolites that deter herbivorous insects (Schulz, 2006; Kuldau and Bacon, 2008) and others improve plant tolerance of insect herbivory (Cosme *et al*., 2016). Fungal endophytes also appear to be able to enhance host plant defences against herbivores and microbial pathogens by 'priming' the plant defence mechanisms (Pieterse *et al*., 2014).

It is evident that fungal endophytes can contribute to improvements in crop production. This has been accepted for AM fungi that have demonstrated that they are effective in a range of beneficial activities including plant growth promotion, heightened fecundity, drought tolerance, disease inhibition and the increased uptake of important nutrients such as nitrogen and phosphate (Ma *et al*., 2005). The various growth enhancing and protective traits that other groups of endophytes can bestow on crop plants is being explored further.

1.7.1 Plant growth promotion by endophytes

Under typical conditions, plant growth is regulated by nutrient availability and plant growth hormones. These are both aspects that can be mediated by fungal endophytes. The endophytes receive the nutrients they require for growth from the host plant and in turn produce chemicals that promote healthy growth in the plant; they can also assist in the retrieval of essential nutrients from the environment (Mayerhofer *et al*. 2012). Plants have been observed using endophytic organisms as a heterotrophic nutrient source (Paungfoo-Lonhienne *et al*., 2010), indicating that fungal endophytes can serve as nutrient reserves.

Endophytic plant growth promoting fungi can produce the growth promoting hormones gibberellic acid and indoleacetic acid which may significantly increase the development of host biomass (Waqas *et al*., 2012). Khan *et al*. (2012) found that many endophytic fungal isolates can promote plant growth; however, some isolates also inhibited growth. It seems there may be trade-offs in the composition of the endophyte mycobiome. Perhaps some endophytes provide other benefits, such as protection from herbivory, which requires the

release of secondary metabolites that are inhibitory to plant growth. Other fungal endophytes might be recruited to mitigate these effects. An example of this reciprocal phenomenon can be seen with the fungal endophyte, *Piriformospora indica* that was discovered by Verma *et al*. (1998). The improved growth of tobacco plants colonized by *P. indica* reduced plant resistance to herbivory (Barazani *et al*., 2005). Therefore, fungal endophyte combinations need to be explored to deliver successful outcomes from endophyte applications.

The varied outcomes of fungal endophyte applications have received scrutiny. In a meta-analysis of existing research Newsham (2011) found that DSE usually have a positive effect on host growth, particularly when most of the nitrogen is available in organic form. Neutral to negative effects arising from applications of ascomycetous root endophytes were found in a meta-analysis by Mayerhofer *et al*. (2012); however, they suggested that the substantial differences between experimental conditions between the studies that were analyzed may have contributed to this finding. It may be that underlying factors, such as the 'competence' of organisms within the environment, need to be considered more closely. Sturz and Nowak (2000) proposed that some biological control agents (BCAs) perform poorly because of poor rhizosphere competence. Similarly, Busby (2015) suggested that variable environmental biotic and abiotic factors can cause varied fungal endophyte performance in terms of plant growth promotion. Therefore, as the numbers of studies increase, the optimal conditions will be revealed for harnessing the growth promotion by endophytes, as well as protection from herbivores and pathogens.

1.7.2 Plant protection

Plants defend themselves against both biotic and abiotic stresses. Biotic stresses include herbivory or infection by pathogens. Abiotic stresses include problems such as excessive heat and insufficient water. Microbes such as fungal endophytes assist in the protection of plants against these challenges (Choudhary, 2012). Fungal endophytes have an advantage when compared to other disease control approaches due to their ability to colonize plant tissue and form relatively stable *in planta* communities (Berretta *et al*., 1998).

Fungal endophytes can be deterrents to herbivores as well as being antagonistic to microbial pathogens (Carroll, 1988), therefore demonstrating potential as BCAs. Interest in biological control has increased due to the rising cost of pesticides, reduced efficacy of these pesticides and the increased demand for pest control agents that do not contain chemical pesticides. In addition, BCAs have the benefit of being regarded as 'low-input practical agents'. (Compant *et al*., 2005). Fungal endophytes can protect against insect herbivores (Azevedo *et al*., 2000; Akello *et al*., 2008; Backman and Sikora, 2008; Vega *et al*., 2008) and this characteristic is often linked to the ability of the fungus to produce toxins that reduce plant palatability (Azevedo *et al*., 2000; Vega *et al*., 2008). There have also been circumstances where fungal endophytes produce alkaloids that deter feeding on plants by undesirable pests but do not appear to affect palatability to farm animals or their well-being (Johnson, 2008). This is a highly desirable symbiosis from an agricultural perspective.

There are several examples of endophytic fungi that are harnessed to protect crops and some popular species of fungal endophytes exist. The entomopathogenic fungus *Beauveria bassiana* has been commercially applied as a microbial insecticide (Gillespie, 1988), protecting cotton and tomatoes against fungal plant pathogens as well as inducing systemic resistance against certain bacterial diseases (Ownley *et al*., 2008). *Beauveria bassiana* is regarded as a 'dual-purpose' agent of biological control (Ownley *et al*., 2004). This naturally occurring endophyte has potential for use against insect pests in the important South African Rooibos herbal tea crop (Hatting *et al.*, 2016). Aside from *Beauveria* sp., a plethora of entomopathogenic fungi exists (Quinlan, 1988) that can exist as endophytes and protect the host plants from insects.

Fungal endophytes can also be antagonistic to microbial pathogens (Carroll, 1988). However, while endophytes can modify plant disease, the extent of this modification is 'context-dependent', i.e., a plant-protecting endophyte of one host species may not be effective in another host; more research is necessary in this regard and blanket allocation of plant-beneficial properties to single endophyte species may not be possible, considering the ecological complexity and interactions within a particular host (Busby *et al*., 2015). Nonetheless, endophytes have been shown to elicit systemic protection against disease,

thereby reducing the incidence and severity thereof (Kloepper and Ryu, 2006). In addition, plant-associated microbes are implicated in priming, where plants have a heightened ability to activate defensive responses to stress or pathogens (Beckers and Conrath, 2007). Furthermore, fungi such as endophytic *Trichoderma* sp. can assist plant defenses by induced systemic resistance (ISR) (Pieterse, 2014). Vallad and Goodman (2004) discussed ISR and systemic acquired resistance (SAR) extensively, as well as the role of beneficial microbes such as endophytic fungi in these forms of induced resistance. Therefore, endophytic fungi fulfill essential roles in plant immunity and resistance against pathogens. Fungal endophytes are considered as the plant equivalent of animal mobile immune cells (Talbot, 2015). This aspect of fungal endophytes put them in a different light, prompting the search for more allies against crop diseases.

It has been noted that while endophytes are largely antagonistic to pathogens, occasionally they can assist pathogens instead (Busby *et al*., 2015). Another negative aspect of plant protection by fungal endophytes is the production of mycotoxins by certain fungi in food crops that are of potential danger to humans and livestock (Bacon and Yates, 2006). These mycotoxins are secondary metabolites that are often very stable. Examples of mycotoxins include ergot poisoning by the sclerotia of *Claviceps purpurea* in grain as well as the mutagenic and carcinogenic substances produced by members of the *Penicillium*, *Fusarium*, *Aspergillus*, *Rhizoctonia*, *Pithomyces*, *Stachybotrys*, *Cladosporium* and *Alternaria* genera. Mycotoxins belong to groups of chemicals such as the ergot alkaloids, aflatoxins, and trichothecenes. Aside from symptomatic diagnosis of humans or animals exposed to mycotoxins, they can be detected in foods by chromatography techniques (Kendrick, 1992) to try and prevent exposure. These mycotoxins are believed to inhibit bacterial quorum sensing, therefore protecting plants against bacterial pathogens (Bacon and White, 2015). While undesirable in terms of food consumption, mycotoxin-producing endophytes could be of use in the production of crops grown for fibre or fuel.

As factors such as climate change cause greater levels of abiotic stress in agricultural environments, endophytes may begin to play an important role because they can protect plants from abiotic stresses (Rodriguez *et al*., 2009b) such as heat and drought (Hubbard *et*

al., 2014). Even the clavicipitaceous fungi protect their hosts from abiotic stress (Kuldau and Bacon, 2008). For example, the fungal endophyte *P. indica* can increase the salt-tolerance of barley in addition to tolerance to several other forms of abiotic stress (Baltruschat *et al*., 2008). Murphy *et al*. (2015) demonstrated how endophytes enabled barley to survive under conditions of abiotic stress. It has been found that beneficial fungal endophytes significantly reduced the levels of abscisic acid to increase abiotic stress tolerance. Depending on the type of stress, the fungal endophytes produced varying effects on levels of jasmonic and salicylic acid. This shows that fungal endophytes synchronize with the endogenous mechanisms of plants for dealing with such abiotic stress (Waqas *et al*., 2012).

The abiotic stress-mitigating mechanisms that fungal endophytes possess enhance the abilities that plants have for dealing with abiotic factors that can affect crop production. Plant breeding addresses such problems but it may be worth considering that fungal endophytes could significantly advance the capacity of crops to be grown under increasingly challenging conditions brought about by climate change and increasing anthropogenic pressures such as water scarcity and pollution (Redman *et al*., 2011).

1.7.3 Phytoremediation

An area that has also received attention is the role of fungal endophytes in phytoremediation. Phytoremediation generally refers to the remediation of environments, often soils, from pollutants such as petroleum-related hydrocarbons or heavy metals (Weyens *et al*., 2009). An investigation by Soleimani *et al*., (2010) revealed that grasses infected with symbiotic endophytes may be more efficient at removing petroleum pollutants. Endophytes are not only able to assist in phytoremediation due to their plant growth promoting and plant protecting properties, but also because they can sequester heavy metals (Porras-Alfaro and Bayman, 2011). The presence of DSE fungi in heavy metal rich soils has been studied and it was found that their levels remained unchanged and they continued to confer beneficial properties such as growth promotion onto the host (Berthelot *et al*., 2016). Research on the role of endophytes in phytoremediation indicates that they assist plants in growing in such hostile environments by producing growth stimulating plant hormones and increasing the uptake of nutrients (Li *et al*., 2012).

1.7.4 Novel chemicals

Fungi produce some of the most important chemicals to human health such as antibiotics, e.g., penicillin, and immunosuppressant drugs, e.g., cyclosporine. As producers of biologically active compounds, fungal endophytes may yield products that could aid in the fight against antibiotic-resistant bacteria, amongst other challenges in human health, such as cancer treatment (Strobel, 2003). The expensive cancer treatment drug, Taxol, is produced by *Paraconiothyrium* sp., a fungal endophyte isolated from yew trees that were the original source of the drug (Talbot, 2015). Kharwar *et al*. (2011) produced an extensive review of the 'anti-cancer' metabolites produced by fungal endophytes, which revealed a great diversity of chemicals with pharmaceutical potential. Fungal endophytes also produce chemicals that have agricultural applications, since they exist within the plants that protective or growth promoting strategies are intended for (Schulz *et al*., 2002). Strobel (2006) discovered a tropical endophyte producing biologically active volatile organic compounds (VOC) of interest to both the pharmaceutical industry and agriculture because they display activity against both human and plant pathogens. The characterization of novel chemicals produced by fungal endophytes may deliver agricultural chemicals, such as the strobilurin fungicides that were discovered to be produced by a saprophytic fungus (Anke *et al*., 1977). Such chemicals are important in the production of food crops such as wheat.

1.8 Wheat, its fungal pathogens, and endophytes

Wheat belongs to the genus *Triticum* and the grass family known as the Poaceae (Mauseth, 2003) of which there are an estimated 10,000 members (Kuldau and Bacon, 2008). Like many other kinds of grass such as maize, rice, and oats, wheat is a major food crop (Gustafson *et al*., 2009). Common or bread wheat is one of many species of wheat and while there are large numbers of cultivars of this species, collectively all AABBDD hexaploid wheat species are referred to as *Triticum aestivum* (Peterson, 1965). Henceforth any use of the word wheat will imply *T. aestivum*.

1.8.1 Wheat biology and cultivation in South Africa

Wheat is a cereal crop that is generally cultivated in the cooler seasons in South Africa. South African spring and winter wheat varieties differ in that the latter require prolonged cold for vernalisation (i.e. the requirement of exposure to cold temperatures that some plants need to start flowering), while the former do not (Scott, 1990). Under appropriate conditions in South Africa, wheat sown to at a depth of around 2.5 to7.5 cm (Peterson, 1965) will go through the growth stages described by Tottman (1987).

Wheat is grown in both the summer rainfall region (Mpumalanga, Gauteng, Limpopo, Eastern Cape interior, Central Free State, Eastern Free State, North Western Free State, South Western Free State and KwaZulu-Natal (Figure 1.5) and the winter rainfall region (Western Cape) of South Africa under irrigated and dryland production conditions, respectively (Burger and Kilian, 2009). Yields in South Africa tend to be lower than those achieved in Europe and America due to poor soils, drought, and disease (Scott, 1990).

Figure 1.5 Wheat grown at Winterton, KwaZulu-Natal, South Africa photographed at various stages during the growing season from June (a) through September (b) to December (c) 2009 (Images were taken by Richard Burgdorf).

Several cultivars are available in South Africa, selected according to their specific disease resistance or tolerance of conditions such as aluminium toxicity or drought. These cultivars are bred by the Small Grains Institute (SGI) of the Agricultural Research Council (ARC), and are produced by seed companies such as PANNAR, Monsanto, and Sensako. A comprehensive report on these cultivars is available from the Agricultural Research Council's Small Grains Institute, which describes the cultivars and their disease resistance and

susceptibility to common wheat diseases (Burger and Kilian, 2009). These diseases also require consideration in relation to the fungal endophytes wheat.

1.8.2 Wheat fungal pathogens

As per the definition of an endophyte by Wilson (1995) and the observations by Photita *et al.*(2004) that latent pathogens can also exist as endophytes, a list of microbes that are known wheat pathogens is provided. Several of these organisms have been detected in plants during research into wheat endophyte diversity and composition (Crous *et al*., 1995). Scott (1990) described the common fungal diseases of wheat in South Africa, as listed in Table 1.2. It should be noted that the root rot caused by *Pythium* spp. was not included in the table as they probably cause immediate disease symptoms and are not members of the fungal kingdom. It would, however, be worth investigating whether there are any oomycetes that exist as endophytes as few reports exist in the literature on this topic.

Table 1.2 A list of fungal diseases of wheat in South Africa (Scott, 1990).

Disease	Causal agent
Basal stem rot	*Sclerotium rolfsii* Sacc.
Common root rot	*Bipolaris sorokiniana* (Sacc.) Schoem. (synonym *Helminthosporium sativum* P.K. and B.; teleomorph *Cochliobolus sativus* (Ito and Kurib.) Drechs.)
Crater disease	*Rhizoctonia solani* Kühn
Crown rot	*Fusarium graminearum* Schw. , *Fusarium* spp.
Eyespot	*Pseudocercosporella herpotrichiodes* (Fron.) Deid.
Glume blotch	*Septoria nodorum* (Berk.) Berk. (teleomorph *Leptosphaeria nodorum* Müll.)
Head blight	*Gibberella zea* (Schw.) Petch. (anamorph Fusarium graminearum Schw.) *Fusarium* spp.
Leaf rust	*Puccinia recondita* Rob. Ex Desm. F. sp. *tritici*
Loose smut	*Ustilago tritici* (Pers.) Rostr. (synonym *Ustilago nuda* var. *Tritici* Schaffn.)
Powdery mildew	*Erysiphe graminis* D.C. f. sp. *tritici* E. Marchal
Speckled leaf blotch	*Septoria tritici* (Rob.) E. Desm. (teleomorph *Mycosphaerella graminicola* (Fück.) Schroeter)
Stem rust	*Puccinia graminis* Pers. F. sp. *tritici* Eriks. and Henn.
Stinking smut	*Tilletia* spp.
Take-all	*Gaeumannomyces graminis* (Sacc.) Arx and Oliv. var. *tritici* Walker; *Magnaporthe rhizophila* Scott and Deacon
Tan spot	*Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph *Drechslera tritici-repentis* (Died.) Schoem. synonym *Helminthosporium tritici-repentis* Died.)

The significant cost of crop losses and pesticide applications encourages the investigation into avirulent forms of these fungal pathogens existing as fungal endophytes. Hypovirulent forms of fungal pathogens can occupy the pathogenic counterpart's niche. Such non-pathogenic forms can be created by exposure to certain viruses (Sneh, 1998). This could be an area of exploration to convert fungal wheat pathogens to endophytes.

1.8.3 Wheat fungal endophytes

Due to its global importance as a food crop, the diversity and function of fungal wheat endophytes have received attention. Several studies have investigated on the diversity of endophytic fungi within wheat (Sieber *et al*., 1988; 1995; Crous *et al*., 1995; Larran *et al*., 2002). Fungal endophytes have been described in some culture-based studies are shown in Table 1.3.

Table 1.3 Previously isolated fungal wheat endophytes from two studies performed in South Africa and Argentina. Genera common to both studies are highlighted.

Crous *et al*. (1995), South Africa	**Larran *et al*. (2002), Argentina**
Alternaria alternata (Fr.) Keisler	*Alternaria alternata*, *Alternaria* spp.
Ascochyta sp., *Acremonium* sp.	*Arthrinium* sp. *Aspergillus* sp.
Bipolaris australis Alcorn	*Bipolaris* sp., *Bipolaris cynodontis* (Marig.) Schoem., *Bipolaris sorokiniana* (Sacc.) Schoem.
Brachysporiella setosa (Berk. and M.A. Curtis) M.B. Ellis	
Chaetomium sp.	*Chaetomium globosum* Kunze *ex* Fries
Cladosporium sp.	*Cladosporium herbarum* (Pers.) Link
Coniothyrium sp. *Cladorrhinum* sp., *Cochliobolus sativus* (S. Ito and Kurib.) Drechsler *ex* Dastur, *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., *Cylindrocarpon destructans* (Zinssm.) Scholten, *Didymella* sp.	*Cryptococcus* sp.
Epicoccum nigrum Link	*Epicoccum nigrum*
Fusarium avenaceum (Fr.) Sacc. *Fusarium acuminatum* Ellis and Everh., *Fusarium culmorum* (W.G. Sm.) Sacc., *Fusarium equiseti* (Corda) Sacc., *Fusarium oxysporum* Schltdl., *Fusarium scirpi* Lambotte and Fautrey	*Fusarium* sp.
Microdochium bolleyi (R. Sprague) de Hoog and Herm.-Nijh., *Nigrospora sphaerica* (Sacc.) Mason, *Periconia* sp.	*Penicillium* sp.
Phoma glomerata (Cda) Wollenw. and Hochapf.	*Phoma* sp.
Phomopsis sp.	*Phomopsis* sp.
Pleospora herbarum (Pers.) Rabenh.	*Pleospora herbarum* (Pers.) Rabenh.
Stagonospora nodorum (Berk.) Cast. and Germ., *Truncatella angustata* (Pers.) S. Hughes, *Geotrichum candidum* Link, *Gliocladium roseum* Bainier, *Gliocladium* sp., *Gnomonia* sp., *Hyalodendron* sp., *Leptosphaeria* sp., *Ophiobolus* sp., *Periconia* sp., *Phialophora* sp., *Pyrenophora tritici-repentis, Pyrenophora* sp., *Phaeoseptoria* sp., *Pyrenochaeta* sp., *Pythium* sp., *Robillarda sessilis* (Sacc.) Sacc., *Septoria tritici, Sporormiella australis* (Speg.) S.I. Ahmed and Cain, *Verticillium sp.*	*Rhodotorula rubra* Harrison, *Stemphylium* sp.

In both the studies listed in Table 1.3 the composition of fungal species from multiple cultivars was investigated but no significant interaction between the microorganisms and cultivars were found. However, using culture-independent methods, Sapkota *et al*. (2015) found that the host cultivar influenced the composition of the phyllosphere microbiome. In addition, a culture-independent study of South African fungal wheat endophytes revealed a significant cultivar x organ x fungal endophyte interaction, indicating that while host genotype effects may not be obvious, possibly diminishing as phenotypic differences become smaller, such effects do exist and may offer routes for harnessing fungal endophytes for increased wheat production (Burgdorf *et al.*, 2016b)

Culture-independent studies have helped develop knowledge on the diversity and functions of fungal endophytes; however, culture-based studies are still relevant in the discovery and application of biological control in wheat. A *Microdochium bolleyi* strain has been shown to inhibit S*eptoria nodorum,* as well as *Fusarium* and *Gaeumannomyces* species (Sieber and Grünig, 2006). In another example, the colonization of wheat roots by *Piriformospora indica* resulted in increased biomass under conditions of low fertility and reduced the severity of infection by *Pseudocercosporella herpotrichoides* under field conditions (Serfling *et al*., 2007) (Note: The field trials were conducted in Germany and the authors of the study recommended that *P. indica* may be better suited to tropical and sub-tropical regions. This organism should be investigated as an endophyte of South African crops, including wheat). Several fungal endophytes have been shown to suppress *Pyrenophora tritici-repentis*, which causes tan spot (Larran *et al*., 2016). Evidently, the discovery of these BCAs and the potential benefits for wheat production encourages further research on fungal endophytes in the growth promotion and the control of wheat diseases. Further research could reveal fungal endophytes that are active against the diseases listed in Table 1.2. They may also benefit crop production in the presence of other environmental challenges. Fungal endophytes have demonstrated the ability to mitigate abiotic stress, i.e., heat stress and drought, in wheat. These endophytes may also enhance the germination of second generation seeds (Hubbard *et al*., 2014). This is an epigenetic effect and Hubbard *et al.* (2014) correctly emphasize that this phenomenon requires further investigation for its future potential in wheat cultivation.

Methods of isolating, identifying and characterizing endophytes continue to produce a greater understanding of their biology. This reveals new areas in which fungal endophytes and their chemical products can be applied.

1.9 The future of endophyte research

Endophytes will play a greater future role in agriculture with the advance of climate change, resulting in raised temperatures, waterlogging and drought. It has been argued that greater attention should be given to the use of fungal endophytes in cereal production because of their great potential for the control of diseases (particularly in the EU with increasing restrictions on the use of chemical pesticides) (O'Hanlon *et al*., 2012). A deeper understanding of the factors that affect the colonization of host plants by fungal endophytes, for example, as demonstrated by the influence of the hosts genome on mycobiome composition (Sapkota *et al*., 2015; Burgdorf *et al*., 2016b) could develop 'integrated plant breeding' as a complementary approach alongside integrated pest management, to enhance crop yields in the presence of increased pesticide resistance, water scarcity and soil infertility.

Further to the development of a better understanding of the factors that govern endophytic biology, it has been suggested that the complex multitrophic interactions between different endophytic taxa, e.g., bacteria and fungi, are not clearly understood despite their significance (van Overbeek and Saikkonen, 2016). The development of NGS and advanced protein analysis, forming the various "meta'omics" (e.g. metagenomics, transcriptomics, and proteomics), should enable better understanding to be developed of these complex biological systems and their interactions, as in the case of wheat endophytes. In addition, it has been advocated that better databases should be developed in conjunction with modern methods that generate such large amounts of data; it has also been advocated that there is a greater global interaction between researchers engaging in this type of research to utilize and make sense of this data (Peršoh, 2015). In the context of this review it is evident that wheat fungal endophyte research spans several continents but there is little evidence of inter-continental collaboration on wheat endophytes, as is the case with rust pathogens that require international co-operation to combat these major threats to food security (Byerlee

and Moya, 1993; Lantican *et al*., 2005). Future consideration should be made for fungal endophytes at international forums for wheat and other important crops.

The knowledge of endophyte diversity and biology will inevitably increase over time. With it will come a new and deeper understanding of the symbioses between living organisms; new chemicals for health, agriculture and industry; and new agricultural practices and paradigms. From what was once regarded as a 'contaminant', endophytes have come a long way and have emerged as an often astonishing area of research that could have profound implications for humans and the world they inhabit. The future of endophyte research holds great appeal for scientists in a wide range of disciplines and exciting discoveries await them.

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Chapter 2 A procedure to evaluate the efficiency of surface sterilization methods in culture-independent fungal endophyte studies[1]

2.1 Abstract

Extraneous DNA interferes with PCR studies of endophytic fungi. A procedure was developed with which to evaluate the removal of extraneous DNA. Wheat (*Triticum aestivum*) leaves were sprayed with *Saccharomyces cerevisiae* and then subjected to physical and chemical surface treatments. The fungal ITS1 products were amplified from whole tissue DNA extractions. ANOVA was performed on the DNA bands representing *S. cerevisiae* on the agarose gel. Band profile comparisons using permutational multivariate ANOVA (PERMANOVA) and non-metric multidimensional scaling (NMDS) were performed on DGGE gel data, and band numbers were compared between treatments. Leaf surfaces were viewed under Variable Pressure Scanning Electron Microscopy (VPSEM). Yeast band analysis of the agarose gel showed that there was no significant difference in the mean band DNA quantity after physical and chemical treatments, but they both differed significantly ($p < 0.05$) from the untreated control. PERMANOVA revealed a significant difference between all treatments ($p < 0.05$). The mean similarity matrix showed that the physical treatment results were more reproducible than those from the chemical treatment results. The NMDS showed that the physical treatment was the most consistent. VPSEM indicated that the physical treatment was the most effective treatment to remove surface microbes and debris. The use of molecular and microscopy methods for the post-treatment detection of yeast inoculated onto wheat leaf surfaces demonstrated the effectiveness of the surface treatment employed, and this can assist researchers in optimizing their surface sterilization techniques in DNA-based fungal endophyte studies.

Keywords: endophyte; fungi; DNA; surface sterilization

[1] This chapter is formatted as per the requirements of The Brazilian Journal of Microbiology, with the exception that the heading, figure and caption numbering has been modified.

2.2 Introduction

Endophytes are microorganisms that live within the tissues of plants without causing damage (Backman and Sikora, 2008). Research which focuses on these organisms must exclude those microbes found on the surfaces of host plants including the lipophilic waxy plant cuticle surface, which is colonized by various fungi and bacteria (Müller and Riederer, 2005). More micro-organisms are to be found on the aerial surfaces of a plant than within plant tissues (Lindow and Brandl, 2003), which emphasizes the importance of extraneous DNA removal as it can affect the conclusions of culture-independent endophyte studies. The removal of these plant surface microbes and their DNA is particularly important when using a PCR-based approach to investigate endophytes. The use of standard surface sterilization techniques employed in culture-dependent research may not guarantee the complete removal of surface organisms (Anand *et al.,* 2006; Manter *et al.*, 2010), so they cannot guarantee the removal of the DNA belonging to these organisms either, in addition to any ambient DNA that may be present. While surfaces are often tested for microbial sterility by plating the post-treated surface onto a nutrient agar (Sessitsch *et al*., 2002), Guo (2010) warned that some surface sterilization methods may not sufficiently denature epiphytic DNA in molecular studies of endophytes.

The efficiency of surface sterilization of plants in culture-based endophyte studies (Schulz *et al*., 1993), the removal of surface fungal DNA from insects (Meyer and Hoy, 2008), as well as DNA removal from the surface of bones and teeth (Kemp and Smith, 2005) has been evaluated. However, the efficiency of surface DNA removal techniques from plant tissues has not been established definitively.

The aim of this investigation was to develop a procedure in which the efficacy of commonly adopted surface sterilization approaches to removing non-endophytic DNA could be evaluated. *Saccharomyces cerevisiae* Meyen ex E.C. Hansen was used as a test organism on winter wheat (*Triticum aestivum* L.), and statistical analysis methods were employed to draw conclusions from the outcomes of surface treatments determined by the presence of PCR-detected *S. cerevisiae* and other microbial DNA. Support for these conclusions was provided by electron microscopy.

2.3 Materials and Methods

2.3.1 Wheat cultivation and sample preparation

Wheat (*Triticum aestivum* L. cv Duzi) was planted in 300 mm pots at a plant density equivalent to 47 kg of seed per hectare, on the 11th of July 2010 in Pietermaritzburg, KwaZulu-Natal, South Africa. Plants were grown outside under 10% shade, in composted pine bark, with drip irrigation providing 4:1:3 NPK (N at 200 ppm) for 10 min 3 times per day. Mean rainfall, mean high and mean low temperatures over the growth period were 0.1 mm, 24.3°C and 10.5°C[2] respectively.

Four replicate pots were used for the treatments, and for the positive control and the negative control (n = 16), arranged in a completely randomized design. On the 22nd of September 2010, at growth stage 60 (Tottman, 1987), leaves in each of the four replicates of the two treatments and the positive control pots were inoculated with 100 mL of yeast broth inoculum per pot. Inoculum was sprayed using a Fragram (Carrara, Queensland, Australia) 1.5 L pressure sprayer, which delivered the broth culture in a fine mist.

The total volume of 1.2 L of inoculum consisted of malt extract broth (MEB), made from 30 g L^{-1} malt extract (Merck, Darmstadt, Germany) and 2.5 g L^{-1} yeast extract (Merck) in distilled water. This was autoclaved for 15 min at 121°C, cooled to room temperature and inoculated with 1.7 g L^{-1} of dried *S. cerevisiae* granules (instant baking yeast from NCP, Johannesburg, South Africa). The broth was incubated in a Model TU-453 shaking incubator (MRC, Holon, Israel) at 25°C for 18 h, reaching a viable cell concentration of 1.02×10^8 cfu mL^{-1}.

2.3.2 Sample collection and surface treatments

After 72 h, during which no rain fell and mean high and mean low temperatures were 24.4°C and 12.7°C respectively, leaves were harvested. Four 0.1 g replicates of sprayed leaves were

[2] All climate data courtesy of Agrometeorology Discipline, School of Agricultural, Earth and Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa

subjected to two treatments (A and B). The experimental controls comprised a positive control (Y), with four replicates of wheat leaf tissue that were sprayed with yeast broth, but not surface treated, and a negative control (N), which consisted of four replicates of wheat leaf tissue that were not sprayed with yeast broth and were not surface treated in any way. The Physical Treatment (A) was a modification of the method used by Sessitsch *et al.* (2002). Leaf samples (0.1 g) were placed in McCartney bottles with 20 mL of a 0.01% water solution of Tween 20 (Merck) and sonicated for 5 min in a Biosonic sonication bath (Colténe/Whaledent, Altstätten, Switzerland). Leaf samples were rinsed once with tap water and once with sterile distilled water. Samples were placed in a 2 mL microtube with 1.5 mL 0.9% NaCl solution and 0.3 g of 0.1 mL acid washed beads (Sigma-Aldrich, St. Louis, MO, USA). The tubes were vortexed on a Disruptor Genie Vortex (Scientific Industries, Inc., Bohemia, NY, USA) for 20 min. Samples were rinsed three times in 1 mL of sterile ultra-pure water and then stored individually in plastic bags and frozen at -80°C before further processing.

The Chemical Treatment (B) was according to the method described by Arnold *et al.* (2007). Leaf samples (0.1 g) were immersed in a 95% ethanol solution for 5 s, followed by 2 min in a NaOCl solution (0.5% free Cl_2) and finally 2 min in 70% ethanol. After that, the samples were dried in a laminar flow hood. Samples were stored individually in plastic bags and frozen at -80°C before further processing.

2.3.3 DNA extraction and amplification

Four replicate leaf samples (0.1 g) for each treatment and controls were ground in liquid nitrogen with the addition of 0.1 mm sterile acid-washed beads (Sigma-Aldrich) and the DNA was extracted using the CTAB protocol for the Nucleospin Plant II Genomic DNA extraction kit (Macherey-Nagel, Düren, Germany). *S. cerevisiae* genomic DNA was extracted from yeast cells pelleted from 1 mL of the same broth used for inoculation, according to the same method as for the plant tissue. Working solutions of all genomic DNA were made up to a final concentration of 10 ng μL^{-1} using nuclease free water (Promega, Madison, WI, USA).

All PCR reactions were performed using the KAPA2G Fast HotStart ReadyMix Kit (Kapa Biosystems, Woburn, MA, USA) and universal fungal primers ITS1F-GC forward primer (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CTT GGT CAT TTA GAG GAA GTA A-3') and the ITS2 reverse primer (5'-TTY GCT GYG TTC TTC ATC G-3') (Wakelin *et al.*, 2007). The dNTP's were at a 0.2 mM concentration, $MgCl_2$ at 1.5 mM, and forward and reverse primers at a final concentration of 800 nM each. The final reaction volume was 20 µL. A sample of 8 ng of genomic DNA template was added to each reaction tube. PCR was performed on a G-storm Goldblock Thermal Cycler (Syngene, Cambridge, United Kingdom). The PCR program consisted of a 2 min denaturation at 95°C followed by 35 cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 10 s. This was followed by a final elongation at 72°C for 30 s.

PCR products and a GeneRuler™ 100 bp ladder marker (Thermo Fisher Scientific, Waltham, MA, USA) were run on 1.5% Seakem LE Agarose (Lonza, Basel, Switzerland) gels containing SYBR® Safe nucleic acid stain (Invitrogen, Carlsbad, California) at 5 V cm^{-1} for 1 h in a 1 x TBE buffer. Gels were visualized and images captured with the GeneSnap Software on the I-chemi G-Box (Syngene) and analyzed using the GeneTools software (Syngene).

2.3.4 Agarose gel analysis

The bands corresponding to the band position of the *S. cerevisiae* amplicons in each sample were quantified relative to the molecular weight marker band representing a molecular weight of 500 bp and a quantity of 115 ng of DNA, in accordance with the manufacturer's instructions (Thermo Fisher Scientific). The quantity of DNA of the band corresponding to the position of the pure *S. cerevisiae* amplicon was therefore used as a measure of the efficiency of the surface treatment. Means of band DNA quantity for the four replicates for Treatments A, B and the positive control (Y) were compared with a one-way analysis of variance (ANOVA), in Genstat (Payne *et al*., 2011).

2.3.5 DGGE gel analysis

PCR products were run on DGGE gels. Gels were run on a Bio-Rad Dcode™ Universal Mutation Detection System (Bio-Rad, Hercules, California, USA). A sample of 20 µL of PCR

product per well was run on a 6% Acrylamide/Bisacrylamide (40%, 19:1, Sigma-Aldrich) gel with a 27 to 44% denaturation gradient, for 16.5 h at 100 V in a 60°C 1 x TAE buffer. The gel was stained in a 1 x SYBR® Gold nucleic acid stain (Invitrogen) for 40 min and the image captured on the I-chemi G-box Gel Documentation system (Syngene). A band presence matrix was produced and band pixel intensity was determined using Quantity One Gel Analysis Software (Bio-Rad).

Contour maps are able to display three-dimensional information in two dimensions: in this case, DGGE band positions and intensity. Band pixel intensity, as a percentage of the pixel intensity value of the brightest band on the gel, was square-root transformed and plotted on a contour map, along with band position a, using the gplots library (Warnes *et al*., 2013) in R (R Core Team, 2013) and edited in Microsoft Windows Paint (2010).

Permutational Multivariate ANOVA (PERMANOVA) implements a flexible non-parametric distance-based analogue of analysis of variance for multivariate data that provides a distribution-free means of testing differences between treatments in their multivariate profile (Anderson, 2001). This was used to test for differences in band composition among treatments.

Non-metric multidimensional scaling (NMDS) provides a robust method of visualizing differences in composition within and between treatments (McCune *et al*., 2002). NMDS projects multivariate distances among samples in low dimensional space so that distances between projected sample points best approximate their original multivariate differences: sites close in the graph are most similar in their overall composition and sites located at opposite ends of the plotted dimensions have a distinct multivariate profile. This was applied to replicate samples within and among treatments. The software package Primer (v. 6) (Clarke and Gorley, 2006) was used for both multivariate analyses.

An unpaired Student's t-test comparing band numbers (excluding the yeast bands) for Treatments A and B, was performed in Microsoft Excel (2010).

2.3.6 Variable Pressure Scanning Electron Microscopy (VPSEM)

A leaf segment was taken for Treatments A, B and the two controls, and viewed under a Zeiss Evo LF-15 Variable Pressure Scanning Electron Microscope (Zeiss, Oberkochen, Germany). Samples did not require treatment before viewing (Stokes, 2008). Sample surfaces were observed at a working distance from 7 mm to 7.5 mm, 15 kGV, between 2000 x and 4350 x magnifications and 0.89 to 0.9 Torr pressure. Representative micrographs were captured to demonstrate differences in surface characteristics due to the treatments.

2.4 Results

2.4.1 Agarose gel analysis

In Figure 2.1 the presence of *S. cerevisiae* from the original inoculum was confirmed by a band corresponding to that of the amplified ITS1 region from pure *S. cerevisiae* genomic DNA that was used as the positive PCR control. There were no native endophytes or epiphytes that shared the same sized ITS1 fragment as *S. cerevisiae* in the non-inoculated control samples. There were visible differences in intensity of the band representing the *S. cerevisiae* amplicon in Treatments A, B and the positive control.

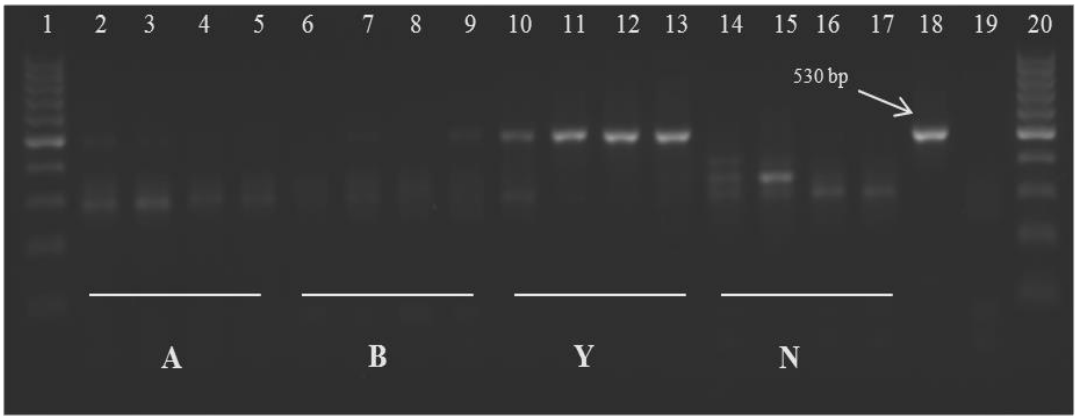

Figure 2.1 PCR of all treatments. Lanes 1 and 20, 100 bp molecular weight marker (Thermo Fisher Scientific); Lanes 2 to 5, Treatment A (physical); Lanes 6 to 9, Treatment B (chemical); Lanes 10 to 13, Control Y; Lanes 14 to 17, Control N; Lane 18, positive PCR control from pure yeast DNA; Lane 19, template-free control. The *S. cerevisiae* band is absent in the negative control samples and the treatments and positive controls show differing intensities of the band corresponding to the 530 bp pure *S. cerevisiae* band.

The raw data of individual band DNA quantities were transformed to the square root of the measured values. ANOVA of the transformed data (Table 2.1) showed no significant difference between Treatments A and B, although they differed significantly from the positive control (Y).

Table 2.1 Results of ANOVA analysis of yeast ITS1 fragment band intensity.

	Mean ± (standard error)	Square-root transformed mean ± (standard error)	CV%
Physical treatment (A)	1.37 ± 0.732	1.00 ± 0.357a	72.39
Chemical treatment (B)	3.43 ± 2.934	1.31 ± 0.755a	115.34
No treatment with Yeast (Y)	60.29 ± 33.331	7.62 ± 0.870b	22.84
$F\text{-value}_{(2,9 d.f.)}$		28.787	
P-value		< 0.001	
LSD (p > 0.05)		2.227	

Means with the same letters are not significantly different (p > 0.05). CV percentages were of transformed data.

2.4.2 DGGE gel analysis

The contour map (Figure 2.2) of DGGE gel data showed differences in the banding patterns, as a result of surface treatments, and in the controls. Region P accommodated the two bands for the yeast ITS1 amplicon (S) in the positive control samples (Y), which were present, to some extent in surface treated samples (A and B) , but absent in the samples that were not inoculated. Region Q consisted of the bands which were greatly reduced by the surface treatments. Region R was populated by bands present in all samples to a varying degree.

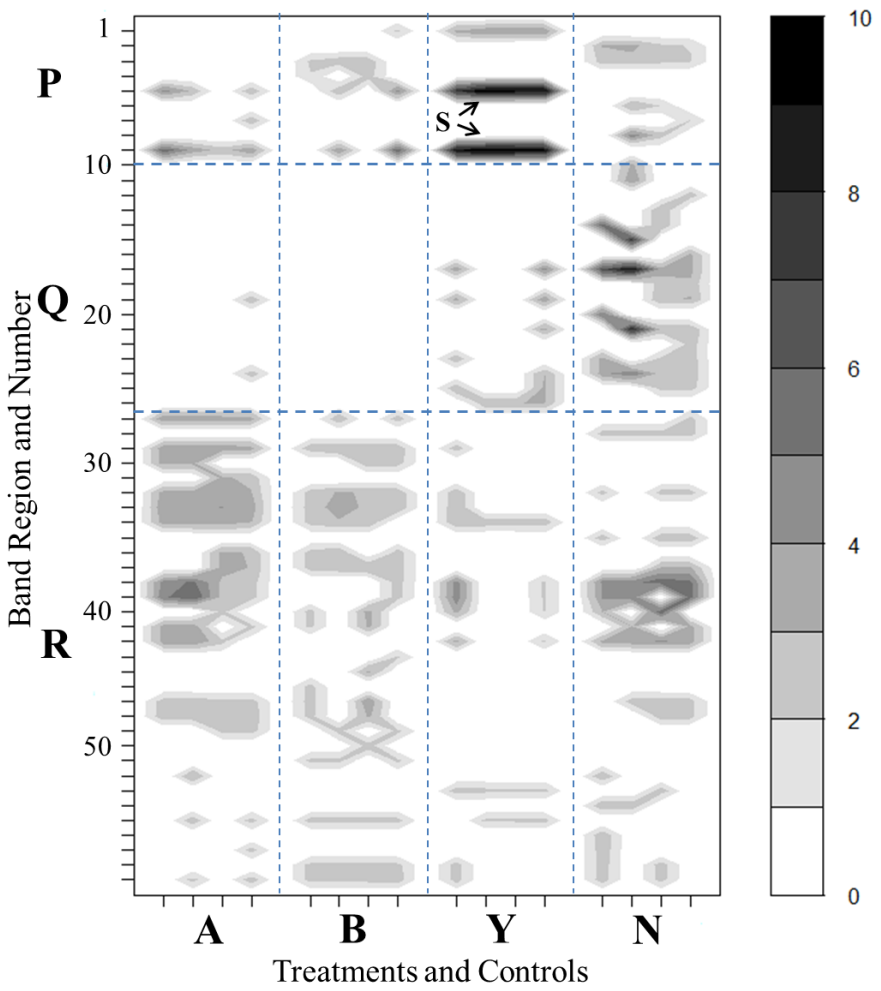

Figure 2.2 Contour map indicating band position and intensities from the DGGE gel data. This shows different regions (P, Q and R) with varying effects of surface and control treatments in the columns A, B, Y and N. The yeast ITS1 fragment is represented by two bands (S) in Region P. The key indicates the increasing intensity of the band with increasing darkness, corresponding to the square root of the percentage of maximum band intensity.

The PERMANOVA analysis (Pseudo-F = 5.7151) indicated a significant difference (P = 0.0001) in band composition amongst treatments. Pair-wise tests established that all treatments differed from one another ($p < 0.05$) in their banding patterns.

Mean similarity values (Table 2.2) provided a measure of the magnitude of the differences in band composition between and within treatments.

Table 2.2 Mean Similarity (%) between/within groups from pairwise PERMANOVA analysis.

Treatment	Physical	Chemical	Yeast	None
A - Physical	59.10			
B - Chemical	38.72	48.46		
Y- Yeast	23.41	18.48	48.67	
N- Negative	19.40	13.86	12.73	39.32

The NMDS plot (Figure 2.3) illustrated the variation within and among treatments quantified in Table 2.2. The proximity of chemically and physically treated samples in the plot confirmed their similarity, though Physical Treatment (A) samples were less dispersed in the plot, than samples subject to Chemical Treatment (B). Both were dissimilar in composition to the samples from the controls. NMDS including peak intensity data for each band showed a similar pattern of treatment effects on DGGE band profiles (result not shown).

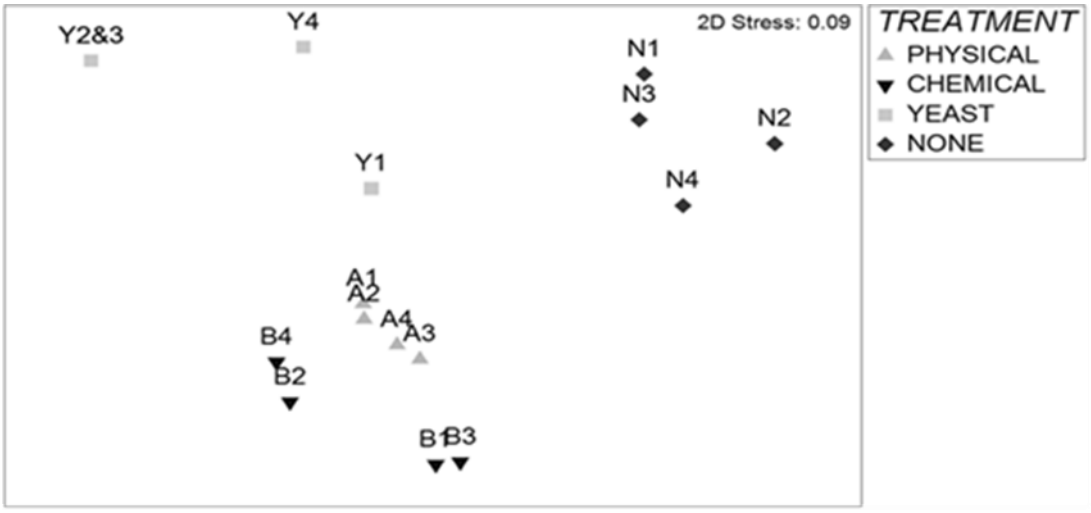

Figure 2.3 An NMDS plot of DGGE bands, showing the clustering of replicate samples from different treatments.

The Student's t-test showed mean band numbers did not differ significantly ($p > 0.05$) between Treatments A and B.

2.4.3 VPSEM

The VPSEM micrographs of uninoculated leaf surfaces of the Negative Control (N) (Figure 2.4 A) indicated extraneous microbial hyphae and debris on the leaf surface, while abundant yeast cells were present on the surfaces of the Positive Control (inoculated and untreated leaf surfaces) (Figure 2.4 B). Micrographs of leaf surfaces exposed to the two treatments indicated the degree of removal of particulates adhering to the surface was more effective with physical abrasion (Figure 2.4 C) than with chemical treatment (Figure 2.4 D).

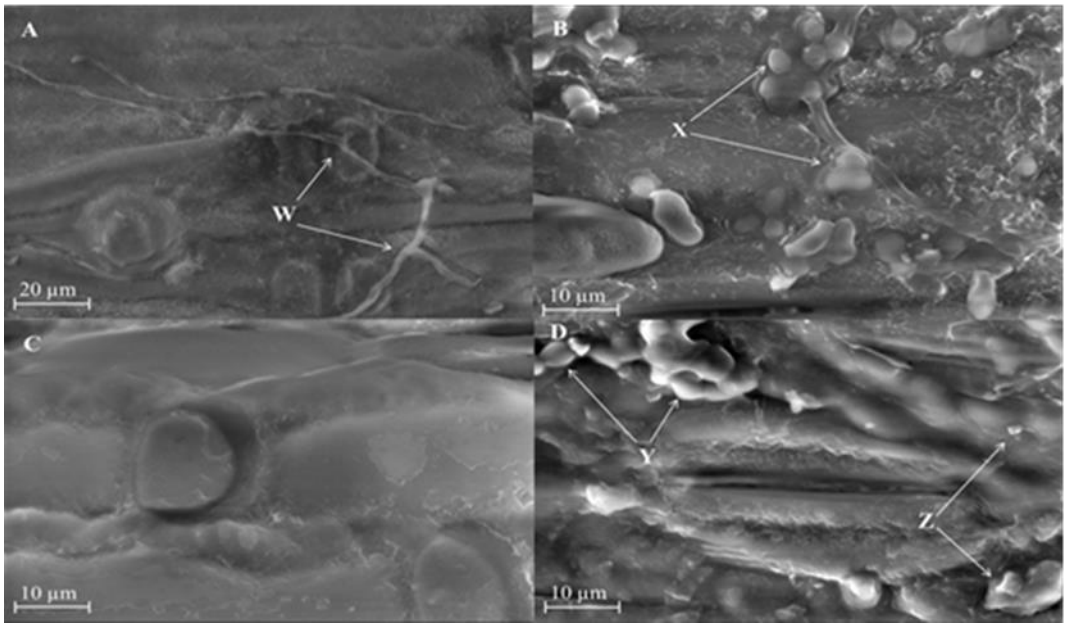

Figure 2.4 VPSEM micrographs of A-an un-inoculated and untreated leaf surface (Negative Control-N) showing fungal hyphae (W), B-inoculated and untreated leaf surfaces (Positive Control-Y) showing abundant yeast cells (X), C-inoculated and physically abraded leaf surfaces (Treatment A) indicating a significant reduction in debris and S. cerevisiae cells, and D-inoculated and chemically surface sterilized leaf surfaces (Treatment B) indicating the presence of *S. cerevisiae* cells (y) and debris (z).

2.5 Discussion

The yeast *S. cerevisiae* was chosen as an epiphytic indicator of surface treatment efficiency because it does not appear to have been isolated in previous culture-based endophyte studies of wheat (Crous *et al.*, 1995; Larran *et al.*, 2002). In addition, PCR amplification revealed that the ITS1 region amplicon derived from *S. cerevisiae* was discernible from native fungal DNA on or within the leaf and it was absent from those leaves which were not

sprayed with yeast (Figure 2.1). This demonstrated that *S. cerevisiae* can serve as a useful inoculant in determining the removal of epiphytic fungal DNA from the leaf.

The goal of a surface treatment method is to remove as much DNA from the surface of the plant while doing minimal damage to endophytic fungal DNA; the relative efficiency of a surface treatment could be inferred from the degree to which yeast DNA could still be detected in DNA extracts from leaf tissue after the treatments.

The components of the chemical treatment (Treatment B) were expected to destroy viable cells on the leaf surface (Arnold *et al.*, 2007), yet the PCR amplification of *S. cerevisiae* DNA sequences (Figure 2.1) from surface treated leaves demonstrated that epiphytic yeast DNA sequences were not eliminated ,as predicted by Guo (2010). PCR is more sensitive in detecting fungi than traditional culture plating (Baek and Kenerley, 1998), which emphasizes the importance of recognizing that microbial sterility does not guarantee the elimination of extraneous or epiphytic DNA.

ANOVA analysis (Table 2.1) of the agarose gel (Figure 2.1) confirmed that Treatments A and B did not eliminate the yeast DNA, but significantly reduced its presence relative to the positive control (Y). The lower CV% value in Treatment A indicated that this was the more consistent method of the two.

The ideal outcome of any surface treatment revealed by DGGE gel data would be no amplification of the yeast or epiphytic DNA with as many other bands as possible while bearing minimal similarity in DNA band composition to the control samples. In the contour map (Figure 2.2) Region P was largely populated by the yeast amplicons, which were strongly represented in the positive control (Y), due to the high presence of yeast DNA. The relative absence of amplicons in Region Q and increased band density in Region R for the two treatments (A and B), compared to the negative control (N), suggested that both surface treatments resulted in an enhanced amplification of endophyte target sequences due to reduced competition for primers (von Wintzingerode *et al*., 1997) by epiphytic target sequences.

The PERMANOVA of implied that the treatments varied in their effect on epiphyte diversity. The mean similarity percentages showed that Treatment A produced the most consistent band profiles (Table 2.2), arguing in its favor as the preferred treatment of the two.

The NMDS plot (Figure 2.3) exhibited compromised consistency of the chemical treatment (B); however, it was less similar to the two controls (Y and N) than the physical treatment (A). Because of this, it was speculated that the chemical treatment damaged target sequences belonging to endophytes as well, even though there was no any significant difference in mean band numbers for the two treatments.

VPSEM images (Figure 2.4) showed qualitative differences between the treatments. The abundance of *S. cerevisiae* cells on the inoculated but untreated control (Figure 2.4 B) correlated with the pronounced PCR amplification of *S. cerevisiae* (Figure 2.1). The VPSEM showed that physical abrasion (Figure 2.4 C) was more efficient in removing microbes and debris than chemical treatment (Figure 2.4 D), although the PCR analysis revealed no significant difference (Table 2.1). VPSEM results alone may not provide a reliable evaluation method in DNA-based studies.

From this data, we would recommend that the physical abrasion technique is superior to the chemical technique along the criteria of greater consistency. This higher-input method may perform better, but larger sample sizes would favor the ease and rapidity of the chemical treatment, which produced the same number of bands found in physically abraded leaves, even though profiles differed slightly.

Since the initial analysis from the agarose gel (Figure 2.1 and Table 2.1) was supported by the subsequent analyses of DGGE gel data (Figures 2.2 and 2.3; Table 2.2), we propose that this procedure alone constitutes adequate investigative effort when striving to optimize surface DNA removal techniques in DNA-based fungal endophyte studies, using *S. cerevisiae* as a control organism.

2.6 Acknowledgments

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Chapter 3 The evaluation of denaturing gradient gel electrophoresis and temporal temperature gradient electrophoresis for fungal wheat endophyte investigations

3.1 Abstract

Denaturing gel electrophoresis (DGE) can be used in culture-independent studies of microbial community composition and the technique has several variants. This work compared two of these variants, namely denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient electrophoresis (TTGE), to establish their relative performance in terms of resolution and detection, as well as cost and preparation time. Per gel reagent and material costs and preparation times were recorded for comparison. Conversion formulae were developed to standardize denaturing conditions for comparison of DGGE and TTGE gels. For all gel samples, band numbers, positions, peak height and base width were recorded. Samples run on DGGE gels tended to be clearer and more distinct from each other and DGGE tended to provide higher band numbers and better resolution. However, TTGE was quicker and cheaper to prepare. The TTGE and DGGE gel data were strongly correlated but DGGE provided more accurate dendrograms for comparisons of pure fungal isolates. Non-metric multidimensional scaling showed that TTGE data profiles were more heterogeneous, while DGGE produced tighter clustering of replicate samples. Although TTGE could be an acceptable technique for resolving DNA sequences in certain applications, DGGE is preferable for fungal wheat endophyte studies.

3.2 Introduction

Denaturing gel electrophoresis (DGE) is a technique used in culture-independent PCR-based microbial studies of microbial community composition. The mixed DNA amplicons derived from the PCR of environmental samples are separated based on their individual DNA sequence composition. This mixture of double-stranded DNA is driven by electrophoresis under increasing denaturing conditions through an acrylamide gel. The increasing denaturation causes structural DNA changes, i.e., becoming larger, resulting in reduced

mobility through the gel matrix (Fromin *et al.*, 2002). Therefore, as the sequences move down the gel they separate in the order of those with lowest to highest guanine-cytosine content (GC-content) because the lower the GC-content of a DNA sequence, the lower the melting temperature (Muyzer, 1999). The variant of this technique known as denaturing gradient gel electrophoresis (DGGE) employs a chemical gradient produced using increasing urea and formamide concentrations. In temporal temperature gradient gel electrophoresis (TTGE), instead of a chemical denaturing gradient, the temperature of the electrophoresis buffer is increased over time. Therefore, as DNA strands move along the gel matrix, the increasing temperature causes the double-stranded DNA to melt, just as in DGGE (Cornejo *et al.*, 2004). Both of these variants can be performed on the Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA).

Based on a protocol evaluation, TTGE was hypothesized to be quicker, easier and cheaper to prepare than DGGE. However, the number of published articles for each method on two internet databases (Figure 3.1) indicated that TTGE is used less frequently than DGGE.

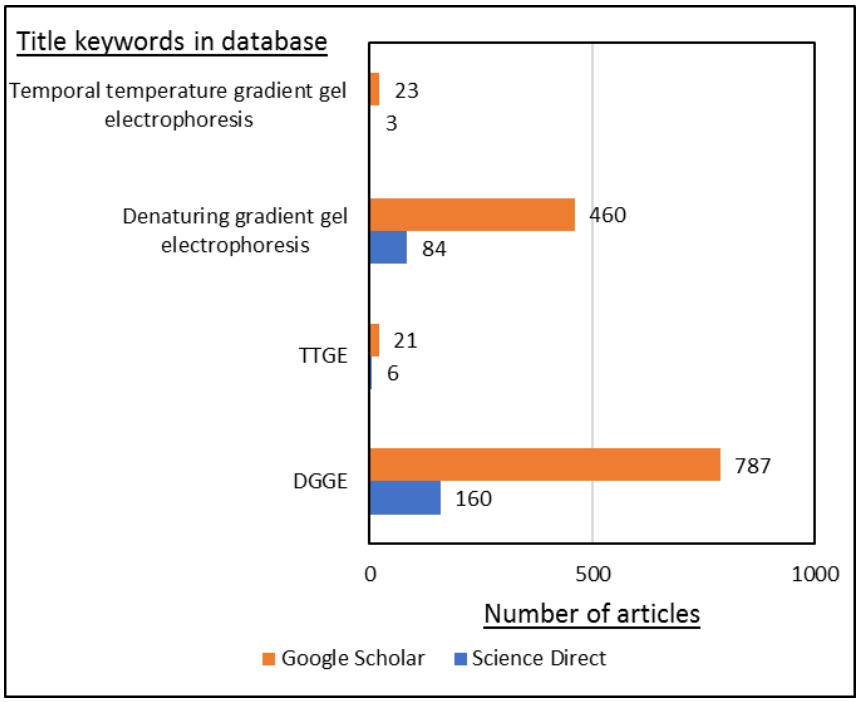

Figure 3.1 A chart of the number of articles published in English found with the specific title keyword searches, 'Temporal temperature gradient gel electrophoresis', 'Denaturing gradient gel electrophoresis', 'TTGE' and 'DGGE', and in Google Scholar and Science Direct from 2003 to 2013 (Search performed 17/04/2013).

On the other hand, both DGGE (Ma *et al.*, 2005; Duong *et al.*, 2006; Miletto *et al.*, 2007; Andreote *et al.*, 2009) and TTGE (Cornejo *et al.*, 2004; Ogier *et al.*, 2004; Nieguitsila *et al.*, 2007; Úbeda *et al.*, 2009) have both been used in successful microbial diversity studies. This indicates that there may be both benefits and disadvantages to each technique.

The aim of this work was to determine whether DGGE or TTGE is more suitable for the study of fungal wheat endophytes.

3.3 Materials and Methods

3.3.1 Sample preparation

All PCR reactions were performed using the KAPA2G Fast HotStart ReadyMix Kit (Kapa Biosystems, Wilmington, Massachusetts, USA) and universal fungal primers ITS1F-GC forward primer (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CTT GGT CAT TTA GAG GAA GTA A-3') and the ITS2 reverse primer (5'-TTY GCT GYG TTC TTC ATC G-3') (Wakelin *et al.*, 2007) synthesized by Inqaba Biotec (Pretoria, South Africa). The dNTP's were at a 0.2 mM concentration, $MgCl_2$ at 1.5 mM, and the forward and reverse primers were at a final concentration of 800 nM each. The final reaction volume was 50 µL. All reactions were performed on a G-storm Thermal Cycler (Syngene, Cambridge, UK) with the following program parameters: 2 min denaturation at 95°C, 35 cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 10 s, with a final elongation step at 72°C for 30 s. Three sets of PCR product were prepared using previously extracted genomic DNA originating from the following sources:

Set 1: PCR products were amplified from 2 ng of genomic template DNA from the first replicate of each of the surface treatments (A and B) and controls (Y and N) from a study on surface decontamination (Burgdorf *et al.*, 2014), as well as a DNA-free no template sterile water control (NTC) and a pure *Saccharomyces cerevisiae* Meyen ex E.C. Hansen genomic DNA positive control. This set of samples compared mixed template samples on each of the gel types.

Set 2: PCR products were amplified from 1 ng of genomic template DNA extracted from 10 previously isolated fungal wheat endophyte cultures as well as *Beauveria bassiana* (Bals.-Criv.) Vuill. and *S. cerevisiae* and an NTC. This set of samples compared the ability of each method to discriminate between fungal isolates.

Set 3: PCR products amplified for analysis by DGGE in a previous study (Burgdorf *et al*., 2014) were retained to be run on a TTGE gel in this experiment. This set was used to evaluate the performance of each method on replicated mixed template samples.

To confirm amplification of products for sets 1 and 2, 5 µL of sample PCR products, along with a GeneRuler^{TM} 100 bp ladder marker (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) were run on a 1.5% agarose gel containing 1 x SYBR® Safe nucleic acid stain (Thermo Fisher Scientific) in 1 x TBE buffer at 5 V.cm^{-1} for 1 h. Gels were visualized and images captured with the GeneSnap Software (Syngene) on the I-chemi G-Box gel documentation system (Syngene).

3.3.2 DGGE and TTGE parameters

The actual denaturing temperature (ADT) values under varying parameters for corresponding sets of TTGE and DGGE gels were determined. In DGGE, the buffer temperature (bT) was constant while urea concentration ([U]) and formamide quantities (% Formamide) increased, raising the ADT by 2°C per mole of urea, as per the DCode™ Universal Detection System user manual (Bio-Rad) and 0.6°C per percent formamide, as per Sadhu *et al.* (1984) (Formula 3.1, Table 3.1).

ADT = bT + ([Urea] x 2) + (% Formamide x 0.6) Formula 3.1

Table 3.1 DGGE denaturing parameters calculated from Formula 3.1, at a constant buffer temperature of 60°C.

Chemical denaturing %	[Urea] (M)	% Formamide	ADT (°C)
50	3.5	20	79
40	2.8	16	75.2
30	2.1	12	71.4
20	1.4	8	67.6

For TTGE gels the ADT values were calculated at constant urea concentrations and varying buffer temperatures (Formula 3.2, Table 3.2).

ADT = ([Urea] x 2) + bT Formula 3.2

Table 3.2 TTGE denaturing parameters calculated from Formula 3.2, at two constant urea concentrations of 8M and 4M.

Buffer Temperature (°C) At 8M Urea	Buffer Temperature (°C) at 4M Urea	ADT (°C)
64	72	80
62	70	78
60	68	76
58	66	74
56	64	72
54	62	70
52	60	68

The calculated running conditions were established for the three samples sets in Table 3.3 to produce a common ADT in each of the DGGE and TTGE gels.

Table 3.3 Reagent and temperature parameters for attaining equivalent denaturing conditions in DGGE and TTGE gels on which three sets of PCR product were run.

Set	Voltage	Run duration	Acrylamide/Bisacrylamide Ratio	DGGE chemical denaturing range	TTGE buffer temperature range/Urea concentration
1	100 V	16 h	37.5:1	30-40%	55-59°C / 8M
2	60 V	16 h	37.5:1	30-50%	55-63°C / 8M
3	100 V	16 h	19:1	25-40%	62-69°C / 4M

All gels were run on the Bio-Rad Dcode™ Universal Mutation Detection System (Bio-Rad). For all three sets, 15 µL of each sample was run on 6% acrylamide/bisacrylamide (Sigma-Aldrich, St. Louis, Missouri, USA) in a 1 x TAE buffer and were post-stained in a 1x SYBR® Gold (Thermo Fisher Scientific) solution for 40 min.

Cost per gel for each set was calculated according to the volume of each gel-specific reagent required to produce a 25 mL gel and run this in a 7 L tank. Costs for other materials, such as water and pH-adjusting buffers, were not included in these calculations. The reagent preparation and gel casting times were recorded to calculate the mean casting time per gel type. The time taken to collect and prepare other materials, such as glassware, was not included in the analysis.

3.3.3 Preparation and casting of DGGE gels

A 1 mL solution of a 10% ammonium persulfate (Merck, Darmstadt, Germany) (APS) solution was prepared by adding 0.1 g of APS to 1 mL of ultra-pure sterile water and stored on ice. The 0% denaturing solution was prepared by mixing 15 mL of 40% acrylamide/bisacrylamide solution (Sigma-Aldrich) to 2 mL 50 x Tris-acetic acid-EDTA (TAE) buffer (pH 8.0) and 83 mL ultra-pure sterile water. The 100% denaturing solution was prepared by mixing 15 mL 40% acrylamide/bisacrylamide solution (Sigma-Aldrich), 2 mL 50 x TAE solution, 40 mL formamide (Sigma-Aldrich) and 42 g of urea (Merck) which was made up to a final volume of 100 mL

with ultra-pure sterile water. These solutions were degassed by sonication and then stored in the dark at 4°C until required.

A 1 mL plug consisting of 100% denaturing solution with 7 µL APS and 1 µL N,N,N′,N′-tetramethylethylenediamine (TEMED) (Merck) was poured into the gel cartridge. This was followed by 22 mL of a chemical gradient solution produced in a gradient mixer with varying volumes of 100% and 0% denaturing solution, as per Table 3.4, to produce the gradients for the three sets shown in Table 3.3. Prior to introduction to the gradient mixer, a volume of 50 µL of APS was added to each solution. In addition, 10 µL of TEMED was added to the higher percentage solution and 5 µL to the lower percentage solution. Each solution was placed in a separate chamber of the gradient mixer and then introduced to the gel chamber. Once the gradient gel solution was poured it was capped with 3 mL of the 0% solution containing 21 µL APS and 1 µL TEMED. The gel comb was inserted and the gel left to polymerize for 1.5 h.

Table 3.4 Quantity of 0% and 100% solutions needed to produce desired DGGE gradient

% Denaturation	**Quantity of 0% solution (mL)**	**Quantity of 100% solution (mL)**
20	8.8	2.2
25	8.25	2.75
30	7.7	3.3
35	7.15	3.85
40	6.6	4.4
45	6.05	4.95
50	5.5	5.5

3.3.4 Preparation and casting of TTGE gels

The preparation of the APS solution was as described previously. Gels for the TTGE reagents for the 3 sets of gel comparisons were mixed according to Table 3.5 and made up to a final volume of 25 mL with ultra-pure sterile water. Solutions were degassed by sonication and stored in the dark at 4°C until required. Prior to pouring the gels, 250 µL of APS and 25 µL of

TEMED were added to the gel solutions. After pouring the gels, the combs were set, and the gels were left to polymerize for 1.5 h.

Table 3.5 Materials used per 25 mL TTGE gel per set of PCR products.

Set	Urea (g)	40% Acrylamide : Bisacrylamide solution (mL)	50 x TAE (mL)
1	12	3.75	0.5
2	12	3.75	0.5
3	6	3.75	0.5

3.3.1 Analysis of DGGE and TTGE gels

Gel images were captured on the I-chemi G-Box gel documentation system (Syngene). Bands were detected, counted and the peak height and peak width were determined using the GeneTools (Syngene) software package.

For peak height and width, all values were background subtracted. Peak height was the background-corrected maximum signal of the peak in grayscale values lying between 0 - 65535. Peak width was the pixel distance between the start and end of the background-corrected peak.

Paired Student's t-test on mean band numbers and for mean band peak height/peak width ratios (as measures of sensitivity and resolution respectively) in each gel pair per set were performed in Microsoft Excel (2010) (Microsoft, Redmond, Washington, USA).

Unweighted pair-group arithmetic average (UPGAMA) clustered dendrograms derived from the DGGE and TTGE gels of the fungal isolates (Figures 3.4 and 3.5 respectively) were produced using GeneTools (Syngene).

DGGE and TTGE gel band matrices were produced from the previously acquired images using Quantity One gel analysis software (Bio-Rad). A Mantel test was performed on the gel matrices in PC-ORD (v 4.25) (McCune and Mefford, 2011).

Non-metric multidimensional scaling plots (NMDS) (based on Jaccard similarity coefficient) for Set 3 gel band profiles were produced using Primer (v. 6) (Clarke and Gorley, 2006).

3.4 Results

3.4.1 Sample preparation

PCR products for fungal isolates and wheat leaf DNA (Figure 3.2) showed bands of suitable quality and size expected for the ITS1 region.

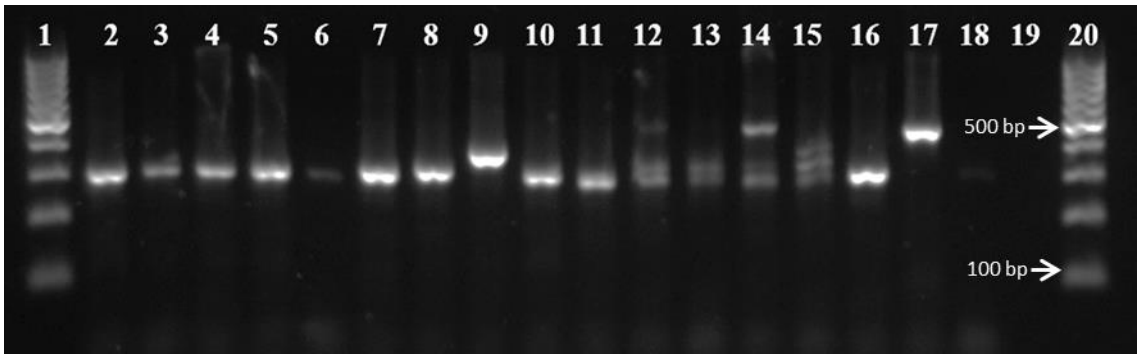

Figure 3.2 Agarose gel of PCR product for fungal wheat endophyte isolates F1-F10 (Lanes 2-11) for Set 1, wheat leaf DNA extracts (Lanes 12-15) for Set 2, *B. bassiana* (Lane 16) and *S. cerevisiae* (Lane 17) used as controls in Set 1. 100 bp ladder markers are in Lanes 1 and 20.

3.4.2 DGGE and TTGE gels

A visual inspection of the DGGE gel of Set 1 sample products (Figure 3.3 A) showed greater numbers of bands, which were clearer and more distinct than bands that were seen after the same samples were run on TTGE (Figure 3.3 B). Bands on the TTGE gel appeared to be more diffuse.

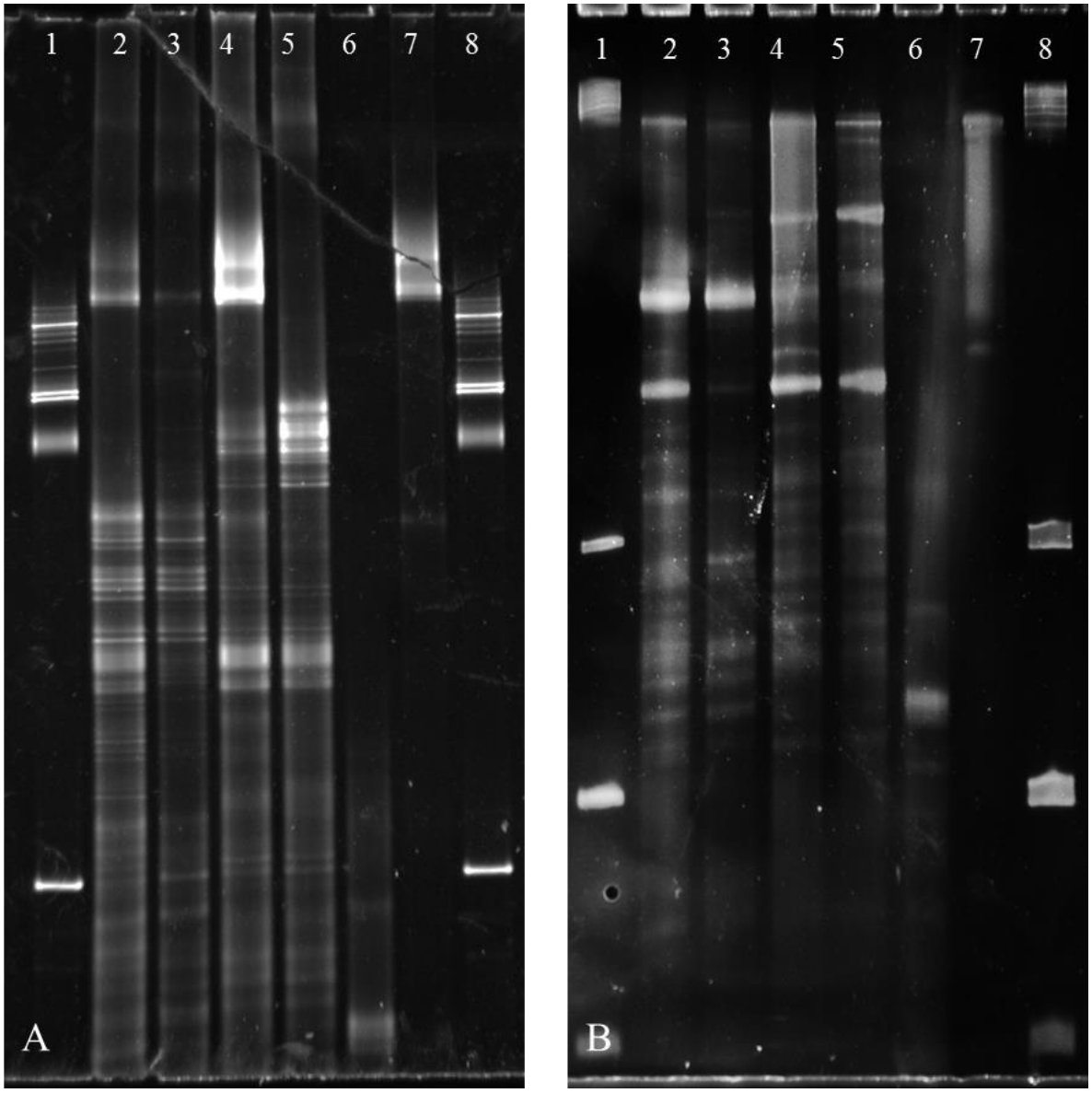

Figure 3.3 DGGE of wheat samples (A) and TTGE gel of wheat samples (B) with 100 bp ladder markers (Lanes 1 and 8), sample A1 (Lane 2), B1 (Lane 3), Y1 (Lane 4), N1 (Lane 5), template free control (Lane 6) and pure *S. cerevisiae* (Lane 7).

A visual inspection of the DGGE gel produced from Set 2 samples (Figure 3.4) showed that the bands appeared more numerous and more distinct than on the TTGE gels (Figure 3.5).

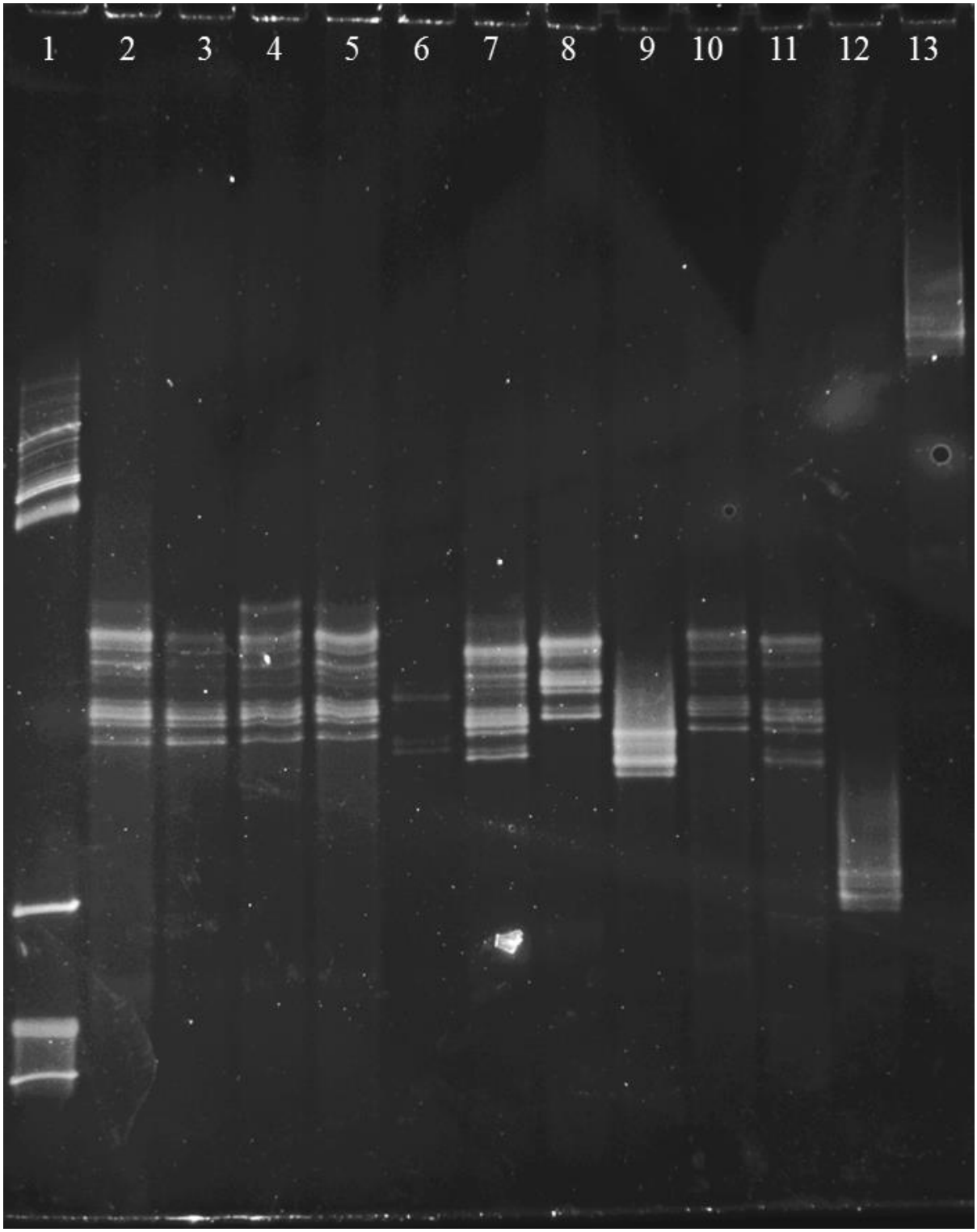

Figure 3.4 DGGE gel of fungal isolate samples with the 100 bp ladder marker (Lane 1), fungal isolates F1-F10 (Lane 2-11), *B. bassiana* (Lane 12) and *S. cerevisiae* (Lane 13).

Bands in the TTGE gel from Set 2 (Figure 3.5) were more widely separated than in the DGGE gel, but were not as distinct (Figure 3.4).

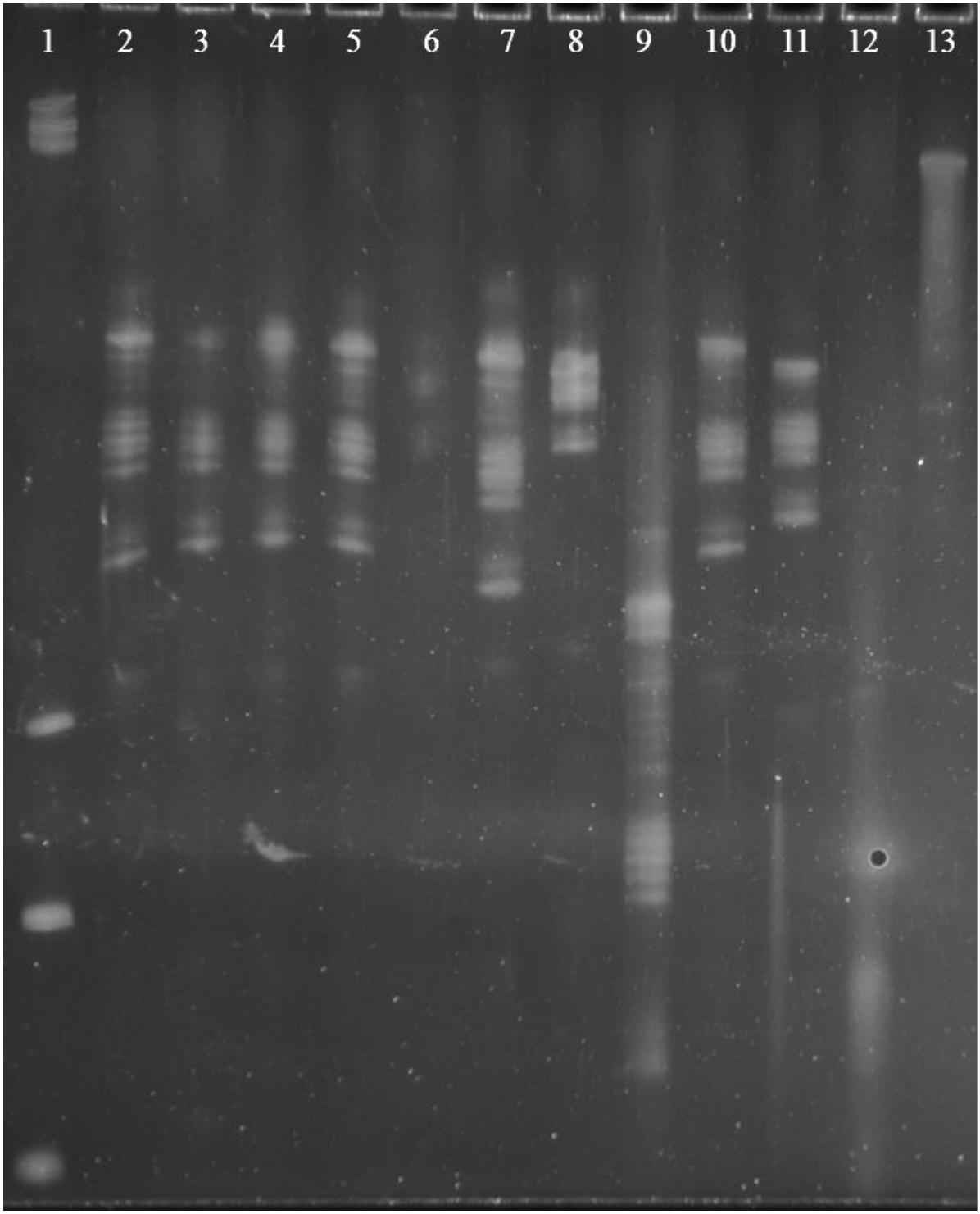

Figure 3.5 TTGE gel of fungal isolate samples with the 100 bp ladder marker (Lane 1), fungal isolates F1-F10 (Lane 2-11), *B. bassiana* (Lane 12) and *S. cerevisiae* (Lane 13).

The PCR products from previous work (Burgdorf *et al*., 2014) used for Set 3 (Figure 3.6) showed bands of suitable size and quantity to be run on DGGE and TTGE gels.

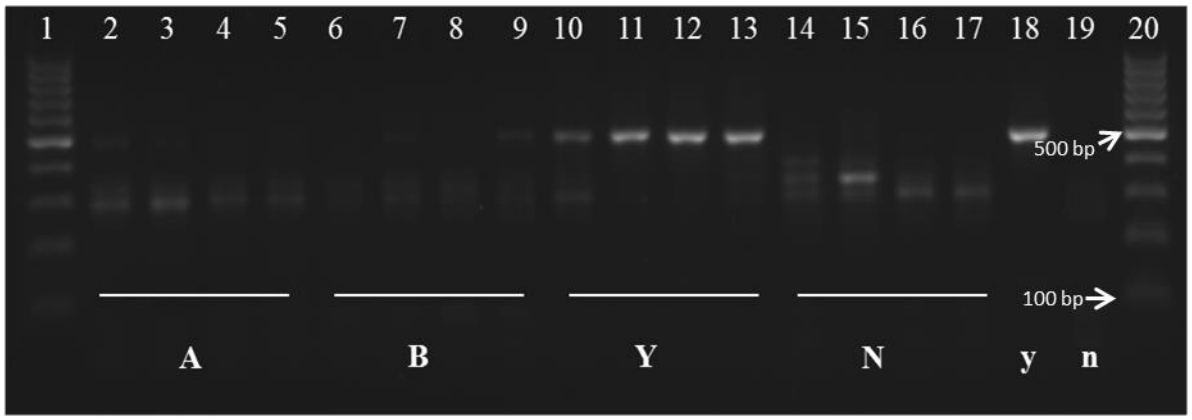

Figure 3.6 The agarose gel of PCR products from wheat leaf extracts after: A–physical treatment (Lanes 2- 5); B–chemical treatment (Lanes 6-9); Y–positive control (Lanes 10-13); N–negative control (Lanes 14-17); y-pure yeast positive PCR control (Lane 18); n-template-free PCR negative control (Lane 19) and 100 bp molecular weight marker (Lanes 1 and 20) (from Burgdorf *et al*., 2014).

The DGGE gel (Figure 3.7) of the Set 3 samples showed band profiles with similarities between samples within treatment replicates.

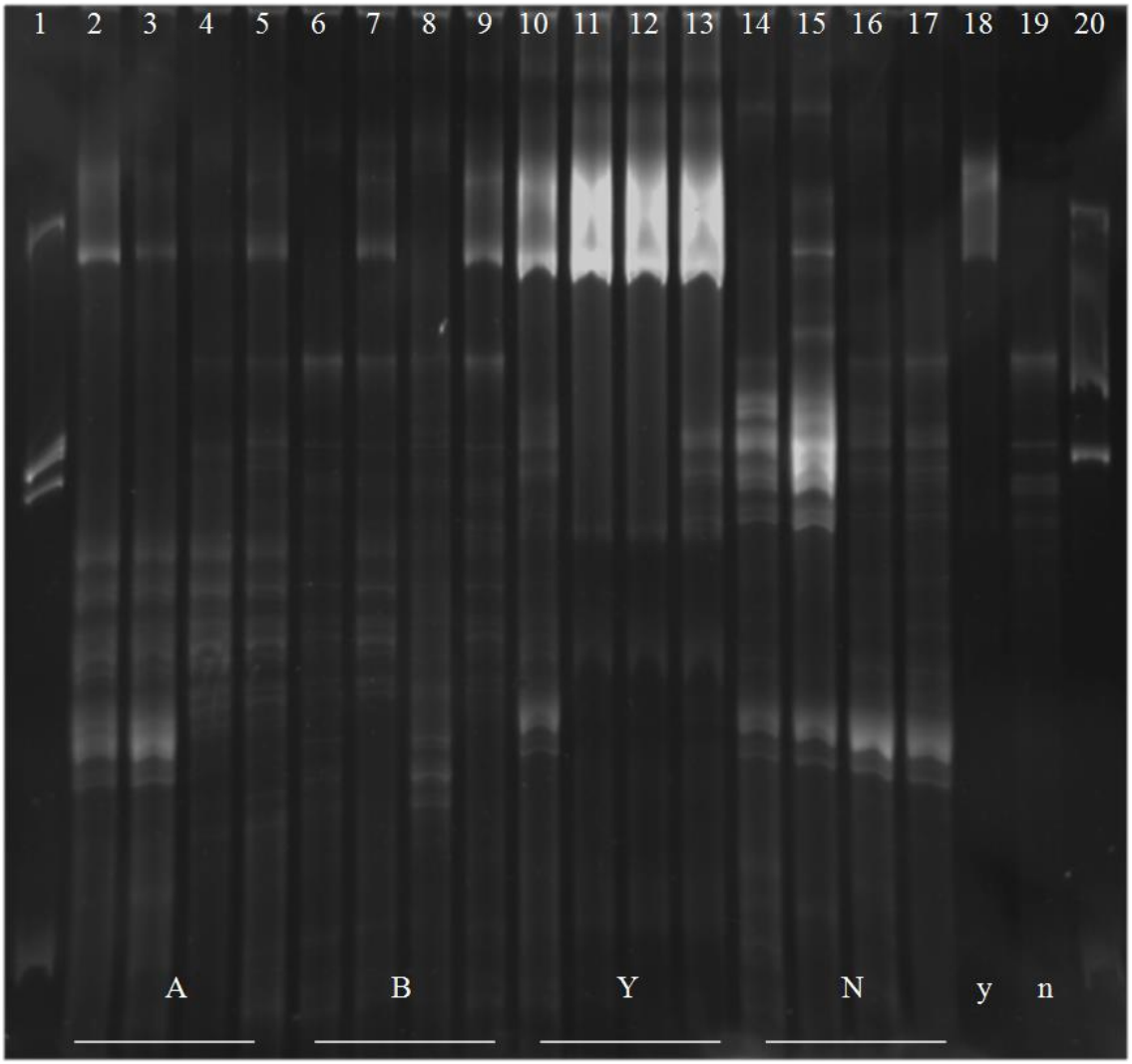

Figure 3.7 DGGE gel PCR products from wheat leaf extracts after: A–physical treatment (Lanes 2-5); B–chemical treatment (Lanes 6-9); Y–positive control (Lanes 10-13); N–negative control (Lanes 14-17); y-pure yeast positive PCR control (Lane 18); n-template-free PCR negative control (Lane 19) and 100 bp molecular weight marker (Lanes 1 and 20) (from Burgdorf *et al*., 2014).

The TTGE gel (Figure 3.8) of the Set 3 samples (Figure 3.6) also showed similarities between samples within treatment replicates; however, the band profiles differed from the DGGE gel (Figure 3.7).

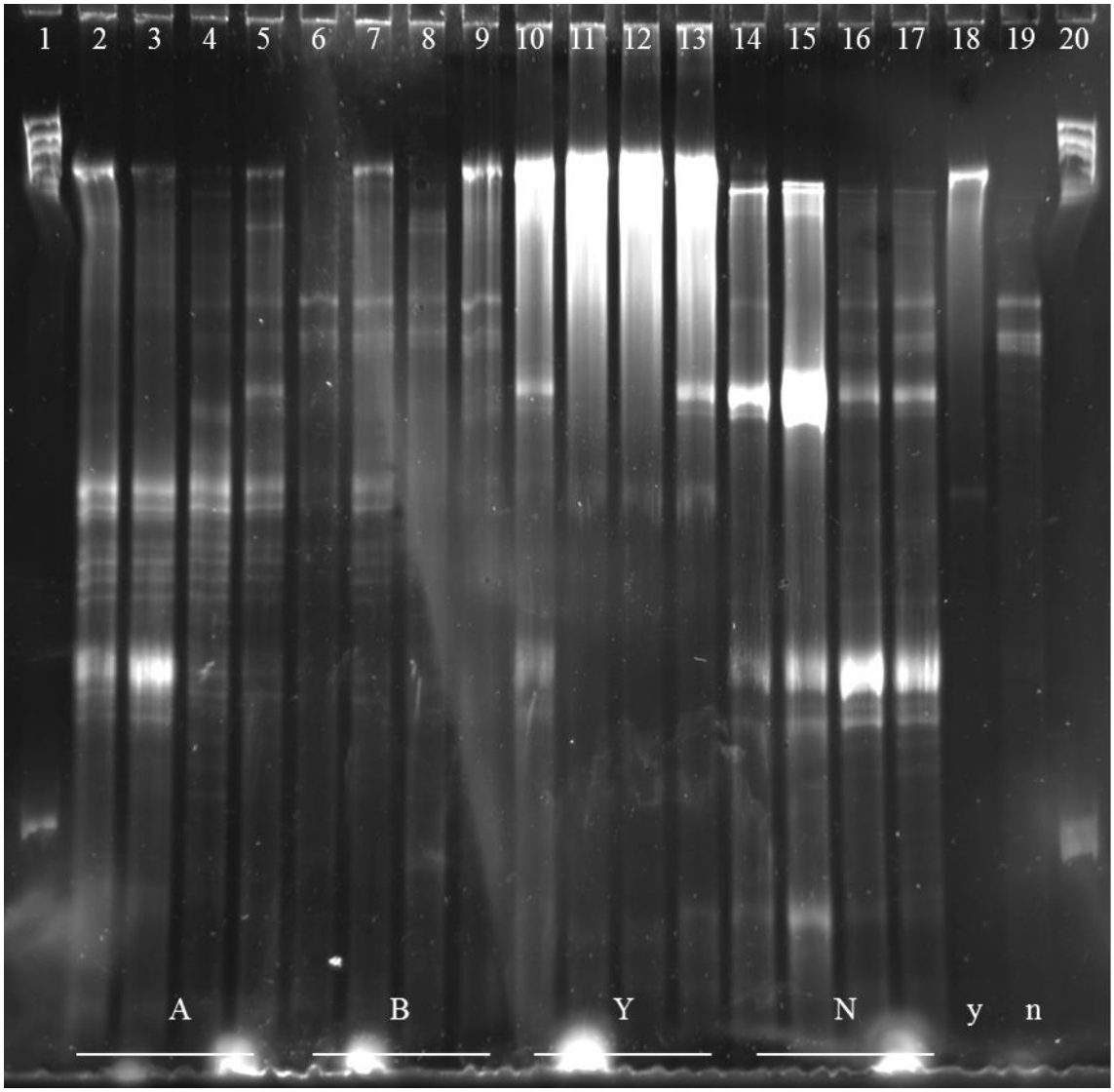

Figure 3.8 TTGE gel of PCR products from Chapter 2 wheat leaf extracts after: A–physical treatment (Lanes 2-5); B–chemical treatment (Lanes 6-9); Y–positive control (Lanes 10-13); N–negative control (Lanes 14-17); y-pure yeast positive PCR control (Lane 18); n-template-free PCR negative control (Lane 19) and 100 bp molecular weight marker (Lanes 1 and 20).

3.4.3 DGGE and TTGE gel analyses

A comparison of the time taken to prepare reagents for each method (Table 3.6) showed that the TTGE reagents were significantly quicker to prepare than for DGGE.

Table 3.6 Mean time required to prepare reagents for each gel for the 3 sets.

Gel type	Mean time to prepare reagents (min)
DGGE	7.81a
TTGE	6.37b

Means with different letters differed significantly ($p < 0.05$)

A comparison of the time taken to cast gels showed a significant and very substantial difference in the time needed to prepare gels for each method (Table 3.7). Casting the TTGE gels required less than a tenth of the time than DGGE gels.

Table 3.7 Mean time taken to cast gels for the 3 sets.

Gel type	Mean time taken to cast gel (min)
DGGE	28.91a
TTGE	2.07b

Means with different letters differed significantly ($p < 0.05$)

A comparison of the cost of reagents required to prepare gels for each method (Table 3.8) showed that TTGE was significantly cheaper than DGGE to prepare. There was a 7.89% difference in mean cost.

Table 3.8 Mean reagent costs for preparing gels as of 02/04/2013.

Gel type	Mean reagent cost to prepare gels (Rand)
DGGE	R 73.05a
TTGE	R 67.29b

Means with different letters differed significantly ($p < 0.05$)

A comparison of the band numbers and h/w ratios for each of the 3 sets for each method (Table 3.9) revealed that in most instances the mean band numbers did not differ significantly, while h/w ratios usually did. DGGE tended to produce more bands with higher h/w ratios.

Table 3.9 Results of Paired Student's t-test for band numbers and h/w ratios for each set of gel comparisons.

Set	**Mean Band Number**		**Mean h/w**	
	DGGE	TTGE	DGGE	TTGE
1	16.50a	9.83a	287.82e	49.25f
2	7.50b	7.33b	125.39g	81.32h
3	17.17c	11.42d	132.20i	152.33i

Means with same letters did not differ significantly ($p > 0.05$)

The Mantel test analysis comparing three sets of the two methods (Table 3.10) revealed a medium to high correlation between band profiles, with correlation decreasing as band numbers increase (seen from mean band numbers in Table 3.9).

Table 3.10 Mantel test statistics for gel matrix comparisons of DGGE and TTGE gels for each set where r = 0 indicates no relationship.

Set	**Standardized Mantel Statistic (r)**	**P-value**
1	0.76	0.00
2	0.99	0.01
3	0.58	0.00

A dendrogram (Figure 3.9) of the Set 2 band profiles from the DGGE gel (Figure 3.4) illustrated the relative band profile similarities between the fungal isolates, with isolate 1, 3 and 4 being identical.

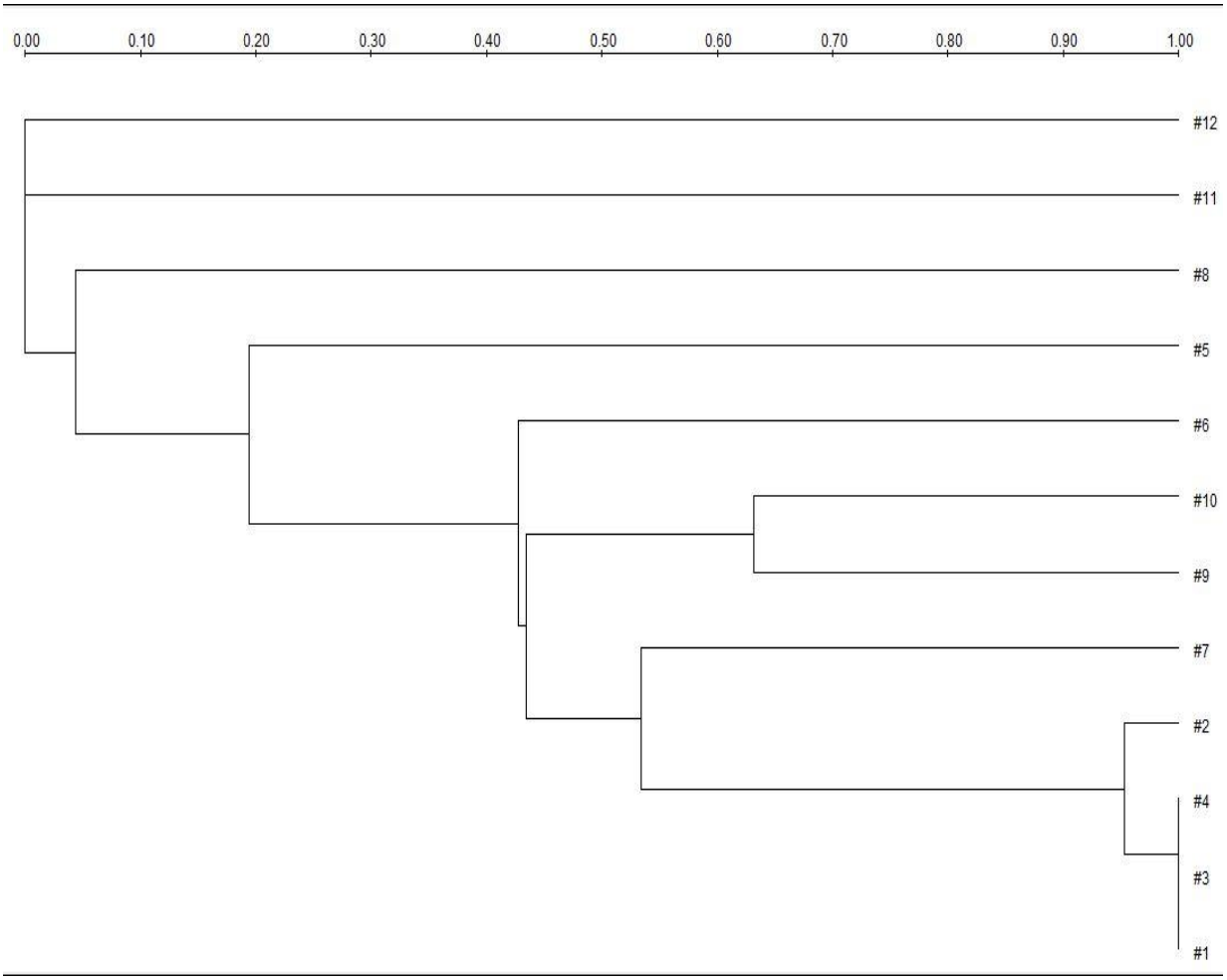

Figure 3.9 An unweighted pair-group arithmetic average (UPGAMA) clustered dendrogram derived from DGGE gel of fungal isolates in Figure 3.4.

A dendrogram derived from Set 2 samples run on the TTGE gel (Figure 3.10) indicated less similarity between isolates as compared to those shown on the DGGE gel (Figure 3.9).

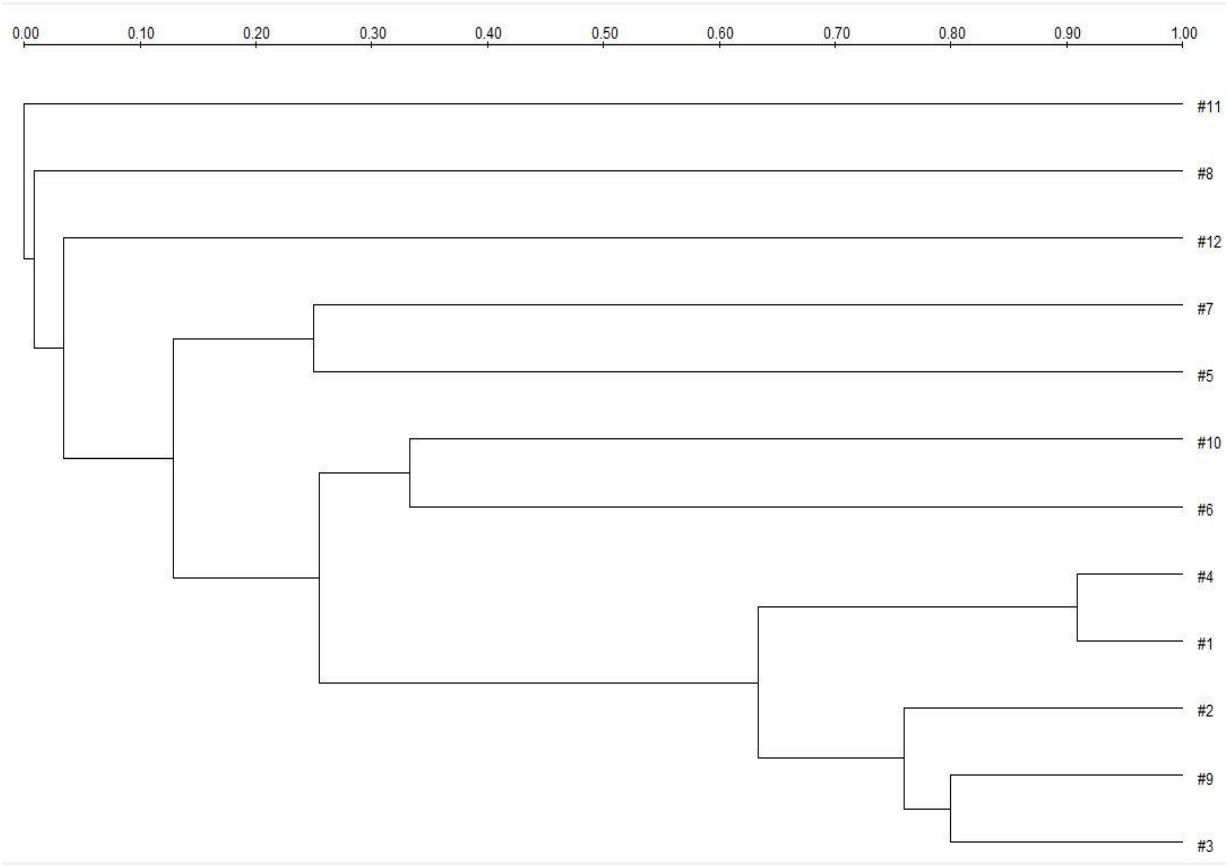

Figure 3.10 An unweighted pair-group arithmetic average (UPGAMA) clustered dendrogram derived from TTGE gel of wheat fungal isolates in Figure 3.5.

An NMDS plot of DGGE bands (Figure 3.11) from the Set 3 samples showed the similarities between the treatments revealed by DGGE gels (Figure 3.7). Samples formed clusters according to their treatments.

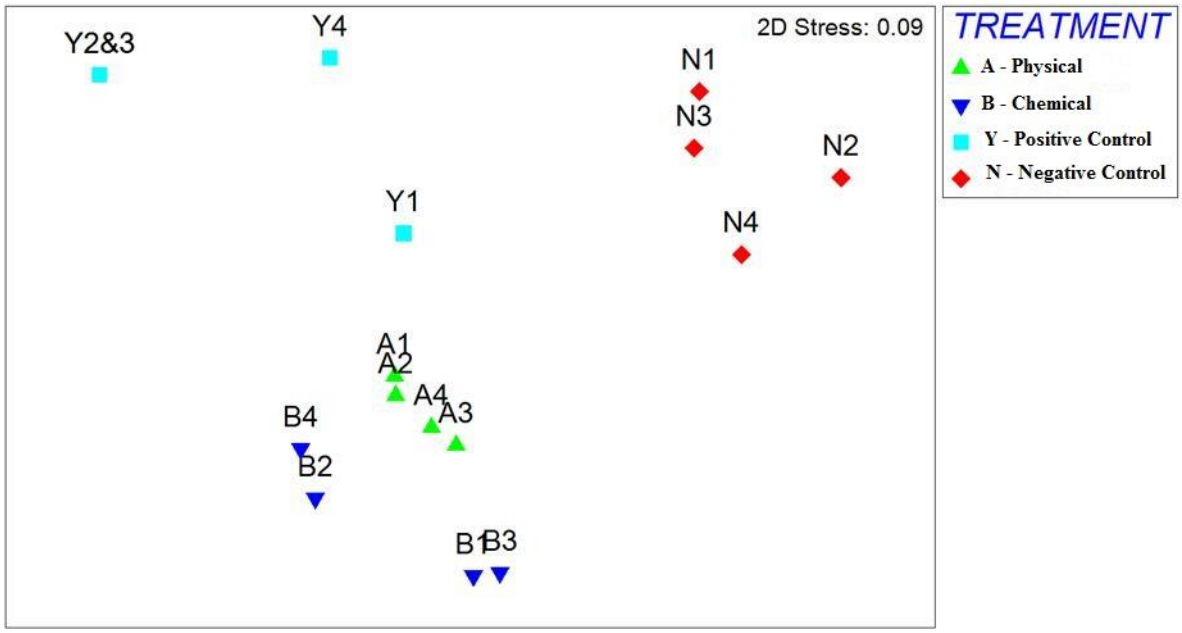

Figure 3.11 An NMDS plot (based on Jaccard similarity coefficient) of DGGE bands from Figure 3.7 (from Burgdorf *et al*., 2014).

An NMDS plot of the TTGE bands (Figure 3.12) from the Set 3 samples showed the clustering patterns of the TTGE gel samples (Figure 3.8). The chemically treated samples (B) were more widely dispersed as compared to the data from the DGGE gel (Figure 3.10).

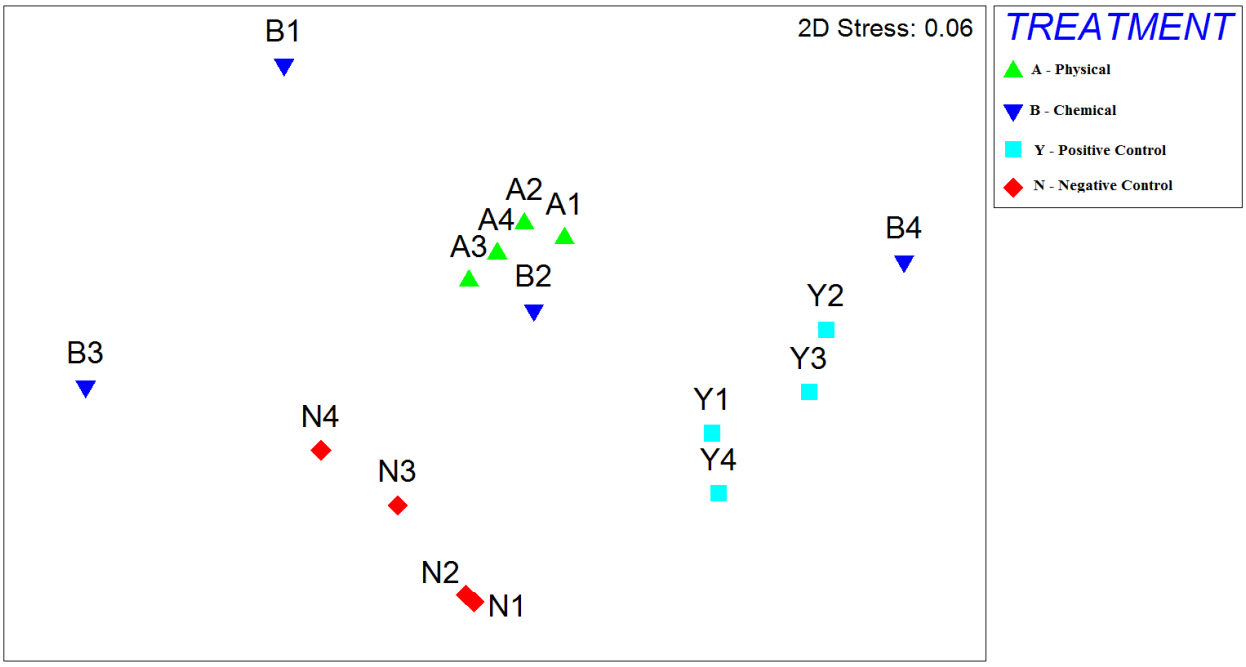

Figure 3.12 An NMDS plot (based on Jaccard similarity coefficient) of TTGE bands from Figure 3.8, showing similar clustering of replicate samples for groups A, Y and N, but significantly more dispersal of group B, compared to DGGE.

3.5 Discussion

There were several evident differences between the methods, demonstrating the merits and disadvantages of each. Despite an initial visual evaluation, after objective analysis of the criteria used for comparison, it was revealed that TTGE performed unexpectedly well. However, it has previously been demonstrated that TTGE can be used in microbial diversity analysis, with results being comparable to those from clone libraries and analysis by fluorescent *in situ* hybridization (FISH) (Bosshard *et al.*, 2000).

The data for Set 1 compared the gel characteristics with a mixture of sample types; Set 2 compared the ability of DGGE and TTGE to distinguish between pure fungal isolates; and Set 3 compared the detection of fungal endophytes in replicated wheat samples. For Set 1 DGGE showed bands which are crisper and more evenly dispersed along the gel (Figure 3.3 A). Band crispness is also demonstrated in Set 2 (Figure 3.4 and 3.5). In the gels from Set 3 (Figures 3.7 and 3.8), the TTGE showed improved crispness, but as in all the other TTGE gels, the clear double banded yeast product seen in DGGE gels was not clearly represented. The

diffused appearance of TTGE bands was observed in images of gels by other researchers (Cornejo *et al.*, 2004; Ogier *et al.*, 2004; Nieguitsila *et al.*, 2007). This was due to the diffusion of mixed DNA in the loading well, as observed by Farnleitner *et al.* (2000), who suggested that TTGE lacks the focusing provided by DGGE with its fixed spatial gradient. The slightly improved crispness of TTGE bands in Set 3 could either be due to the increased focusing from better sample loading or because of the smaller pore size of the lower acrylamide/bisacrylamide ratio (19:1) for that gel. The mixed DNA template moves quickly down the liquid of the well but meets resistance as it hits the gel. With the greater pore size of the 37.5:1 matrix, DNA template can start moving into the gel more immediately, resulting in greater distance between homogenous sequences in the original heterogeneous mixture. In the 19:1 ratio the DNA migration into the gel is slower, resulting in a smaller gap between leading and trailing homogeneous DNA sequences in the mixture.

The improved TTGE band clarity in Set 3 (Figure 3.8) demonstrated that the TTGE gels could be optimized further, while the DGGE method was more robust because DGGE performed satisfactorily under the varying conditions for all three sample sets. Therefore, less optimization effort is required in performing DGGE, relative to TTGE. DGGE is usually optimized in terms of establishing the best upper and lower denaturing conditions, while TTGE requires optimization of the temperature gradient and the urea concentration.

The calculated ADTs (Formulae 3.1 and 3.2) were intended to provide the same denaturing conditions between across the DGGE and TTGE gels per sample set (Tables 3.1 and 3.2). However, it appeared that the effect of the chemical and temperature gradients did not correspond between methods. This was visible also in the differences between band profiles of the ladder markers between DDGE and TTGE gels in each set (Figures 3.3, 3.4, 3.5, 3.7 and 3.8). This highlighted a discrepancy in the reported 2°C per mole change in denaturing temperature for urea indicated in the Bio-Rad user manual compared to the 2.25°C per mole change that was reported by Hutton (1977). In addition to this, the chemical denaturing effect on DNA by formamide is not constant (Sadhu *et al*., 1984) and can vary according to the GC-content of the DNA (Blake and Delcourt, 1996). Therefore, sequence separation conditions were not linearly correlated between the two methods. In the TTGE gels the

actual gradient was wider than was calculated due to the underestimated effect of the urea, although this issue could be rectified by increasing the concentration accordingly. Furthermore, the inconsistent effect of the formamide in the DGGE gels implied that TTGE can potentially perform more predictably because formamide is not used in these gels.

The significant difference in preparation times of each method was highlighted in the substantial difference in time taken to cast the gel (Tables 3.6 and 3.7). This is an important determinant in which method to use, especially if basic screening is all that is required. In this case, TTGE would be the preferable method, in addition to being cheaper.

For Sets 1 and 2 (Figures 3.3 to 3.5), DGGE gels visibly appeared to produce more distinct bands than the TTGE gels. However, statistical analysis revealed that mean band numbers were not significantly different (Table 3.9). This was because the gel analysis technology (hardware and software) overcame the limitations of visual band discrimination. However, in Sets 1 and 2, the h/w ratios for all the bands were significantly higher for DGGE (Table 3.9), explaining why the mean band numbers tended to be higher for DGGE gels. This was because the resolution of these samples was better in the DGGE gels, so bands with very similar sequences were more likely to separate in the DGGE gels than in TTGE gels. The third set of gels demonstrated the greater sensitivity of the DGGE gel, without a significant difference in resolution. Therefore, the results showed that DGGE tended to have greater sensitivity and resolution than TTGE. Farnleitner *et al.* (2000) observed that DGGE had up to eight times greater spatial separation ability than TTGE and that TTGE was sometimes not able to separate certain bands, detecting 29% fewer bands than DGGE. This partly explains why culture-based methods could detect a greater number of fungal taxa than TTGE in a previous study comparing the two approaches (Nieguitsila *et al*., 2007).

The greatest correlation between methods was found when comparing the band profiles of the fungal culture isolates (Table 3.10), which indicated that the TTGE could provide similar data to that of DGGE in such an application. However, comparing the dendrograms of the same two gels for Set 2 (Figures 3.9 and 3.10) showed that TTGE and DGGE had important differences in terms of comparing the identities of the isolates. Sequence analysis of these

isolates (data not shown) supported the conclusions of the DGGE dendrogram (Figure 3.9), which showed that the isolates represented in Lanes 1, 3 and 4 were identical. Therefore, the phylogenetic conclusions from the TTGE gel were less accurate than those from the DGGE gel. This indicated that TTGE inadequately distinguished between fungal isolates.

The NMDS plots of the two sets of data from the gels in Set 3 (Figures 3.11 and 3.12) showed that the TTGE gel produced more widely dispersed data points, complicating the interpretation of this data. Marie *et al.* (2006) found that while DGGE and TTGE profiles were comparable, DGGE provided a greater number of bands and the data produced from DGGE and TTGE did not correspond entirely. However, in contrast, they opted to use the data from the TTGE gels because the DGGE data was more complex. Therefore, the selection of which method to use can be a compromise between data quality and complexity. Empirical comparisons, such as those performed in this work, must determine which method is preferable for a specific study subject.

This work revealed that DGGE was preferable to TTGE for the study of fungal wheat endophytes because TTGE could underestimate species richness and fail to confirm sequence similarities. Considering the time and cost benefits, the optimization of TTGE could be explored further for use in other environmental diversity studies. However, DGGE proved to be the more robust and consistent method for use in fungal wheat endophyte studies.

3.6 References

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Chapter 4 Molecular analyses of field grown wheat reveal a cultivar x organ interaction on fungal endophyte biomass and a significant chytrid presence

4.1 Abstract

Fungal endophyte community composition can be affected by various factors, such as the host genome. Research into the host genome effects on fungal endophyte composition can assist in harnessing the potential benefits of such relationships in agro-ecosystems. Several culture-based studies have investigated the presence of a cultivar effect on endophyte composition. However, a culture-based approach can only detect organisms that can be isolated and grown. Culture-independent methods can detect both culturable and non-culturable fungal endophytes for comparisons of fungal endophyte community composition (ECC) between wheat cultivars. Denaturing gradient gel electrophoresis (DGGE), high-resolution melt (HRM) analysis of community profiles, quantitative PCR, and sequence analysis were used to analyse and compare the fungal ECC of four wheat cultivars grown under field conditions. A significant organ and cultivar x organ interaction effects on fungal biomass were observed. A chytrid, namely *Olpidium brassicae* formed a significant component of the fungal endophyte community across all tissues in wheat. This finding highlighted the utility of the culture-independent in revealing cryptic interactions and endophytes, and raised questions about the factors that influence the organisms that reside within field-grown wheat.

4.2 Introduction

Microbial endophytes, which occur within plants without causing disease, have been likened to the human gut microbiome in function and impact on host health and are of increasing interest for agriculture (Sessitsch and Mitter, 2015). These organisms are being studied to reveal the relationship between host plant and microbe and to develop new biological control strategies for crop production (Porras-Alfaro and Bayman, 2011). To harness the benefits of endophytes in agriculture, several studies have sought evidence of a selective

influence on endophyte diversity by the plant host genotype (Sessitsch *et al.*, 2002; Hoffman and Arnold, 2008; Pan *et al.*, 2008; Andreote *et al.*, 2010; Manter *et al.*, 2010; Wearn *et al.*, 2012).

Wheat is a major global food crop (Mayer *et al.*, 2014), and several culture-based studies have investigated the wheat endophyte community composition to detect a cultivar-based effect. Sieber *et al*. (1988) reported that cultivar did not affect the composition of endophytes isolated from four wheat cultivars. Other researchers also observed that wheat cultivar did not influence the detected wheat endophyte diversity (Crous *et al*., 1995; Larran *et al.*, 2002), although another study detected several interactions between microorganisms, tissues, growth stages and cultivar (Larran *et al.*, 2007). Subsequently, host genome effects on wheat-associated microbes were revealed by culture-independent studies (Sapkota *et al.*, 2015), suggesting that a culture-independent approach may reveal cultivar-based effects or interactions that were not readily discovered by traditional culture-based methods.

While culture-based methods can identify many fungal endophytes, such methods cannot identify organisms that do not grow on artificial media (Sánchez Márquez *et al*., 2012). On the other hand, culture-independent methods can detect both culturable and non-culturable endophytes (Saito *et al.*, 2007). PCR amplification of DNA sequences that can be used to identify fungi, followed by denaturing gradient gel electrophoresis (DGGE), can be used to identify endophytic fungi sequences and compare host profiles of endophyte composition by separation of these sequences on DGGE gels (Garbeva *et al.*, 2001). PCR primers amplifying the internal transcribed spacer (ITS) regions have optimal sizes for DGGE (Fromin *et al*., 2002). These primers amplify the ITS region which can be used for sequence identification of fungal species and for the characterization fungal endophyte composition (Blaalid *et al.*, 2013) across a broad range of fungal taxa (Schoch *et al.*, 2012). Quantitative PCR (qPCR) would be able to compare relative quantities of fungal biomass within host plant (Tellenbach *et al*., 2010). In addition, community fingerprinting by high-resolution melt (HRM) analysis, which has been used to compare microbial communities (Hjelmsø *et al.*, 2014; Kim and Lee, 2014), could reveal further endophyte community characteristics.

This research employed culture-independent methods, including PCR-DGGE, qPCR, HRM and sequence analysis, to investigate fungal endophyte community composition of different tissues in different wheat cultivars, to determine whether genomic differences in wheat cultivars can affect fungal endophyte composition and which types of fungal endophytes associate wheat under field conditions.

4.3 Materials and Methods

4.3.1 Sample collection

Samples were collected from a field trial run by the Agricultural Research Council's Small Grains Institute (SGI) (Blydskap Road, Lindley Direction, Bethlehem-District, 9700) in South Africa. The trial was located at 28°50'56.72''S and 29°28'5.22''E in the KwaZulu-Natal Province, within a commercial irrigated wheat field, subject to pesticide and fertilizer applications by the farmer. It was arranged in a randomized blocks design with 18 cultivars (4 replicate blocks per cultivar), including the commonly cultivated, national cultivars Duzi, Kariega, Krokodil and Olifants that were selected for fungal endophyte analysis in this study. These cultivars were selected because their rust disease resistance profiles differed, according to the ARC's Guidelines (Burger and Kilian, 2009). Blocks consisted of 8 rows of wheat, which were 5 m long and spaced 0.017 m apart at a planting density of 225 plants.m^{-2}. The total fertilizer application reached a target of 220, 30 and 20 kg.ha^{-1} NPK, with micronutrient application between the tillering and the stem elongation stages.

The wheat was planted on 05 June 2009. Seeds were treated with Anchor® (Chemtura, USA) fungicidal seed treatment (carboxin + thiram (200 +200 g.L^{-1})) before planting. Insecticides and herbicides were applied as necessary. Personal communications with the farmer indicated that the fungicides Folicur® (Bayer CropScience Ag, Germany) (tebuconazole) and Amistar® (Syngenta, Switzerland) (azoxystrobin) were each applied three times to control wheat rusts, as per the manufacturer's recommendations. Whole plant samples were collected on the 16th of September 2009 from wheat plants at the Feekes Growth Stage 11.1 (Large, 1954) because this stage was considered to harbour the highest diversity of endophytes (Larran *et al*., 2007). Single plants for each of the cultivars, Duzi, Kariega, Krokodil and Olifants (n = 16), were randomly sampled from the central region of each

replicate plot to ensure spatial independence from all other samples. Whole plants were washed in running tap water, removing soil from the roots and then stored for at -20°C to reduce DNA degradation (Allentoft *et al*., 2012) before further processing. Sample sections were taken from the same regions in the respective tissues, weighing approximately 0.2 g each. For the leaves, single segments were taken from the middle section of the leaf at the midpoint of the plant. Stem segments were taken from the midsection of the first internode after the second visible stem node from the base. Root segments were taken from each plant, from the middle of the largest roots.

4.3.2 Surface sterilization and DNA extraction

The leaf, root and stem sections were surface sterilized using the physical abrasion method described by Burgdorf *et al*. (2014). Tissue segments were placed in 30 mL McCartney bottles with 20 mL of a 0.01% water solution of Tween 20 (Merck, Darmstadt, Germany) and sonicated for 5 min in a Biosonic sonication bath (Coltene, Altstätten, Switzerland). Samples were then rinsed once with tap water, sterile water and then placed in a 2 mL microtube with 1.5 mL 0.9% NaCl solution and 0.3 g of 0.1 mm glass beads (Sigma-Aldrich, St. Louis, Missouri, USA). The tubes were vortexed on a Disruptor Genie Vortex (Scientific Industries, Bohemia, New York, USA) for 20 min, rinsed three times in 1 mL of sterile ultra-pure water and stored at -80°C for further processing.

DNA was extracted from all tissues according to a modified version of the extraction process described by Kang *et al*. (1998): Samples consisting of 0.2 g of plant tissue were ground in liquid nitrogen with 0.1 g of 0.1 mm glass beads (Sigma-Aldrich) using decontaminated ceramic mortars and pestles. The ground tissue was placed in a 2 mL microtube with 400 µL Extraction Buffer 1 (0.5% SDS, 0.2 M Tris (adjusted to pH 8.0 with 1 M HCl), 0.2 M NaCl and 0.025 M EDTA) and 50 µg of Proteinase K (Sigma-Aldrich) and then incubated at 37°C for 1 h. To this was added 400 µL of Extraction Buffer 2 (2% CTAB (w/v), 0.1 M Tris (pH 8.0), 0.02 M EDTA (pH 8.0), 1.4 M NaCl, 1% polyvinylpyrrolidone MW 40,000) and then 400 µL of 24:1:1.25 chloroform: isoamyl alcohol: phenol (pH 8.0) . The tubes were mixed by gentle inversion and then centrifuged at 14,000 x *g* for 10 min at 4°C. The supernatant was transferred to a 1.5 mL microtube with 400 µL of chloroform and centrifuged before. The

supernatant was transferred to a 2 mL microtube, 2/3 of the supernatant volume of isopropanol was added and then stored at -20°C overnight. The tubes were then centrifuged at 16,000 x *g* for 10 min at 4°C and the supernatant was discarded. The pellet was washed twice with 70% ethanol, dried in a laminar flow hood and then resuspended in 50 µL of 1 x TE buffer. DNA purity and quantity was confirmed on a Nanodrop 1000 (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA).

4.3.3 PCR, DGGE, gel analysis and band identification

Three sets of PCR (root (n=16), stem (n=16) and leaf (n=16) samples) were performed on the G-Storm Thermal Cycler (Syngene) using the GC-clamped universal fungal ITS1F-GC forward primer (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CTT GGT CAT TTA GAG GAA GTA A-3') and the ITS2 reverse primer (5'-TTY GCT GYG TTC TTC ATC G-3') (Wakelin *et al.*, 2007) with the Kapa 2G Fast PCR kit (Kapa Biosystems, Wilmington, Massachusetts, USA). Primer concentration was at 400 nM, and template DNA concentrations were standardized at 0.4 ng, 2 ng and 4 ng per reaction for root, leaf and stem tissue respectively, in a final reaction volume of 25 µL. DNA from unidentified fungal endophytes previously isolated from the respective organs of wheat samples was used as PCR positive controls. The PCR parameters consisted of initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 15 s, with a final elongation step at 72°C for 1 min. The quality of PCR products and product sizes were confirmed by agarose gel electrophoresis.

All root, stem and leaf PCR products (15 µL per lane) were run along a 30-45% urea/formamide denaturation gradient on 6% gels (37.5:1 acrylamide/bisacrylamide (Sigma-Aldrich)) at 100 V for 16 h in 60°C 1 x TAE buffer on the Bio-Rad Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc., USA). Gels were stained in a 1 x SYBR® Gold nucleic acid stain (Thermo Fisher Scientific) for 40 min and the images were captured on the I-chemi G-box gel documentation system (Syngene). Band presence / absence matrices with band pixel intensities were produced using Quantity One gel analysis software (Bio-Rad).

Differences in band composition (presence / absence of bands resolved by DGGE) among cultivars and tissue were tested by Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) using Primer (v.6) (Clarke and Gorley, 2006).

A Mantel test (Mantel, 1967), using Mantel's asymptotic approximation (Douglas and Endler, 1982), was performed to correlate band profiles between organs using PC-ORD (v. 4.25) (McCune and Mefford, 2011).

Based on the banding profile data from DGGE gels of all the cultivar organs, it was decided to identify some of the band sequences in the Duzi cultivar that were commonly observed in the other cultivars as well. Replicate samples of root, stem, and leaf PCR products were pooled according to tissue type and run on a DGGE gel as before. The gel was stained with 1 x SYBR® Safe (Thermo Fisher Scientific) and selected bands of suitable quality were excised, rinsed in sterile water and placed in a 1.5 mL tube with 100 µL of 0.5 x TE buffer and three 2 mm zirconia beads (Biospec Products, Inc., Bartlesville, Oklahoma, USA). Samples were homogenized on a Biospec 16 bead beater (Biospec Products, Inc.) for 30 s and incubated overnight at 4°C. The samples were then centrifuged briefly and the supernatant used as PCR template at 1 µL per 25 µL reaction. The PCR was performed under the same conditions as before. Products were sent to Inqaba Biotec (Pretoria, South Africa) for sequencing using the Big Dye® Terminator kit (V3.1, Thermo Fisher Scientific) on the ABI 3500 XL Genetic Analyzer (Thermo Fisher Scientific). Sequences were checked for quality and submitted for BLAST searches (Altschul *et al*., 1990) on the NCBI database. Sample identity was assigned according to highest percentage matches that were found.

4.3.4 Quantitative PCR (qPCR) and high-resolution melt (HRM) analysis

PCR of samples of the replicates of root, stem and leaf DNA extracts from the four wheat cultivars was performed on a Rotor-Gene 6000 (Qiagen, Hilden, Germany) using the same primers as before, using the Kapa SYBR Universal qPCR kit (Kapa Biosystems). The PCR parameters consisted of a 10 min denaturing step at 95°C, followed by 40 cycles of 95°C for 20 s, 52°C for 30 s and 72°C for30 s. This was followed by a high resolution melting step ranging from 75°C to 95°C at 0.1°C increments. Template DNA was at the same

concentration as before in a 25 µL reaction volume. A series of duplicate reactions from serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of previously extracted genomic DNA from *Beauveria bassiana* (Bals.-Criv.) Vuill. at a starting template concentration of 0.02 ng.µL^{-1} was included on the PCR run for the purposes of developing a standard curve for relative fungal DNA quantification, as an indicator of relative fungal biomass.

Relative quantities of original DNA template for each template were calculated using the Rotor-Gene 6000 Series Software (v.1.7 (Build 75), Corbett Research, Mortlake, Australia). A two-way ANOVA was performed comparing $\log_{10}$ transformed relative DNA quantity between tissues and cultivars in Genstat (Payne *et al*., 2011).

Principal Component Analysis (PCA) was performed on HRM profiles (dF / dT values per 0.1°C increment) in Canoco 5 (Ter Braak and Šmilauer, 2012) and PERMANOVA and PERMDISP on the same data were performed in Primer 6 (Clarke and Gorley, 2006).

4.3.5 Clone library sequence analysis

Based on the DGGE gel observations root, stem and leaf tissue replicate PCR products were pooled according to organ type for the cultivar Duzi as a representative of general fungal endophyte composition for all cultivars. The products were also pooled to produce a clone library of the most common fungal endophytes within the replicated samples. Pooled products were purified using the Zymo SV Gel and PCR cleanup kit (Zymo Research, Irvine, California, USA). Clone libraries were produced using the Clonejet PCR Cloning Kit (Thermo Fisher Scientific) and E. cloni® 10G Chemically Competent Cells (Lucigen, Middleton, Wisconsin USA). Colonies were randomly selected and checked for suitably sized inserts and then ten PCR amplification products from each clone library per organ were sent to Inqaba Biotec for sequencing. Sequences were submitted for BLAST searches on the NCBI database for identification.

A cladogram was produced to provide support for the identification of sequences identities that were returned from the BLAST search, as follows: a total of 54 sequences, including the 30 from the 3 clone libraries and another 24 selected from the NCBI database were used to

develop a neighbour-joining tree (Saitou and Nei, 1987) in MEGA5 (Tamura *et al.*, 2011) using the p-distance method (Nei and Kumar, 2000) after removing ambiguities. The tree was tested with 1000 bootstraps.

4.4 Results

4.4.1 DGGE gel profiles

The DGGE gels showed profiles that appeared largely consistent between cultivars and their replicates. Groups of bands could be seen to occur in all cultivars and organs, and the identities of these recurring groups were determined from the sequences of excised bands, shown in Figure 4.1.

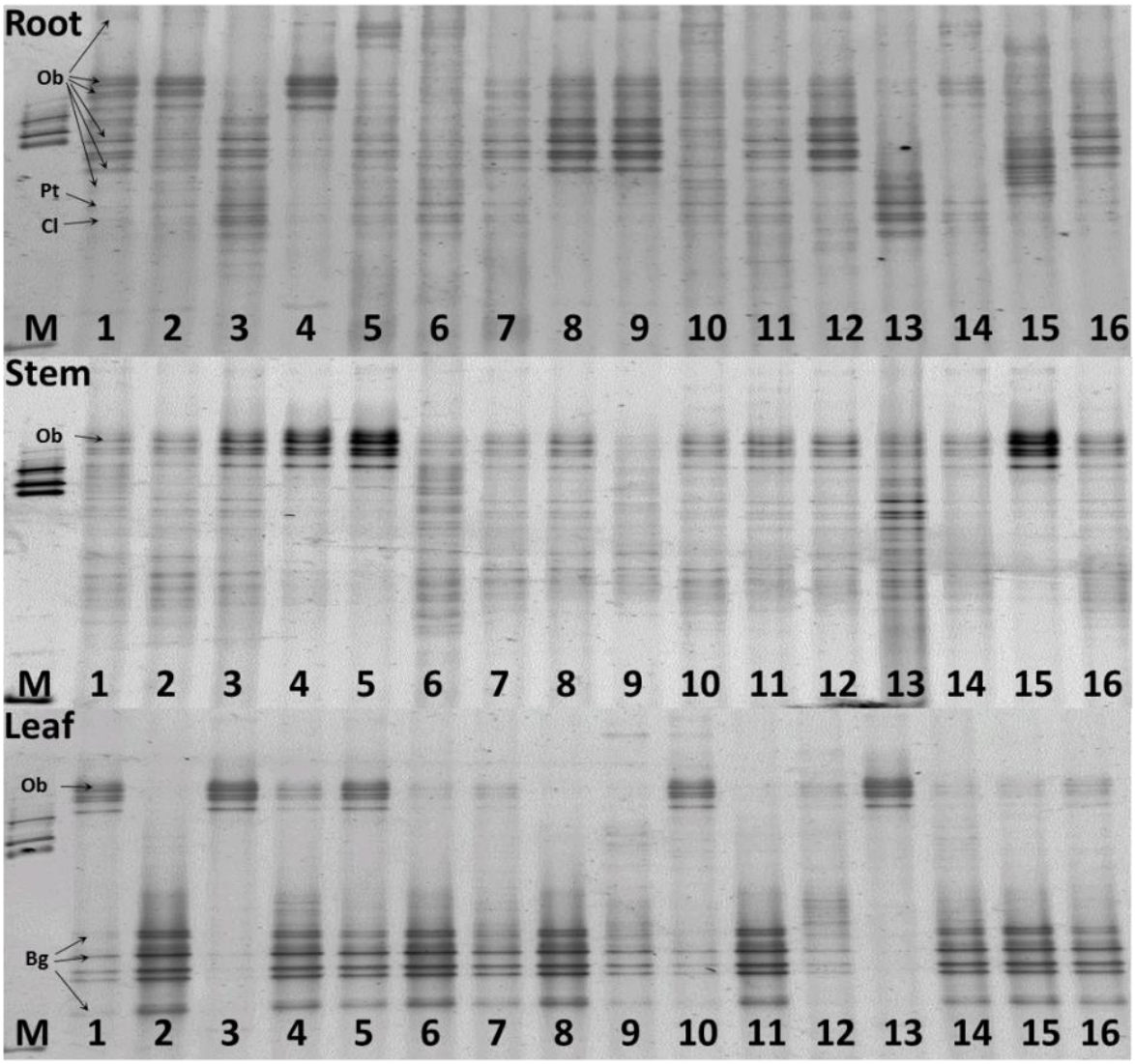

Figure 4.1 Fungal ITS PCR products for wheat root, stem, and leaf tissue separated on DGGE gels with each of the replicates for cultivars, Duzi (Lanes 1-4), Kariega (Lanes 5-8), Krokodil (Lanes 9-12) and Olifants (Lanes 13-16) and a marker (M). Excised band identities are indicated, representing *Olpidium brassicae* (Ob), *Phoma terrestris* (Pt), *Cochliobolus lunatus* (Cl) and *Blumeria graminis* (Bg).

4.4.2 Gel analysis

PERMANOVA of root, stem, and leaf band profiles showed no significant differences between cultivar profiles ($p > 0.05$).

The Mantel test of DGGE band profiles showed negligible correlations ($r \sim 0$) and supported the null hypothesis of no relationship ($p > 0.05$) between the DGGE profiles of fungal

endophytes for different organs. This indicated that the fungal endophyte gel profiles differed significantly between roots, stems, and leaves.

4.4.3 qPCR analysis

A plot of the mean log_{10} transformed relative fungal DNA values for the four cultivars (Figure 4.2) illustrated how differences in the fungal biomass per organ interacted with cultivar.

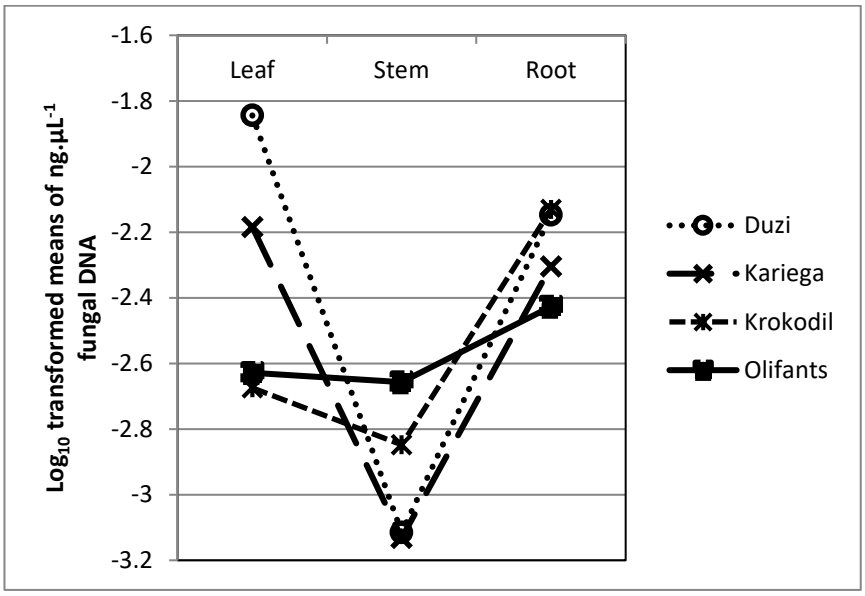

Figure 4.2 Log_{10} transformed means of replicate leaf, stem, and root sample values for relative quantities of fungal DNA for the different cultivars, showing a switch in the pattern of organ colonization preference that was influenced by cultivar.

The two-way ANOVA of log_{10} transformed relative DNA quantities (Table 4.1) revealed a cultivar x organ interaction effect on relative fungal DNA quantity, i.e., fungal biomass ($p = 0.026$). There was also a significant difference in relative amounts of fungal biomass between organs ($p < 0.001$). The fungal biomass in roots and leaves did not differ, but both differed significantly from the amounts in stems (see Table 4.1).

Table 4.1 Results of a two-way ANOVA of log_{10} transformed relative DNA quantities for cultivar, organ and cultivar x organ effects.

Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**f-ratio**	**p-value**
Cultivar	3		0.3147	0.1049	f-ratio	0.545
Organ	2		4.4968	2.2484	0.72	<.001
Cultivar x Organ	6		2.4058	0.4010	15.48	0.026
Residual	35	(1)	5.0834	0.1452	2.76	
Total	46	(1)	12.2857			
Grand mean	-2.507					
Cultivar	Duzi	Kariega	Krokodil	Olifants		
Mean	-2.369	-2.540	-2.551	-2.571		
Organ	Leaf	Root	Stem			
Mean	-2.333f	-2.252f	-2.938g			
Cultivar x Organ	Leaf	Stem	Root			
Duzi	-1.844d	-3.115ab	-2.147cd			
Kariega	-2.184bcd	-3.132a	-2.304abcd			
Krokodil	-2.674abcd	-2.848abc	-2.131cd			
Olifants	-2.629abcd	-2.657abcd	-2.427abcd			

Means with the same letters were not significantly different ($p > 0.05$)

4.4.4 HRM analysis

The PERMANOVA of the HRM profiles found no significant effects or interactions for cultivars; however, DNA profiles between organs differed significantly ($p = 0.001$).

The PERMDISP analysis of HRM profiles found a significant difference in heterogeneity between organs ($p = 0.001$), but not between cultivars ($p = 0.344$). There was no significant difference in heterogeneity between roots and leaves ($p = 0.327$), but they both differed from stem tissue ($p = 0.001$), which had the most homogeneous profiles.

The PCA biplot of the HRM profile data (Figure 4.3) showed homogeneity among the samples from tissues, but not among cultivars. Root samples differed the most from leaf and stem samples.

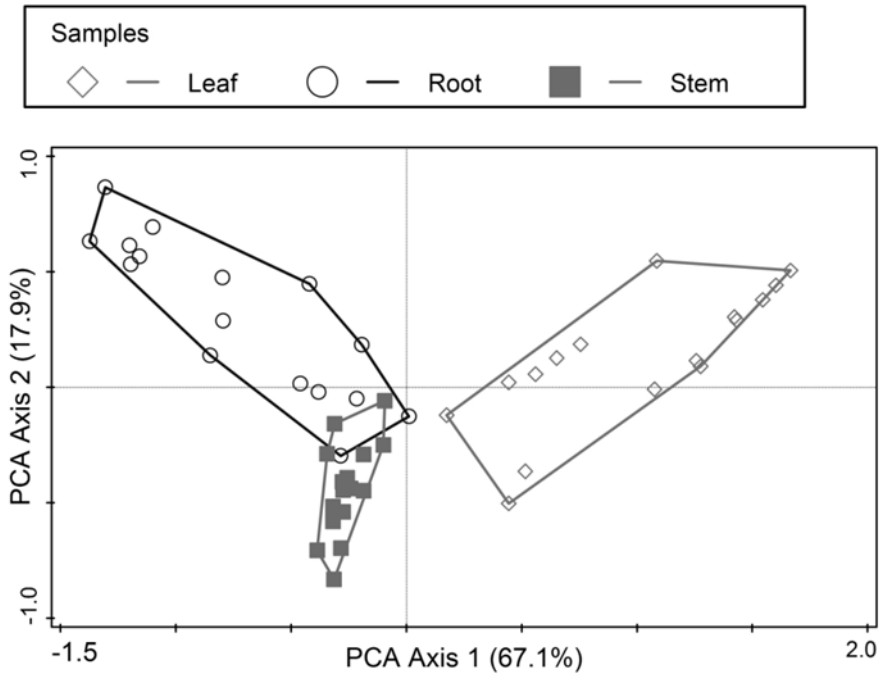

Figure 4.3 PCA biplot of HRM profile data for all replicates from all organs of each cultivar, showing the greatest variation explained by differences between organ-type, with no significant variability attributed to cultivar differences.

4.4.5 Sequence analysis

BLAST sequence identities of root (R1-10), stem (S1-10) and leaf (L1-10) fungal sequences were presented on a neighbour-joining tree (Figure 4.4) and revealed a high incidence of the obligate biotroph, *Olpidium brassicae* (Woronin) P.A. Dang. in all tissues. Except for a few matches with wheat DNA sequences, the sequences corresponded to fungal ITS sequences on the NCBI database. The sequences from root tissue included saprophytic fungi known to exist in soil and to associate with plant roots. The sequences from leaf tissue included sequences belonging to fungal species known to be endophytes or pathogens of wheat. Some typically root-associated organisms were also detected.

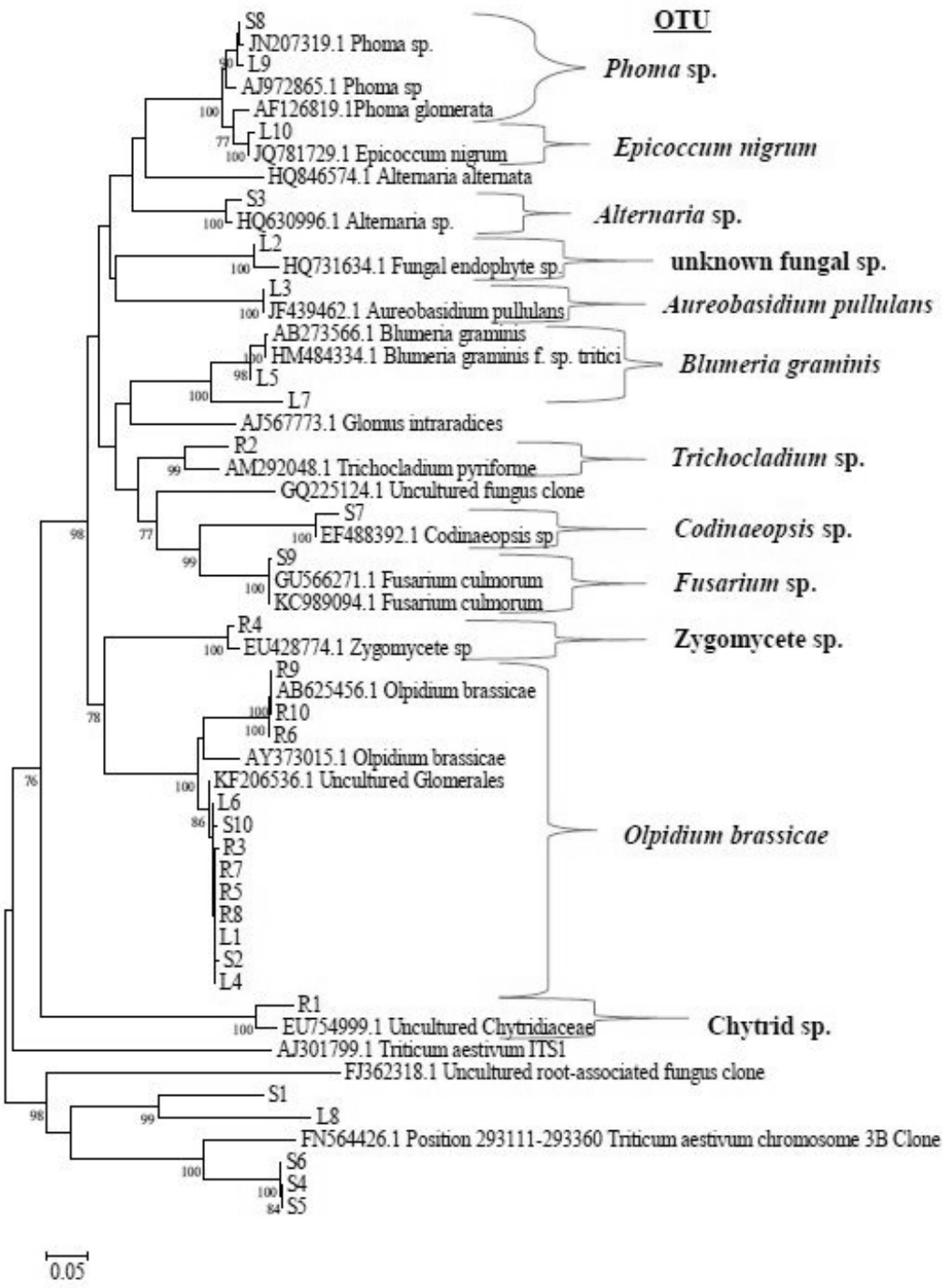

Figure 4.4 A neighbour-joining tree for 54 sequences including clone sequences, nearest Genbank matches, and selected sequences from Genbank. Bootstrap values above 70% are shown at nodes. The scale indicates the number of base differences per site. Cloned sequence clusters are assigned to fungal operational taxonomic units in bold.

4.5 Discussion

The cultivation-independent approach in this study aimed to reveal culturable and non-culturable fungal endophytes, and any host genotype effects or interactions that have not previously been revealed by culture-based studies of wheat endophytes. The root, stem and leaf fungal endophytes detected by PCR were visualized on DGGE gels, which were then analysed for evidence of a cultivar effect on fungal endophyte composition.

Some taxa were represented by multiple bands (Figure 4.1), referred to as paralogues (Woo *et al*., 2010; Lewis *et al*., 2011). This phenomenon confounds diversity estimates from these gels and suggests that there were relatively few organisms detected by PCR and visualized by DGGE. Instances have occurred where DGGE has detected fewer species relative to culturing methods (Duong *et al.*, 2006). In this study the application of systemic, broad-spectrum fungicides to the wheat plants in the field by the farmer would have reduced species richness, since Riesen and Close (1987) noted a reduction in the abundance of certain fungal endophytes in fungicide-treated barley leaves, and Karlsson *et al.* (2014) found that fungicides reduced fungal OTU richness in the wheat phyllosphere.

PERMANOVA of DGGE band profiles indicated that host cultivar did not affect endophyte community composition, i.e., diversity of fungi was the same for all cultivars under these field conditions, as concluded in studies by Sieber *et al*. (1988) and Crous *et al*. (1995). Similarly, the tissue specificity revealed by the Mantel test was also supported by Crous *et al*. (1995), who found that fungal endophyte profiles differed between plant organs, and by Sieber *et al*. (1988), who reported that the plant organ had the greatest influence on fungal endophyte composition, while cultivar had the least effect.

The two-way ANOVA of DNA quantities (Table 4.1) showed a cultivar x organ interaction for relative fungal endophyte biomass, although overall biomass between cultivars did not differ. The interactive effect of cultivar x tissue on fungal biomass showed that while total biomass remained constant, certain cultivars favoured denser endophytic colonization of certain tissues (Figure 4.2). This suggests that there is a controlled level of endophyte colonization within a plant, limiting the extent to which each organ is colonized by fungal

endophytes. The control of colonization by organisms in distal tissues is supported by the observation that signaling is essential to plant-microbe symbioses (Witzany, 2006) and that fungal leaf endophytes affect metabolic activity in host plant roots (Malinowski *et al.*, 2000).

Larran *et al.* (2007) attributed their findings of a microorganism x wheat cultivar interactions to the frequency of endophyte isolation. This could be indicative of differences in the extent of colonization, i.e., the relative biomass, of the microbes that were isolated. The quantitative nature of NGS (Amend *et al.*, 2010) could have contributed to the discovery of a host genotype effect on fungal community composition in different wheat cultivars by Sapkota *et al*. (2015), who found significant differences in certain fungal OTU abundances. Therefore, the use of quantitative analysis by qPCR may be necessary for future investigations of host genotype effects on microbial endophyte composition, especially where differences in diversity are not evident and agronomic practices, e.g. fungicide applications, may influence endophytic association with host plants.

The PERMANOVA of HRM data supported the findings of the DGGE, with no significant effect by cultivar on the HRM profiles, nor any interaction. The latter may seem to contradict the findings of the two-way ANOVA; however, this was because HRM profiles were derived from end-point PCR reactions, which did not provide quantitative information on fungal biomass. The reporting of this finding is mainly to recommend the use of HRM as a less labour-intensive screening tool in fungal endophyte studies for comparing community profiles, as proposed by Hjelmsø *et al.* (2014). PCA of HRM data also provided graphic depiction of the homogeneity of stem samples relative to the leaves and roots, where there was greater heterogeneity. This information was confirmed by the PERMDISP analysis, which implied that the stems were a more restrictive environment for endophyte colonization than leaves and roots.

The DNA sequence data emphasized the strengths of the molecular approach to endophyte studies. Considering that only 0.2 g of material per sample was analysed, the detected organisms probably represented the dominant systemic population within the plants,

representing the dominant organisms and the general fungal endophyte community composition within tissues and cultivars.

The Genbank database has many erroneous taxonomic assignments to sequence data (Hyde and Soytong, 2008). Many of the uncultured *Glomerales* sequences were closely matched to *Olpidium brassicae*. It is unlikely that arbuscular mycorrhizal (AM) fungi are found in stems and leaves (see clade membership of root, stem and leaf OTU's with *O. brassicae* in Figure 4.4). Therefore, several NCBI sequences assigned to *Glomerales* are probably misidentified *Olpidium* sp. isolates. The identities of the detected sequences were inferred from clade membership on the neighbour-joining tree (Figure 4.4) because of such uncertainties in the BLAST search.

Alternaria, *Fusarium*, *Phoma* and *Epicoccum* spp. are frequently isolated from wheat (Crous *et al.*, 1995; Larran *et al.*, 2002; Larran *et al*., 2007). *Alternaria* spp. and *Fusarium* spp. are economically important pathogens of wheat and produce mycotoxins (Magan *et al.*, 1984; Snijders and Perkowski, 1990). *Phoma* spp. have been described as endophytes of certain grasses (Wang *et al.*, 2007). *Phoma glomerata* (Corda) Wollenw. and Hochapfel has been reported as a pathogen of wheat (Hosford, 1975), but it has also been described as a mycoparasite of a powdery mildew (Sullivan and White, 2000). Its presence and role as a wheat endophyte may also be worthy of further investigation. *Aureobasidium pullulans* (De Bary) G. Arnaud ex Cif., Ribaldi and Corte and *Epicoccum nigrum* Link have been reported to be plant endophytes with biological control potential (Martini *et al.*, 2009). *Trichocladium* spp. have been identified as grass endophytes (Márquez *et al.*, 2010) and an endophytic *Codinaeopsis* sp. is the source of a compound named codinaeopsin, which has significant pharmacological properties (Rosa *et al.*, 2011). *Blumeria graminis* (DC.) Speer causes powdery mildew of wheat (Bélanger *et al.*, 2003). Therefore, the detected fungal endophytes were likely to have had a range of interactions that influenced wheat growth and health. The presence of the obligate biotroph, *O. brassicae*, seemed to be inversely correlated to the presence of *B. graminis* in leaf samples (see leaf gel in Figure 4.1), suggesting that these organisms may be antagonists. *O. brassicae* cannot presently be detected by a culture-based approach, which confirmed that the culture-independent

approach can reveal the presence of organisms and interactions that evade detection by culture-based methods. It is noteworthy that a substantial proportion of endophyte sequences, i.e., at least 33% of clones in the stem, 56% in the leaf tissue and as much as 90% in the root tissue (Figure 4.4) belonged to *O. brassicae*. While *O. brassicae* is known to associate with wheat (Zhang *et al.*, 1994; Mozafar *et al*., 2000), the chytrids are a group of fungi rarely considered to be prevalent in agricultural environments, particularly as endophytes. Chytrids are reported to inhabit disturbed environments, (a definition which can describe a commercial wheat field), as well as being parasites of other fungi such as arbuscular mycorrhizal fungi (Klein, 2006). The absence of AM fungi in this study supports the notion that the *O. brassicae* displaced the AM fungi or parasitized them. The dominant endophyte, *O. brassicae*, may have filled a niche left by the suppression of AM fungi due to the application of phosphate-containing fertilizers (Graham and Abbott, 2000). These scenarios merit further investigation regarding the potential benefits or losses to wheat caused by this organism. Furthermore, the biology of such an unexpected endophyte invites speculation on the conditions that resulted in this organism associating with the host plant and the effect that it had.

The culture-independent work in this study revealed that, under the study field conditions, the fungal endophytes detected were equally capable of colonizing all wheat cultivars, although there were differences in the endophyte composition of roots, stems and leaves. Therefore, while host genotype influences fungal endophyte composition, there may be a spectrum of influence, which can be overwhelmed by environmental factors, such as pesticide applications and land management. The agronomic practices that affect endophyte composition need to be explored further before the beneficial properties of such organisms can be harnessed.

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Chapter 5 Next generation sequencing analysis of the effect of tebuconazole on wheat flag leaf eukaryotic endophyte composition

5.1 Abstract

Systemic fungicides used in wheat production are pathogenic to many plant-inhabiting fungi such as fungal endophytes. The aim of the study was to reveal the effect of tebuconazole on the eukaryotic endophytes of wheat flag leaves using next generation sequencing (NGS). Treated and untreated leaves were surface sterilized prior to metagenomic DNA (mDNA) extraction. NGS was performed on DNA amplified using universal ITS primers. SCATA analysis was used for operational taxonomic unit (OTU) assignment of sequences, which were identified against CBS, UNITE and Genbank databases. A maximum likelihood (ML) tree was developed for taxonomic assignment of key genera. OTU mean read numbers and OTU richness were compared. The treatment effects were analysed using Principal Component Analysis (PCA), permutational multivariate ANOVA (PERMANOVA), distance-based test for homogeneity of multivariate dispersions (PERMDISP) and similarity percentage analysis (SIMPER). With one exception, non-wheat OTUs belonged to the Dikarya. *Puccinia* read numbers differed significantly (P=0.01) between treatments and fungicide treatment tended to reduce total OTU read numbers and OTU richness. The variability of most key OTUs correlated positively with unsprayed samples. Treatment influenced OTU composition. Treated samples had the greatest homogeneity in endophyte composition and *Puccinia* made the greatest contribution to variation, with low contribution from the other OTUs. Dikarya were the dominant wheat flag leaf endophytes, and while the fungicide suppressed *Puccinia* and reduced fungal endophyte abundance, it did not significantly alter the community assemblage.

5.2 Introduction

Agronomic conditions, such as fungicide application, determine the composition of the endophytic eukaryotic organisms that reside within wheat (Burgdorf *et al*., 2016). Studies on

the effects of fungicides on wheat found that several fungicides, including tebuconazole, delayed wheat senescence and increased grain yield (Ruske *et al*., 2003; Zhang *et al*., 2010). A high-throughput sequencing study of wheat reported the reduced fungal species richness resulting from fungicide application and suggested that the suppression of phyllosphere saprotrophs was responsible for the delayed senescence and increased yield (Karlsson *et al*., 2014). Further exploration using the power of next generation sequencing (NGS) may reveal additional factors that could explain these effects. For example, NGS has revealed that fungicide application is a major determinant of the fungal community composition (epiphytes and endophytes) on and within cereal leaves (Sapkota *et al.*, 2015)

NGS produces many thousands of reads per sample at an affordable price and is reducing the cost of DNA sequencing (Izard and Rivera, 2014). This makes the technology available to a wider range of scientists, including those studying endophytes by culture-independent methods (Akinsanya *et al.*, 2015). The increased sampling depth offered by NGS allows for greater insight into the diversity and dynamics of endophyte communities (Bullington and Larkin, 2015).

A PCR-based NGS approach requires the use of high-coverage primers to describe fungal community assemblages. Numerous primers have been recommended for the characterization of fungal endophyte communities and many of these try to provide a degree of taxon specificity; however, this introduces a level of bias to the PCR (Manter and Vivanco, 2007). This bias can be reduced by using classic universal primers with high-range species coverage (Martin and Rygiewicz, 2005, Tedersoo *et al.*, 2010, Toju *et al.*, 2012). In the case of eukaryotic endophytes, host plant DNA would be expected to co-amplify and then be sequenced as well. However, the high sequence depth of NGS (Smith and Peay, 2014) would be expected to detect the most abundant endophytic DNA sequences present in the metagenomic DNA (mDNA) derived from host plant samples.

The aim of this study was to investigate the effect of tebuconazole on both the fungal and non-fungal eukaryotic endophyte community composition of wheat flag leaves. This would be performed using universal eukaryotic PCR primers and the deep sequencing power of

NGS to establish the effect of fungicides on the dominant eukaryotic wheat flag leaf endophytes, which may include non-fungal organisms.

Materials and methods

5.2.1 Wheat cultivation, fungicide application, sampling, surface sterilization, and storage

Wheat (*Triticum aestivum* cv Duzi) was planted on the 12th of July 2013 at a rate of 54 kg.h^{-1} in three 2.5 x 4.0 m plots at the Controlled Environment Facility at the University of KwaZulu-Natal, Pietermaritzburg. Each plot was broadcast fertilized with 1 kg of general purpose 2:3:4 (NPK) fertilizer (Gromor (PTY) Ltd, Cato Ridge, South Africa) at planting.

Just prior to flag leaf emergence, the three plots were each divided into two on the longest side and separated using plastic sheeting to form a total of six sub-plots. Therefore, each full plot consisted of one fungicide treated (samples 1, 4 and 5) and one untreated (samples 2, 3 and 6) sub-plot. Using a knapsack sprayer, plants in the treated sub-plots were sprayed to run-off with Folicur (25mL/10L), at the emergence of the flag leaves (Feekes Growth Stage 8) on the 21st of August 2013, and again at flowering (Feekes Growth Stage 10.5.1) on the 12th of September 2013. Mean temperature and total rainfall over the growth period are presented in Table 5.1.

Table 5.1 Mean minimum and maximum temperatures and total monthly rainfall for July to October 2013. Data provided by the AgMet department at UKZN.

Month	Mean minimum temperature (˚C)	Mean maximum temperature (˚C)	Total rainfall (mm)
July	4.9	22.2	5.6
August	6.3	23.7	14.7
September	7.4	25.7	18.0
October	9.2	24.2	137.4

A total of thirty-six flag leaves from individual plants were randomly collected per sub-plot on the 12th of October 2013 at Feekes Growth Stage 11.1. Dried leaf tips were removed to

exclude senesced material. Leaves were surface sterilized, using a modification of the technique described by Arnold *et al.* (2007), because of the large number of leaves and their size (Burgdorf *et al*. 2014). Leaves were rinsed for 1 min under tap water, and then using clean 10 L buckets (washed thoroughly with soap decontaminated with 0.5% NaOCl and rinsed with sterile autoclaved ultra-pure water), leaf samples were immersed in 95% ethanol for 5 s, 0.5% NaOCl for 2 min and 70% ethanol for 2 min. Samples were then rinsed in ultra-pure water, dried in a laminar flow cabinet and then stored at -80˚C.

5.2.2 DNA extraction

All extraction processes took place in a laminar flow using equipment that had been washed with soap, decontaminated with 0.5% NaOCl, rinsed with sterile ultra-pure water and autoclaved where possible. For each sample (n = 6) the 36 leaves were frozen in liquid nitrogen and homogenized in a Waring 8009L stainless steel blender (Conair Corporation, East Windsor, NJ, USA). A 1.5 g subsample of the pulverized mixture was placed in a sterile plastic 50 mL conical tube. The DNA was extracted as per a modified version of the method described by Kang *et al.* (1998): 15 mL of Buffer 1 (0.5% SDS, 0.2 M Tris, 0.2 M NaCl and 0.025 M EDTA, pH 8.0) was added to the tubes. The samples were mixed gently and frozen overnight at -20˚C. The tubes were then incubated at 80˚C for 2 hours. This was followed by the addition of 15 mL of Buffer 2 (2% CTAB (w/v), 0.1 M Tris, 0.02 M EDTA, 1.4 M NaCl, 1% polyvinylpyrrolidone MW 40,000, pH 8.0). The samples were frozen again overnight at -20˚C. To each of the tubes 15 mL of chloroform: isoamyl alcohol (24:1) with 5% phenol (pH 8.0) was added. The samples were shaken at 40 rpm on an MRC benchtop orbital shaker (MRC, Holon, Israel) for 1 h. The tubes were then centrifuged at 10,000 x *g* for 10 min. For each sample, 600 µL of supernatant was removed and mixed with 400 µL of isopropanol in a 2 mL microtube. The tubes were inverted several times and then centrifuged at 16,000 x *g* for 10 minutes at 4˚C. The supernatants were discarded and the pellets were washed twice with 70% ethanol and then dried in a laminar flow cabinet. The pellets were re-suspended in 100 µL of 1 x TE buffer and the purified DNA was checked for quantity and quality on a Nanodrop 1000 (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) and by agarose gel electrophoresis.

5.2.3 Sequencing

The extracted DNA was sent to Inqaba Biotec (Pretoria, South Africa), where genomic DNA was standardized to 30 ng of template DNA per PCR reaction, and amplified using the universal primer pair ITS5 and ITS4 (White *et al.*, 1990) with the addition of indexing adapters on the 5' end of each primer. Gel purified amplicons of size range 500-700bp were individually indexed and sequenced using the MiSeq V3 sequencing kit on the Illumina MiSeq platform (Software version 1.8) (Thermo Fisher Scientific).

5.2.4 Sequence analysis and OTU assignment

Raw files were received from Inqaba Biotec and sequence quality of raw data for each of the samples was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The indexed and Q30 quality filtered files also supplied by Inqaba Biotec were processed further, using the SCATA pipeline (Durling *et al.*, 2011) as follows: All six forward read FASTQ files were merged in Bio Linux 8 and uploaded as a single FASTQ file to the SCATA server for clustering and operational taxonomic unit (OTU) assignment. Quality data was ignored and OTUs were assigned with a proportional primer match of 0.7, 3% clustering distance, a 200bp minimum length of pairwise alignment, and using the 'CBS isolates' and 'UNITE Dec. 2014' reference databases to tentatively identify OTUs. All other settings were left at default. After the OTU assignment and identification had taken place, discarding any singletons (Karlsson *et al.*, 2014), the sequence clusters were labeled (OTU1 to OTU86) and paired with their respective sequences in BioEdit (version 7.2.0) (Hall, 1999) to produce a FASTA file. This file was uploaded to SCATA as a reference database to produce OTU tables for all samples. Individual samples were then run against this reference database, using the same parameter settings as before. The previously assigned OTUs were identified for each sample and OTU tables produced in Microsoft Excel 2010 (Microsoft, Redmond, Washington, USA) for further comparison. As before, all singletons in individual samples were discarded.

5.2.5 Phylogenetic tree and taxonomic assignment

NGS read abundance is semi-quantitative (Amend *et al*., 2010); therefore, the wheat sequence read numbers were used as an internal quantity standard for relative individual OTU abundance among species and samples. OTU numbers were standardized by scaling down all sample wheat read numbers to rounded relative proportions of the lowest recorded wheat sequence read number value. The data from the OTU tables were then filtered to exclude all wheat sequences and those that were represented by standardized read numbers of less than 1% of the total non-wheat sequence reads. Since the SCATA OTU identification yielded multiple identities for many of the remaining OTUs, the sequences were matched to the nearest identities by performing a BLAST search (Altschul *et al.*, 1990) on the NCBI Genbank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For each of the OTUs, the highest scoring sequence matches with identities at greater than 97% coverage in genera proposed by SCATA, were downloaded from Genbank and used to produce the phylogenetic tree. The sequences were aligned in BioEdit using ClustalW (Thompson *et al.*, 2002). Molecular phylogenetic analysis was performed in Mega6 (Tamura *et al.*, 2013), a model test was performed and evolutionary history was inferred by the maximum likelihood (ML) method based on the Tamura 3-parameter model (Tamura, 1992). The initial tree for the heuristic search was obtained automatically by applying the maximum parsimony method. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.4807)), eliminating all positions containing gaps and missing data. The ML tree that was produced was used to establish the most probable taxonomic identity of the OTUs that were assigned within the samples.

5.2.6 Statistical analyses of effects of the fungicide treatment

A paired t-test (Microsoft Excel 2010) compared the mean $\log_{10}(1 + x)$ transformed standardized OTU read numbers individually for each of the eight dominant OTUs between the two treatments. A paired t-test also compared mean total OTU richness between treatments and $\log_{10}$ transformed mean total standardized OTU read numbers, including those which occurred at frequencies less than 1% of total OTU read numbers, between treatments. For Principal Component Analysis (PCA), only standardized $\log_{10}(1 + x)$ transformed read numbers for OTUs representing more than 1% of total read numbers were

included. The PCA triplot was produced in Canoco 5 (Ter Braak and Šmilauer, 2012). Permutational ANOVA (PERMANOVA), using a Monte Carlo permutation test (recommended for small sample numbers); Euclidean distance-based test for homogeneity of multivariate dispersions (PERMDISP); and similarity percentage analysis for species contributions (SIMPER) were performed using Primer 6 (Clarke and Gorley, 2006) on standardized $\log_{10}(1 + x)$ transformed read numbers for OTUs representing more than 1% of total read numbers.

5.3 Results

5.3.1 Sequence analysis and OTU assignment

Mean raw data file size was 281.5 ± 59.3 Mb in size. The mean Q30 filtered file size was 239.2 ± 52.3 Mb for the forward reads and 7.8 ± 1.7 Mb for the reverse. The FastQC analysis showed that while the forward reads were suitable for further analysis, but the reverse reads had substantially higher error levels; therefore, only the forward reads were considered for further analysis in this study.

After SCATA analyses and the removal of singletons from the forward reads, total sequences numbers were 2,198,001 reads, of which wheat sequences accounted for 2,191,146 (99.69%) and 6855(0.31%) non-wheat OTUs. Individual sample sequence read numbers ranged from 286,380 to 483,052 reads (mean = 366,333.5 ± 78760.8 reads). In these samples, wheat sequences represented 99.85 to 99.94% of reads. A total of 86 OTUs were assigned by SCATA from the merged sample data. Of the 86 OTUs, 35 were identified as wheat sequences. Only 44 of the original OTUs were identified in the analysis of the six samples against the merged file OTU reference database. An additional two read clusters that were not assigned OTUs in the merged data used to produce a reference database were found separately in two of the individual samples. Of the 46 OTUs, four could not be matched to any known sequences and one OTU sequence found only in one sprayed wheat flag leaf sample matched that of the bird cherry-oat aphid, *Rhopalosiphum padi* L. The rest were all members of the Dikarya. Non-wheat sample read numbers for the 46 OTUs ranged from 2 to 4,370 reads per OTU.

The SCATA derived OTU identities in several cases resulted in multiple species within multiple genera (Table 5.2). However, a search of Mycobank (www.mycobank.org) for some of the genera, such as *Stagonosporopsis , Peyronellaea*, *Boeremia* and *Didymella* indicated that these were synonymous with *Phoma* sp., and that some *Botryotinia* sp. are teleomorphs of a *Botrytis* sp. Therefore, not all genera were included in the phylogenetic analysis to avoid duplication or redundancy. Additionally, Genbank searches of genera such as *Sphaerothyrium* and *Myriosclerotinia* did not yield sequences for inclusion as matches were not found that had more than 97% coverage. There were a total of 6855 reads for the OTUs that were represented by more than 1% of the sequences in the total non-wheat read sequences. These were regarded as the most abundant OTUs, which were subjected to further analyses.

Table 5.2 Taxonomic assignments by SCATA from the UNITE/CBS databases for the most abundant OTUs represented by more than 1% of non-standardized wheat reads.

OTU	**Genera**	**Total read numbers per OTU**
2	*Puccinia*,	4370
3	*Aspergillus*	536
4	*Cladosporium*, *Mycosphaerella*	525
5	*Articulospora*	312
6	*Phoma*, *Epicoccum*, *Stagonosporopsis*, *Peyronellaea*, *Boeremia*, *Didymella*, *Macroventuria*, *Sphaeriothyrium*	257
7	*Sclerotinia*, *Botryotinia*, *Dumontinia*, *Ciboria*, *Myriosclerotinia*, *Monilinia*, *Botrytis*	207
8	*Pleurotus*, *Lentinus*	159
9	*Alternaria*	154

5.3.2 Phylogenetic tree and taxonomic assignment

The ML tree that was produced (Figure 5.1) had 173 positions in the final dataset. Apart from OTU5, all the other eight most abundant OTUs had 100% matches to sequences in the Genbank database, several of which were type material (Figure 5.1). A pairwise alignment

search on the CBS database confirmed a 99% match for OTU5 with *Articulospora proliferata* A. Roldán and W.J.J. van der Merwe (Accession no. SH148516.06FU).

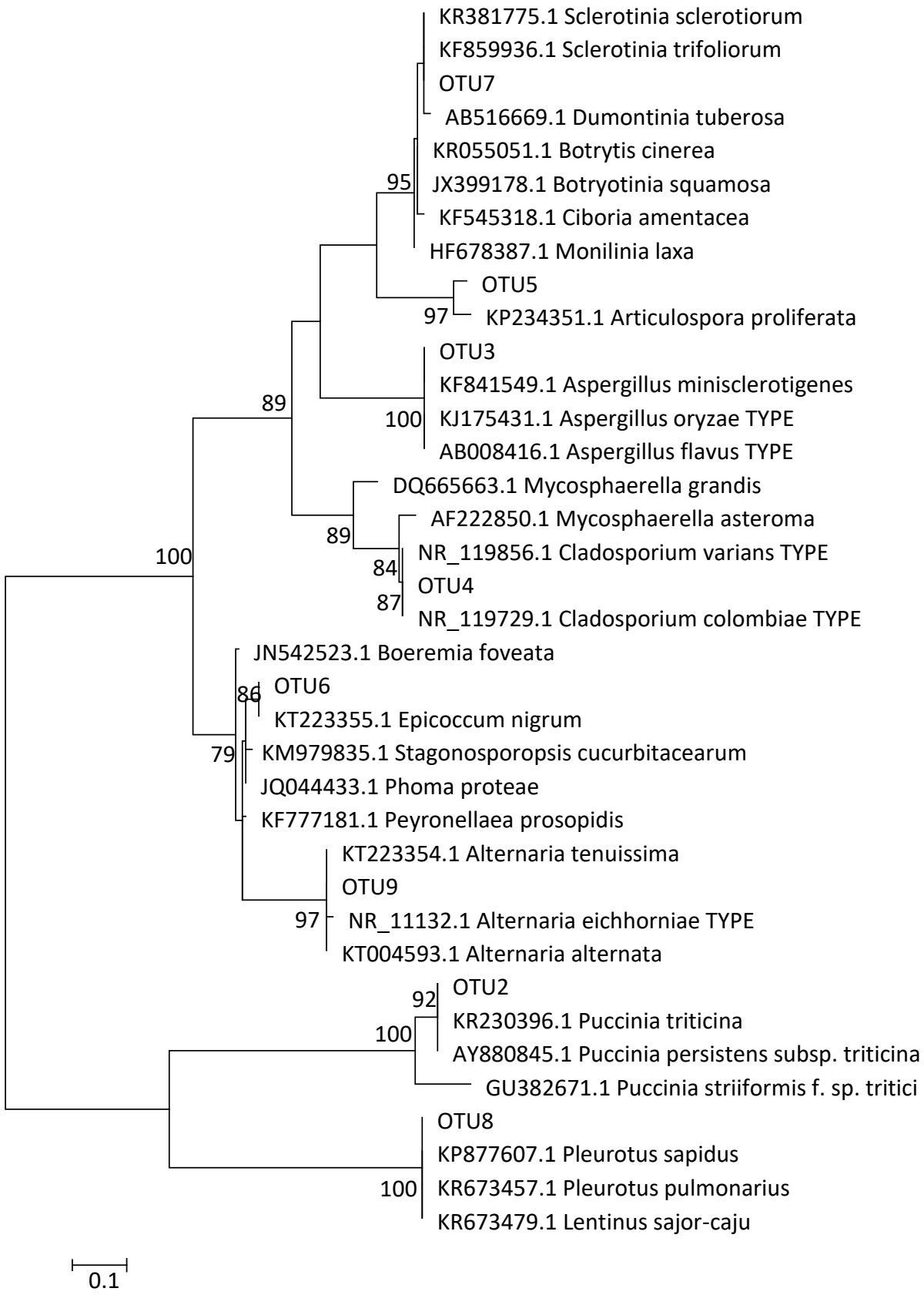

Figure 5.1 A maximum likelihood tree with bootstrap values above 70 shown at nodes and the scale indicating the rate of base pair substitution.

From the ML tree (Figure 5.1) and searches on the CBS database, taxonomic assignments were proposed for each of the eight dominant OTUs. The species identity was mostly uncertain, as several OTUs shared 100% sequence matches with multiple taxa, e.g., OTU3 matched type material sequences for both *Aspergillus flavus* Link and *Aspergillus oryzae* (Ahlb.) Cohn and OTU4 matched type material sequences for both *Cladosporium varians* U. Braun, Melnik and K. Schub. and *Cladosporium colombiae* K. Schub. and Crous. In most cases, taxonomy could be proposed with a degree of confidence to at least the genus level, as shown in Table 5.3. The table shows that all major taxa were members of the Dikarya and most taxa belonged to the Phylum Ascomycota. The highest read numbers were for *Puccinia* Pers., a genus containing known biotrophic wheat pathogens (Scott, 1990). The rest of the genera contain species that have been recorded as endophytes of plants, including wheat, as well as those that have been classified as wheat pathogens.

Table 5.3 Proposed taxonomic assignment to genus level for OTUs represented by more than 1% of reads as inferred from the ML tree in Fig 5.1.

OTU	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**% of non-wheat reads**
2	Basidiomycota	Pucciniomycetes	Pucciniales	Pucciniaceae	Puccinia	65.5
3	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	7.0
4	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium	7.7
5	Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Articulospora*	4.7
6	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Epicoccum	3.9
7	Ascomycota	Leotiomycetes,	Helotiales	Sclerotiniaceae	Sclerotinia	2.5
8	Basidiomycota	Agaricomycetes	Agaricales	Pleurotaceae	Pleurotus/Lentinus	1.9
9	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	2.3

* As inferred from the CBS database sequence match

The stack plot of the sample OTU composition (Figure 5.2) using the $\log_{10}(1+x)$ transformation of standardized read numbers suggested that *Puccinia* dominated the community composition of unsprayed wheat.

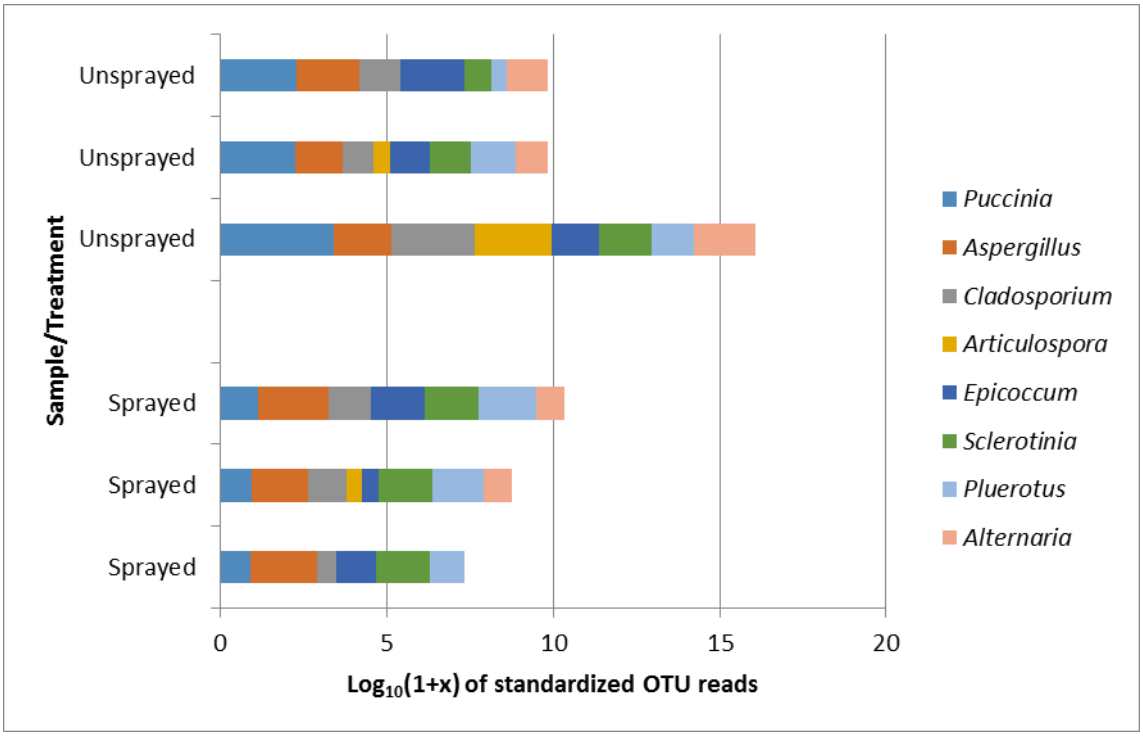

Figure 5.2 A stack plot $\log_{10}(1 + x)$ transformed standardized read numbers showing OTU proportions of total reads for samples that were unsprayed or sprayed with fungicide.

5.3.3 Statistical analyses of effect of fungicide treatments

The paired t-tests for individual OTU read numbers that were presented graphically in the stack plot (Figure 5.2) found that only *Puccinia* ($p = 0.04$) and *Alternaria* ($p = 0.02$) differed significantly between samples.

The graphical comparison of the $\log_{10}$ of mean sequence numbers per treatment (Figure 5.3 a) showed total OTU read numbers tended to be higher in unsprayed samples, although the paired t-test indicated that the differences were not significant ($p = 0.15$). A graphical comparison of mean OTUs per treatment (Figure 5.3 b) suggested that OTU richness tended to be greater in unsprayed wheat flag leaf samples, although the paired t-test did not find the difference to be significant ($p = 0.44$).

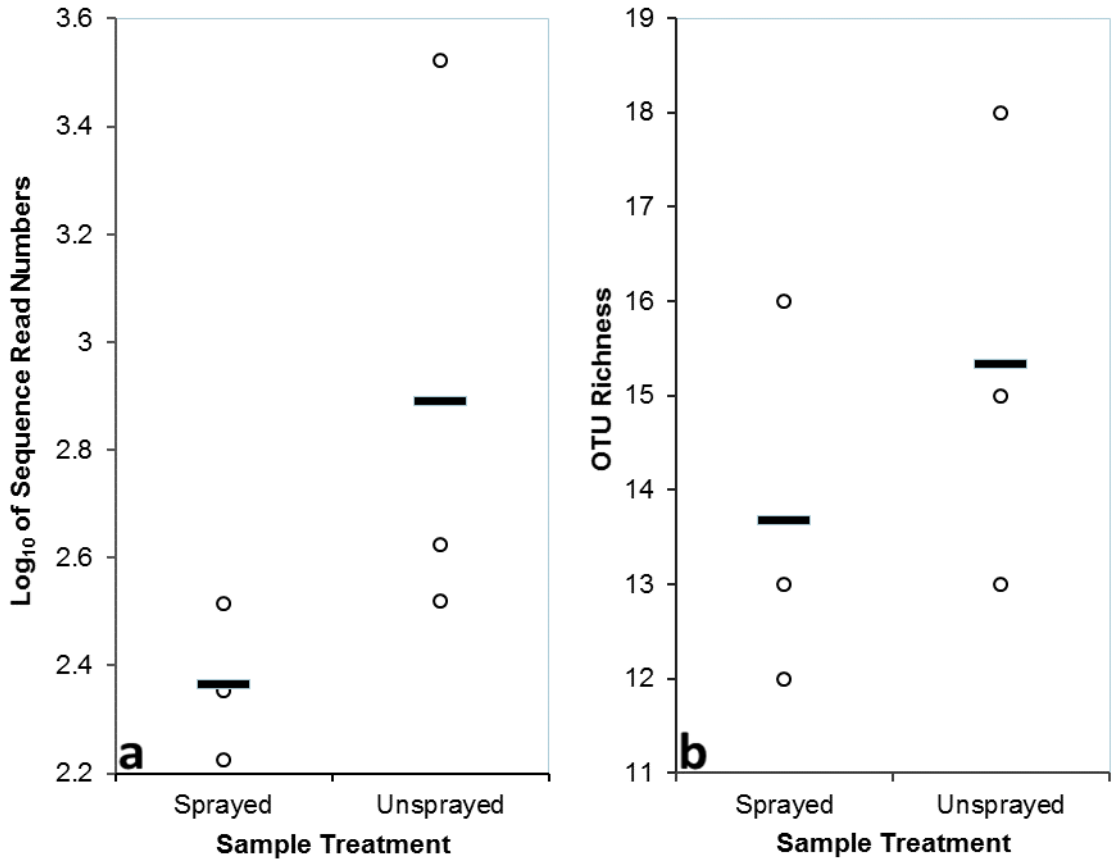

Figure 5.3 (a) A scatter plot of sample log_{10} transformed fungal OTU sequence read numbers per treatment, with a bar showing the mean value. (b) A scatter plot of sample OTUs per treatment, with a bar showing the mean values, for samples that were unsprayed or sprayed with fungicide.

The PCA triplot (Figure 5.4) demonstrated a spraying effect on OTU composition. Most OTU read numbers were positively correlated with the unsprayed samples, displaying strong correlations between increases in OTU2, OTU4 and OTU9 and no fungicidal application, and a weak correlation between either treatment and OTU7 and OTU8. Therefore, spraying reduced the abundance of OTU2, OTU9, OTU4, and to a lesser extent, OTU5, whereas OTU6, OTU3, OTU7 and OTU8 appeared to be relatively unaffected.

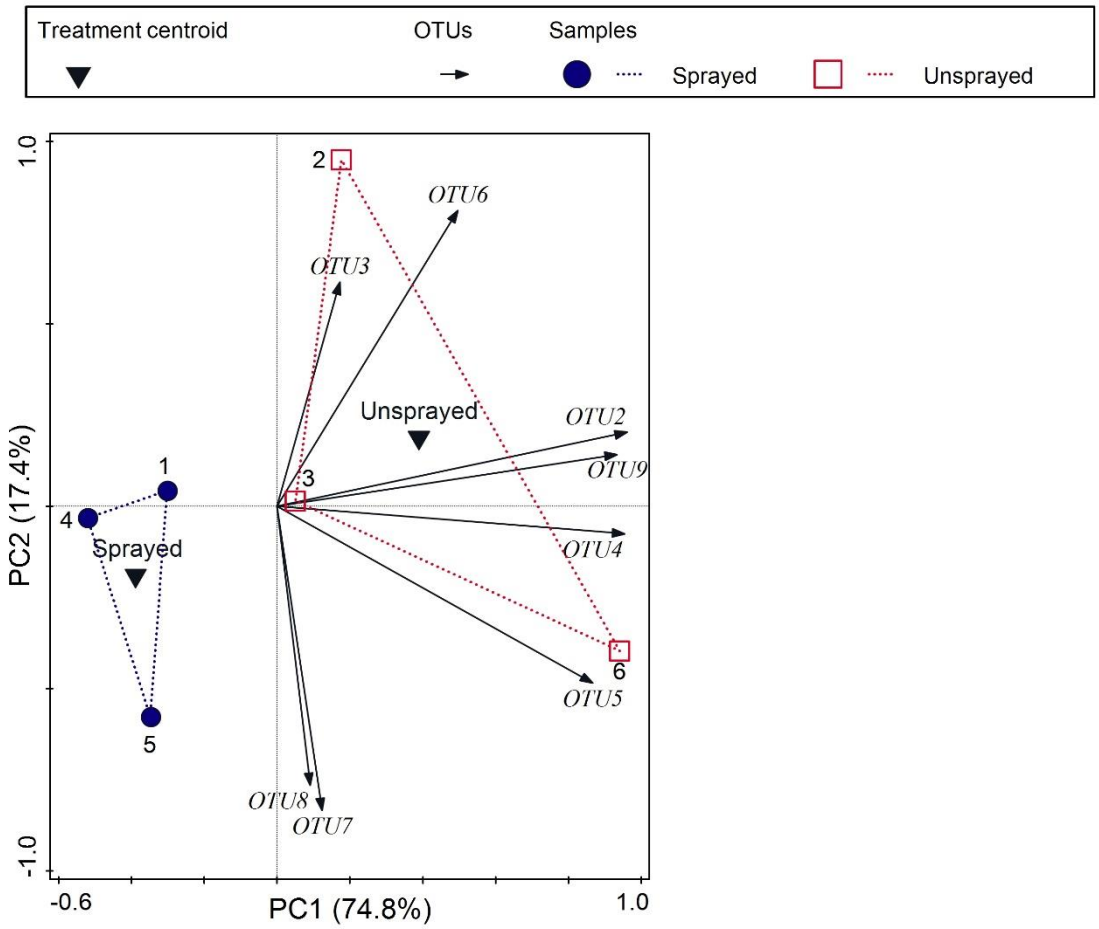

Figure 5.4 PCA triplot showing variation in the abundance of OTUs (arrows), samples (points) and treatment centroids (triangles) for $\log_{10}(1+x)$ transformed standardized OTU read numbers.

PERMANOVA analysis results (Table 5.4) suggested that treatment with the fungicide tended to alter community composition (Pseudo-F = 3.7765, p(MC) = 0.057).

Table 5.4 Results of PERMANOVA analysis for $\log_{10}(1+x)$ transformed standardized OTU read numbers for OTUs represented by more than 1% of non-wheat sequences, revealing a discernible effect on sample community composition caused by fungicide application.

Source	**df**	**SS**	**MS**	**Pseudo-F**	**p(perm)**	**Unique perms**	**p(MC)**
SP	1	57.586	57.586	4.3141	0.109	10	0.057
Res	4	53.393	13.348				
Total	5	110.98					

The PERMDISP pairwise comparison results indicated that sprayed treatment tended to be more homogenous in OTU composition than non-sprayed samples (Table 5.5), but because of the small sample size, dispersions were not significantly different (p(perm) = 0.115).

Table 5.5 Means and standard errors for dispersions of $\log_{10}(1+x)$ transformed standardized OTU read numbers for OTUs represented by more than 1% of non-wheat sequences, showing greater mean dispersion for unsprayed samples, which indicates that they had a greater composition heterogeneity.

Group	Size	Mean	SE
Sprayed	3	1.79	0.94
Unsprayed	3	3.73	0.57

The SIMPER analysis indicated that OTU2 (*Puccinia*) contributed most of the variability between treatments (97.7%), followed by OTU4 (*Cladosporium*) (1.4%), while the remainder of the most abundant OTUs (OTU3 to OTU9) collectively contributed less than 1% to variation in Euclidean distance between treatments.

5.4 Discussion

The combined depth of sequencing by NGS and the broad taxonomic range of universal PCR primers was used to establish what the dominant eukaryotic endophytes were within the flag leaves of wheat, and to observe how they were affected by a systemic fungicide. A greater number of sequences were produced than by traditional clone library sequencing approaches, although a substantial portion of the data from the reverse reads was not suitable for further analysis.

The poor quality of the reverse reads on the Illumina MiSeq platform has been reported as a problem (Quail *et al.*, 2012, Bolger *et al.*, 2014). The exclusion of the reverse read data from the analysis was further justified because of the great difference in magnitude between the final quality filtered forward and reverse read data, and the sequence composition variation that would result from this. However, the use of the forward reads was justified because the

difference between the raw data and Q30 filtered data for forward reads was low and the FastQC analysis indicated that the data quality was good.

The high incidence of wheat sequence reads was expected because the ITS5 and ITS4 primers developed by White *et al.* (1990) had few internal mismatches with the wheat priming site, and no terminal 3'-end mismatches. In PCR, mismatches at the 3'-end of the primer inhibit (but do not prevent) the amplification of non-target sequences, while internal mismatches can have a negligible effect on amplification (Stadhouders *et al.*, 2010). To illustrate the problem of low primer specificity, in an NGS study of soil fungi using fungal-specific primers it was found that almost half of the sequences amplified from soil were of non-fungal origin, almost half of those were of plant origin, and from 16 soil samples, almost 80% of the total data was discarded after data filtering (Schmidt *et al.*, 2013). This also demonstrated the extent to which NGS can harness the depth of sequencing to analyze microbial community composition, even when the great majority of sequences are not from target organisms.

The impartial amplification of both wheat and insect ITS1 regions demonstrated the capacity to amplify a broad taxonomic range due to the lack of specificity of the primers used in this study. However, all the detected microbial eukaryote sequences belonged to the Dikarya (Tables 5.2 and 5.3), which partially validates the development of Dikarya specific primers by, for example, Toju *et al*. (2012). Despite the relatively low number of non-wheat sequences detected, the number of sequence reads produced (Table 5.2) in this work would have required far greater effort using a traditional clone library approach, where PCR amplicons are ligated to plasmids, *E.coli* transformed, and individual colonies selected at random to represent individual sequences. Using this approach to produce as many sequences as shown in Table 5.2 would be prohibitive in terms of time and cost.

The ML tree (Figure 5.1) was developed to provide the most likely taxonomic assignments for the most abundant OTUs. The major clades formed by the ML tree (Figure 5.1) separated the OTUs according to phyla. OTU2 and OTU8 were both Basidiomycetes and formed a distinct branch from all the other OTUs, which were Ascomycetes. The more detailed

taxonomy of the proposed genera (Table 5.3) was well supported by the clades formed in the ML tree. OTU5 and OTU7 are correctly grouped together in the order *Heliotales*; OTU6 and OTU9, which are grouped closely with one another (Figure 5.1), are also members of the family *Pleosporaceae* (Table 5.3). This provided confidence in the model used to produce the tree, as well as the taxonomic assignment to the genera proposed in Table 5.3.

The identities inferred from the ML tree (Figure 5.1) were considered in terms of the taxonomic and biological likelihood of the taxonomic assignment. OTU2 was identified as a member of the genus *Puccinia* Pers., a group of rust-causing fungal diseases of wheat (not an endophyte *sensu stricto*), identified as either *P. triticina* Erikss., or *P. persistens var. triticina* (Erikss.) Z. Urb. and J. Marková. However, according to the MycoBank database (www.mycobank.org) these two are synonymous. *P. triticina* is one of the most widespread wheat leaf rust species (Huerta-Espino *et al.*, 2011) and it was recognized in KwaZulu-Natal, South Africa after 2007 (Terefe *et al.*, 2009), which validates this taxonomic assignment of OTU2 to *P. triticina*.

OTU3 was found to be a member of the genus *Aspergillus* P. Micheli ex Haller. *A. flavus* is a mycotoxin-producing opportunistic plant pathogen and *A. oryzae* is a domesticated fungus used in food production. These are closely related species within the same sub-genus (Machida *et al.*, 2005). This explains the 100% match of the OTU3 sequence to both type specimens on Genbank. *Aspergillus* spp. are not typical wheat pathogens and Larran *et al.* (2002) isolated an endophytic Aspergillus sp. from wheat leaves. While *A. oryzae* has been reported as an endophyte of coffee (Chaves *et al.*, 2012), assignment to species level was uncertain.

The two *Cladosporium* type-specimens matched with OTU4 suggested that this OTU is a member of the genus *Cladosporium*; however, the Mycobank database indicated that these were distinct organisms. Members of this genus have been reported as endophytes, most notably a Taxol-producing species (Zhang *et al.*, 2009). *Cladosporium* spp. have been identified as wheat phyllosphere inhabitants (Karlsson *et al.*, 2014). Along with *Alternaria* spp., *Cladosporium* spp. have been reported as the dominant fungal species within the

phyllosphere of cereal crops, including wheat (Sapkota *et al.*, 2015). A member of this genus, *C. herbarum* (Pers.) Link has been described as a pathogen of wheat. While a BLAST search of the Genbank database limited to this wheat pathogen yielded a 99% match with 100% coverage, considering the erroneous multi-species matches in non-curated databases (Nilsson *et al*., 2006), the species identity was inconclusive.

OTU5 matched *Articulospora proliferata*, which has been reported as a grass endophyte (Martin and Dombrowski, 2015) and it has been described as an aquatic hyphomycete in South Africa and Spain (Jooste *et al.*, 1990). It has probably not been reported as an endophyte more frequently because it belongs to a group of fungi that are difficult to isolate and culture (Sugahara *et al.*, 2008).

Epicoccum nigrum Link is the likely identity of OTU6 as it has frequently been associated with wheat, often as an endophyte (Sieber *et al.*, 1988, Crous *et al.*, 1995, Larran *et al.*, 2007, Blixt *et al.*, 2010, Vujanovic *et al.*, 2012, Karlsson *et al.*, 2014, Nicolaisen *et al.*, 2014).

A literature search did not yield any reports of *Sclerotinia* spp.as either endophytes or pathogens of wheat. The same was true for the closely related *Botrytis cinerea* Pers.; therefore, this is a first report of *Sclerotinium*/*Botrytis* as a wheat endophyte or pathogen, although species identity was uncertain.

OTU8 that was identified as a *Pleurotus*/*Lentinus* sp. A *Pleurotus* sp. has been identified as a root endophyte of barley (Lopez-Llorca *et al.*, 2006) and another species was isolated from grape leaves (Brum *et al.*, 2012). The detection of this fungus as a dominant endophyte of wheat was unexpected and worthy of further investigation; however, it was beyond the scope of this study.

The genus *Alternaria* Nees has been associated with wheat. *A. alternata* (Fr.) Keissl. has been isolated from wheat leaves with high frequency (Crous *et al.*, 1995, Larran *et al.*, 2002); and the pathogen *A. triticina* Prasada and Prabhu causes a foliar blight in wheat (Chaurasia *et al.*, 2000). The 100% ITS1 matches to multiple *Alternaria* species (Figure 5.1) could infer that the

species proposed as identities from the ML tree are closely related. However, the previous description of *A. alternata* as a frequently isolated wheat leaf endophyte in Argentina and South Africa, as well as the sample leaves having been asymptomatic, suggests that OTU9 represented *A. alternata*.

The organisms associated with the eight OTUs, OTU2 to OTU9, were considered the key species because they were abundant enough to be ecologically meaningful in the endophyte community of the sampled wheat flag leaves. Another NGS study of the wheat phyllosphere mycobiome also found fewer than ten dominant fungal species in the endophytic and epiphytic community combined (Sapkota *et al.*, 2015). As a percentage of total reads, the most abundant genus detected was *Puccinia* (Table 5.3). This was influenced by the high read numbers for OTU2 in the unsprayed samples (see Figure 5.2). The remainder of the key OTUs were at substantially lower levels, indicative of a quantitative difference between pathogens and endophytes.

From the results of the paired t-tests comparing OTUs and the stack plot (Figure 5.2), OTU2 (*Puccinia*) and OTU9 (*Alternaria*) were the only OTUs that had a significant difference in presence. The stack plot also showed that all OTUs were represented in both treatments and in most samples. Therefore, the fungicide treatment did not affect the species assemblages and that the most observable effect was in suppressing rust, which is a disease that tebuconazole is intended to control (Milus, 1994), and *Alteraria*, which is a source of mycotoxins in wheat (Patriarca *et al*., 2007).

It has been proposed that the lack of difference in endophyte species composition could be due to the presence of DNA from non-living fungi that had been killed by the fungicide (Karlsson *et al*., 2014), which requires further investigation; however, the substantial differences in *Puccini* sp. reads between sprayed and unsprayed leaves challenged this explanation, because this substantial difference was not observed in the other endophytes.

Comparisons between treatments indicated that unsprayed samples had higher standardized read numbers (Figure 5.3 a) and more OTUs (Figure 5.3 b). Previous NGS

studies have also found that mean OTU richness of the phyllosphere was greater for unsprayed leaves (Sapkota *et al.*, 2015; Karlsson *et al.*, 2014). The lower standardized read numbers for non-wheat OTUs (Figure 5.3 b) demonstrated the suppression of fungal growth within the flag leaves by the systemic fungicide. The reduced OTU abundance due to fungicidal activity has also been observed in other studies (Sapkota *et al.*,2015; Karlsson *et al.*,2014).

The PCA triplot (Figure 5.4) summarized the data, showing that there was a clear fungicidal effect on the endophytic community composition. The unsprayed samples had not been treated with a systemic fungicide; therefore, the observation that there were positive correlations between these and the variation in most of the key OTUs (Figure 5.4) was expected. The weak effect on variation in OTU7 and OTU8 was because the organisms they represent were less sensitive to the fungicide (Karlsson *et al.*, 2014). The PERMANOVA analysis supported what is apparent in the stack plot of genera (Figure 5.2), where community composition was altered in terms of relative abundance of the key OTUs; and the PERMDISP analysis showed that, as is visible from the dispersion of samples in the PCA triplot and Table 5.5, OTU variation was greatest in unsprayed samples, because the *Puccinia* flourished in the absence of the fungicide, with only the plant defences to suppress it. This is evident from the SIMPER analysis, which showed that 97% of the variation between treatments was due to OTU2. OTU4 (*Cladosporium* sp.), contributed the next largest amount of variation (1.4%), which was negligible in comparison, as were the rest of the OTUs. From this, it is apparent that the fungicide had the greatest effect on the pathogen it was targeted at, although it did not have a significantly detrimental effect on the other endophytes. This supports the potential use of fungal endophytes as biological control agents, even in commercial wheat cultivation where tebuconazole is applied. It also supports the suggestion that the available environmental candidate endophytes have a predominant influence on fungal endophyte community composition (Burgdorf *et al*, 2016).

Our studies regarding the effects of the fungicide on the fungi associated with wheat leaves were supported by the conclusions of Karlsson *et al.* (2014). However, the bulk of the dominant non-pathogenic organisms they detected were categorized as yeasts. None of the

most abundant organisms in this study were yeasts (Figure 5.2) because the surface sterilization removed epiphytes and their DNA (Burgdorf *et al.*, 2014). This was an example of the greater abundance of epiphytes compared with endophytes (Lindow and Brandl, 2003).

This study demonstrated that the universal eukaryotic primers confirmed Dikarya to be the dominant endophytes residing within the flag leaves of wheat, regardless of whether they were sprayed with a systematic fungicide or not. It also showed that tebuconazole slightly reduced the number and abundance of fungal species, as well as the community composition, but that the types of organisms that were present did not alter significantly. NGS analysis can be used to determine the effects of fungicides on endophytic community composition, particularly during co-infection by pathogens, such as *Puccinia*. This may help with developing new integrated pest management strategies based on the effects of the fungicides.

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Chapter 6 Enhanced PCR detection of *Puccinia* and fungal endophytes of wheat using non-extendable blocking primers

6.1 Abstract

PCR-based studies of plant pathogen and endophyte community composition are constrained by primer problems such as variable amplification efficiency and non-target sequence amplification. This study developed non-extendable blocking primers (NEBPs) for use with universal eukaryotic ITS-PCR primers, to enhance target *Puccinia* Pers. and endophyte sequence amplification while suppressing host wheat DNA amplification. These NEBPs were 100% complementary to the priming site and flanking regions for wheat. ITS-PCR products under increasing concentrations of NEBPs were compared on agarose gels. Diluted ITS-PCR products were used as template in qPCR assays of wheat host and *Puccinia* amplicon production under increasing NEBP concentrations. Gel analysis showed the suppression of wheat DNA amplification, while non-host target sequence amplification was enhanced as NEBP concentrations increased. The qPCR assay of wheat amplicons from ITS-PCR products showed a linear decrease in wheat amplicons as NEBP concentration increased. *Puccinia*-specific qPCR of ITS-PCR products showed a non-linear association between *Puccinia* sequence quantities and increasing NEBP concentrations. *Puccinia* sequence amplification increased up to a specific NEBP concentration after which amplification was suppressed. It was shown that universal primers used with optimal NEBPs concentrations successfully suppressed host wheat DNA amplification with enhanced *Puccinia* and eukaryotic endophyte DNA amplification.

6.2 Introduction

PCR, followed by next generation sequencing (NGS), can reveal the species composition of fungi that infect plants, either as pathogens or endophytes (Knief, 2014). However, bias introduced during PCR amplicon formation can have a great impact on subsequent NGS data (Shokralla *et al.*, 2012). Bellemain *et al*. (2010) showed that PCR primers are a significant source of such bias in the amplification of fungal sequences. While considering this problem,

Ihrmark *et al*. (2012) explained that it is difficult to develop primers that are both wide-ranging, i.e., 'universal', as well as specific to a desired group of organisms. In other words, it may not be possible to design primers that specifically amplify fungi but exclude all other non-fungal organisms. Further to this, Toju *et al*. (2012) found that certain primers that amplify the fungal ITS region also amplify non-target sequences in mixed-template metagenomic DNA (mDNA). Clearly this is a significant problem in PCR-based detection of fungal pathogens and endophytes where the overwhelming majority of the DNA available belongs to the host plant.

Vestheim and Jarman (2008) offered a solution to this problem. They proposed that universal primers that target conserved regions could be used in mixed template PCR, provided that they are prevented from annealing to matching sequences of non-target host DNA. This is achieved by including a non-extendable blocking primer (NEBP) at the annealing site for each of the extending primers. These oligonucleotide sequences are perfectly complementary to the region spanning the primer annealing site for non-target template DNA but have been modified to prevent extension by the polymerase enzyme. These NEBPs are typically longer than the amplifying primers and consequently, have higher annealing temperatures. The NEBPs bind to the priming region before the amplifying primers can anneal and, as a result, they prevent the amplification of that sequence (Vestheim and Jarman, 2008).

This technique was originally developed by Vestheim and Jarman (2008) for a PCR-based study of the stomach contents of krill. Subsequently, Boessenkool *et al*. (2012) employed the same technique to block human contaminant DNA in a study of rare ancient mammal DNA. Leray *et al*. (2013) also showed that such NEBPs were more effective than predator DNA digestion in PCR-based DNA identification of prey species from the gut contents and faeces of coral fish. Further utilizing the enhanced detection capability of this approach, Wang *et al*. (2013) designed NEBPs to detect rare cancer mutations in tumours. Applied to plants, NEBPs were utilized to study bacterial endophyte communities in a species of North American prairie grass, resulting in a 300-fold increase in bacterial sequence detection with no plant chloroplast sequences detected (Arenz *et al.*, 2015).

In this work, we designed NEBPs complementary to the sequences flanking the wheat ITS region. We aimed to establish whether the use of these NEBPs in combination with universal eukaryotic primers would inhibit the amplification of wheat sequences and enhance the detection of *Puccinia* Pers. species and other fungi in wheat mDNA. The use of NEBPs in combination with universal primers in PCR would obviate the need for fungi-specific primers in studies that explore fungal presence and diversity within host plants, and at the same time, reduce primer-related taxonomic bias in future NGS investigations on the dynamics of fungal community composition during infection by pathogens such as *Puccinia* spp.

6.3 Materials and Methods

6.3.1 Primer design

Wheat and fungal ITS sequences obtained from a previous NGS study of wheat mDNA samples (Burgdorf *et al*., 2016) were used to find identical matches in the Genbank database. The region from the 3' end of the small subunit (SSU) ribosomal DNA to the 5' end of the large subunit (LSU) ribosomal DNA sequence, up to, and in the case of wheat, including the ITS5 and ITS4 (White *et al.*, 1990) priming sites, was downloaded for each of the wheat and fungal sequences. These were aligned in BioEdit v7.2.0 (Hall, 1999). All blocking and amplifying primers were developed using PerlPrimer v1.1.21 (Marshall, 2004) in the Bio-Linux 8 operating system. All primers were synthesized by Inqaba Biotec (South Africa) and each of the NEBPs had a 7-carbon amino acid added to the 3' end, which prevented extension by the DNA polymerase but did not prevent annealing to the corresponding DNA target sites.

The design of the NEBPs followed the guidelines proposed by Vestheim *et al*. (2011). The NEBPs were designed to have a 100% match to wheat sequences at the 3' ends of the ITS5 (forward) and ITS4 (reverse) priming regions, respectively. The NEBPs were designed to anneal specifically to the highly variable sections of the wheat ITS region within the forward and reverse primers, to prevent annealing to other eukaryotic sequences. At the same time, they were designed to overlap slightly onto the universal primer annealing site to prevent

the 3’ end of the amplifying primer from binding to the site, thereby preventing the DNA polymerase from extending.

These NEBP target sites are shown in Figure 6.1 and Figure 6.2, which illustrate where the blocking and extending primers anneal relative to one another. The forward NEBP (TaITS5blk, Figure 6.1) was designed with a 3 bp overlap with the 3’ end of the ITS5 primer and included a relatively conserved region which corresponds to the annealing site of the ITS1 primer (White *et al.*, 1990), although that primer was not used in this study. The reverse NEBP (TaITS4blk, Figure 6.2) was designed with a 4 bp overlap with the 3’ ITS4 primer and had several fungal sequence mismatches distributed relatively evenly across the NEBP.

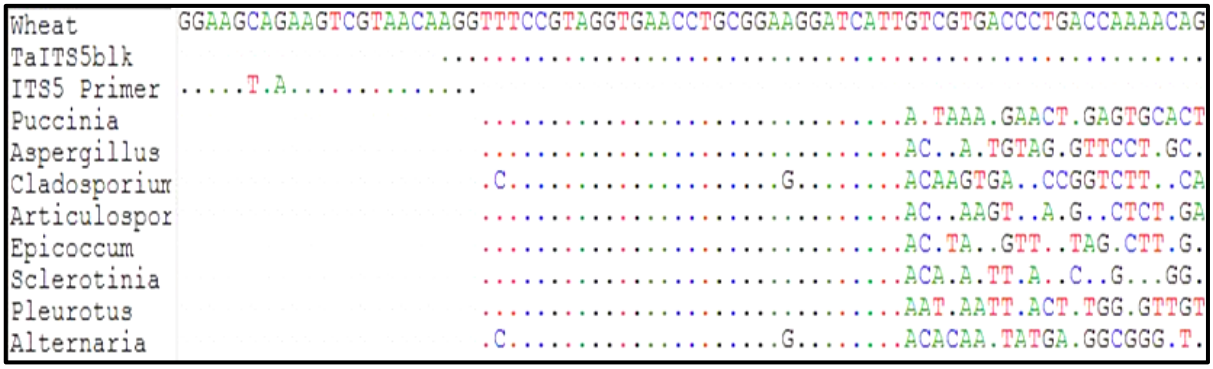

Figure 6.1 Position and coverage of the forward primer and NEBP, TaITS5blk, in relation to the wheat target area and the priming sites of sequences from previously identified fungi associated with wheat. Dots represent bases that are identical to the corresponding bases in the wheat sequence.

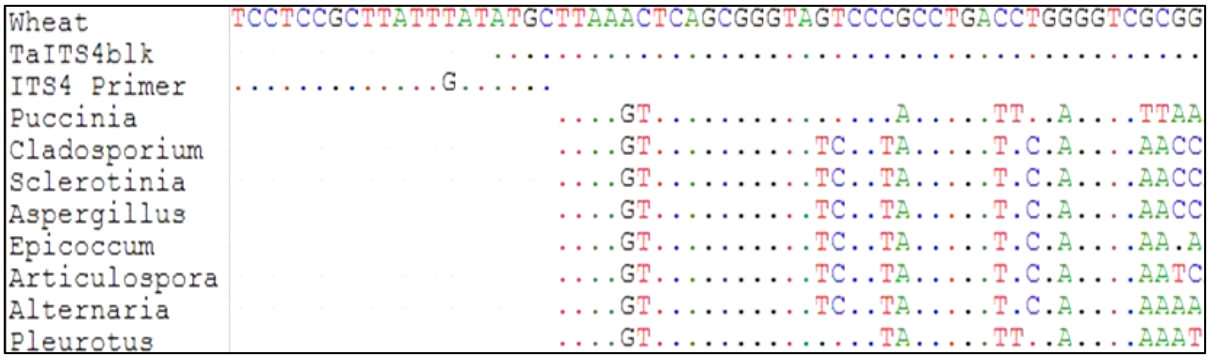

Figure 6.2 Position and coverage of reverse primer and NEBP, TAITS4blk, in relation to the wheat target area and the priming sites of sequences from previously identified fungi associated with wheat. Dots represent bases that are identical to the corresponding base in the wheat sequence.

Two additional primers pairs were developed for qPCR. The first primer pair was designed to anneal internally to the NEBPs and specifically amplify wheat ITS sequences, therefore being able to amplify any wheat sequences that were produced by the ITS5 and ITS4 primers during the ITS-PCR. The second primer pair specifically amplified a *Puccinia* sequence that had previously been detected in the wheat mDNA samples used as template in the PCR reaction with the universal eukaryotic primers and the NEBPs. A Primer-BLAST search was performed on the NCBI website to confirm the specificity of the wheat and *Puccinia* primers and gel analysis of PCR products was used to confirm that they produced amplicons of the expected size.

6.3.2 qPCR and agarose gel electrophoresis

All PCR reactions were performed on a Rotor-Gene 6000 (Qiagen, Hilden, Germany) using the Kapa SYBR® Fast qPCR Universal kit (Kapa Biosystems, Wilmington, Massachusetts, USA). The final PCR reaction volume for all samples was 25 µL.

To investigate the effect of increasing NEBP concentrations on total ITS region amplification from wheat mDNA (ITS-PCR), the universal eukaryotic ITS5 and ITS4 primers (Table 6.1) were used at a final reaction concentration of 400 nM for each reaction. Duplicate samples were prepared for increasing NEBP concentrations, with 0 nM, 100 nm, 200 nM, 400 nM, 600 nM and 800 nM concentrations of each of the two NEBPs, TaITS5blk and TaITS4blk (Table 6.1). Template DNA consisted of 30 ng per reaction of surface-sterilized wheat leaf mDNA that had been used in a previous study of fungal wheat endophytes. Included in the run were positive controls consisting of genomic *Beauveria bassiana* (Bals.-Criv.) Vuill. DNA at 2.2 ng and 0.022 ng per reaction respectively. There was also a 'no template' control (NTC) included in the run, which consisted of a reaction mixture with the ITS5 and ITS4 primers, but no template DNA or NEBPs. The NEBPs had been previously tested with the ITS5 and ITS4 primers to ensure they did not form any spurious PCR products.

The ITS-PCR parameters comprised of an initial denaturation step of 3 min at 95°C, followed by 40 cycles of 10 s at 95°C, 20 s at 54°C and 10 s at 72°C. Threshold cycle (C_T) values, represented by the cycle number at which a significant change in fluorescence across all

qPCR samples occurs, indicative of the change in target template DNA concentration (where low C_T values are associated with higher template concentration and high C_T values with lower ones) (Schmittgen and Livak, 2008), were used to analyse the effects of increasing NEBP concentrations (Boessenkool *et al.*, 2012). The C_T values were determined using the Rotor-Gene Series 6000 Software (Qiagen).

Products were run on a 2% agarose gel containing 1 x SYBR® Safe (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in 1 x TBE at 6 $V.cm^{-1}$ for 1 h. The gel was viewed and captured to detect bands using the GeneSnap software (Syngene, Cambridge, UK) on an I-chemi G-box gel documentation system (Syngene). The gel was analysed using the GeneTools software (Syngene) to determine band molecular weights and DNA concentrations relative to those of a 100 bp GeneRuler™ Plus molecular weight marker (Thermo Fisher Scientific).

For the evaluation of wheat sequence amplification at increasing NEBP concentration, a 10^{-5} dilution was made from the PCR products of the ITS-PCR with increasing NEBP concentrations; 1 µL volumes from these solutions were used as the templates for the qPCR determination of relative wheat amplicon quantity using the wheat-specific primer pair, TaSPITS1F and TaSPITS1R (Table 6.1). A two-step qPCR was performed with an initial denaturation step of 1 min at 95°C, followed by 40 cycles at 95°C for 5 s and 60°C for 15 s. The C_T values were determined in the Rotor-Gene Series 6000 Software. The sample products were run on a 1.5% agarose gel containing 1 x SYBR® Safe in 1 x TBE at 6 $V.cm^{-1}$ for 1 h, with a 100 bp GeneRuler™ Plus molecular weight marker to confirm the formation of a single band of the appropriate size.

For the evaluation of *Puccinia* sequence amplification at increasing NEBP concentration, a 10^{-4} dilution was made from the PCR products of the ITS-PCR with increasing NEBP concentrations; 1 µL volumes from these solutions were used as the template for the qPCR determination of relative *Puccinia* amplicon quantity using the *Puccini*-specific primer pair, PuSPITS1F and PuSPITS1R (Table 1). A two-step qPCR was performed with an initial denaturation step of 1 min at 95°C, followed by 40 cycles at 95°C for 5 s and 57°C for 15 s.

The C_T values were determined in the Rotor-Gene Series 6000 Software. The sample products were run on a 1.5% agarose gel with 1 x TBE containing 1 x SYBR® Safe at 6 V cm^{-1} for 1 h, with a 100 bp GeneRuler™ Plus molecular weight marker, to confirm the formation of a single band of the appropriate size.

Table 6.1 A list of the primers used in this study. The T_m of each primer was calculated using the nearest neighbour method on http://simgene.com/OligoCalc . The number '7' in the NEBP sequences TaITS5blk and TaITS4blk represents the C7 amino acid on the 3' end which serves to prevent extension from the primer.

Name	Sequence (5'-3')	Tm (˚C)	Reference
ITS5 (F)	GGAAGTAAAAGTCGTAACAAGG	58	White *et al*. (1990)
ITS4(R)	TCCTCCGCTTATTGATATGC	56	White *et al*. (1990)
TaITS5blk (F)	AGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTC GTGACCCTGACCAAAACAG**7**	74	This study
TaITS4blk (R)	ATGCTTAAACTCAGCGGGTAGTCCCGCCTGACCTGGGG TCGCGGT**7**	75	This study
TaSpITS1F (F)	ACGCGTCATCCAATCCGT	53	This study
TaSpITS1R (R)	CGGGTTAGGCACAGTGTTC	52	This study
PuSpITS1F (F)	CCTGCGGAAGGATCATTA	49	This study
PuSpITS1R (R)	TGCCACGTATACTTAATCAC	47	This study

6.3.3 Statistical analysis

All qPCR data was exported to and analysed using Microsoft Office Excel 2013 (Microsoft, Redmond, Washington, USA). Mean fluorescence values versus cycle number were used to

present all duplicate qPCR samples. Linear and curvilinear (quadratic) regression models were fitted to the response of C_T values to increasing NEBP amounts. Where applicable, values were calculated from quadratic formulae to establish maximum or minimum concentrations of the NEBPs at which changes in amplicon production characteristics took place.

6.4 Results

6.4.1 Primer-BLAST

A Genbank BLAST search of the designed NEBPs indicated that they were also 100% identical to the same region of the ribosomal/ITS DNA region for a wide variety of plant species, particularly grasses. The forward NEBP had only a single complete match to a purported uncultured fungal sequence. The reverse NEBP completely matched only four purported fungal sequences.

6.4.2 ITS-PCR gel analysis and qPCR

The agarose gel analysis (Figure 6.3) showed the increase in concentration of the NEBPs (TaITS5blk and TaITS4blk) in the ITS-PCR reaction with the ITS5 and ITS4 primer pair used to amplify all eukaryotic ITS target sequences resulted in a decrease in the ~700 bp-sized wheat ITS DNA band intensity and an increase in non-wheat ITS DNA band numbers, although the intensity and presence of these bands decreased in most cases after 400 nM. The bright 700-730 bp band was assumed consist of wheat ITS amplicons, since the amplification of this wheat mDNA with the ITS5 and ITS4 primers, in a previous study (Burgdorf *et al*., 2016), had revealed that more than 99% of the sequences belonged to the wheat, which, according to the Genbank sequences used for the NEBP design (a consensus sequence produced from the *T. aestivum* L. sequences AY049040.1, FJ609737.1, and KJ131565.1), was 714 bp long. This is the closest size to the band assigned to wheat. The band F3 was assumed to represent *Puccinia*, which was the most numerous fungal sequence present in the same previous study, and a Genbank Primer-BLAST showed that *Puccinia* fragments amplified using ITS5

and ITS4 were approximately 680-700 bp long, which corresponds to the position of the band F3.

In total six non-wheat bands were seen (F1-F6, Figure 6.3), with the highest number of non-wheat bands observed at 400 nM concentration of NEBP (F1, F2, F3, F4, and F6). Beyond 400 nM a band (F5) formed that was only present at 600 nM and 800 nM NEBP concentrations.

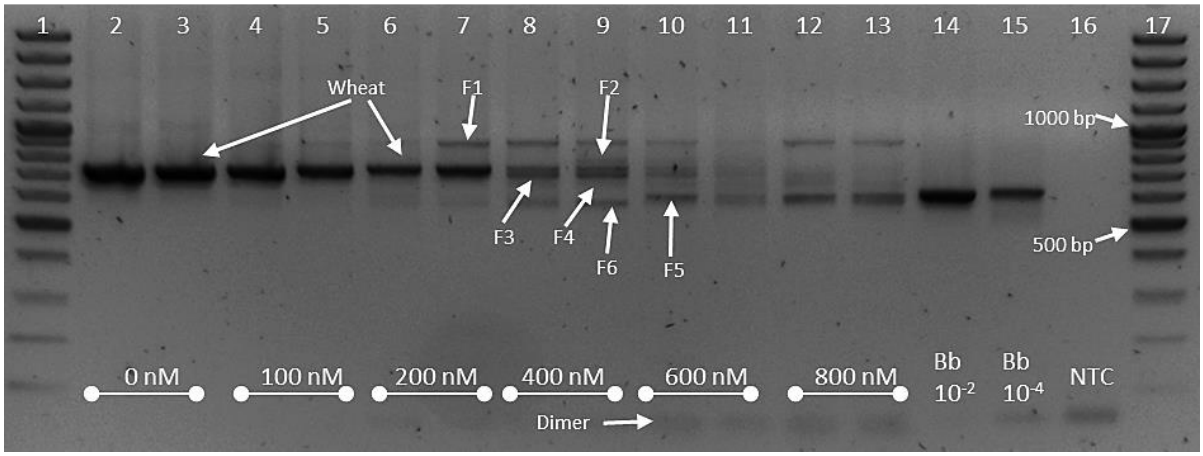

Figure 6.3 Agarose gel of ITS-PCR products from qPCR amplification of ITS from wheat mDNA with increasing concentrations of NEBPs (lanes 2 to 13); lanes 1 and 17 contain 100 bp molecular weight ladder markers, while lanes 14 and 15 contain PCR product from two different dilutions of genomic *B. bassiana* DNA without any NEBPs and lane 16 consists of a no template control with only the ITS5 and ITS4 primers.

The graph in Figure 6.4 was derived from the analysis of the gel shown in Figure 6.3. The graph showed that the DNA quantity of the band representing wheat ITS DNA decreased, initially quite rapidly, as the NEBP concentrations were increased. It was also shown that the sum of the DNA quantity for non-wheat bands (F1 - F6, Figure 6.3), excluding primer dimer DNA, increased as NEBP concentration increased. The relative change in DNA quantity in both instances appeared to diminish at NEBP concentrations beyond 400 nM.

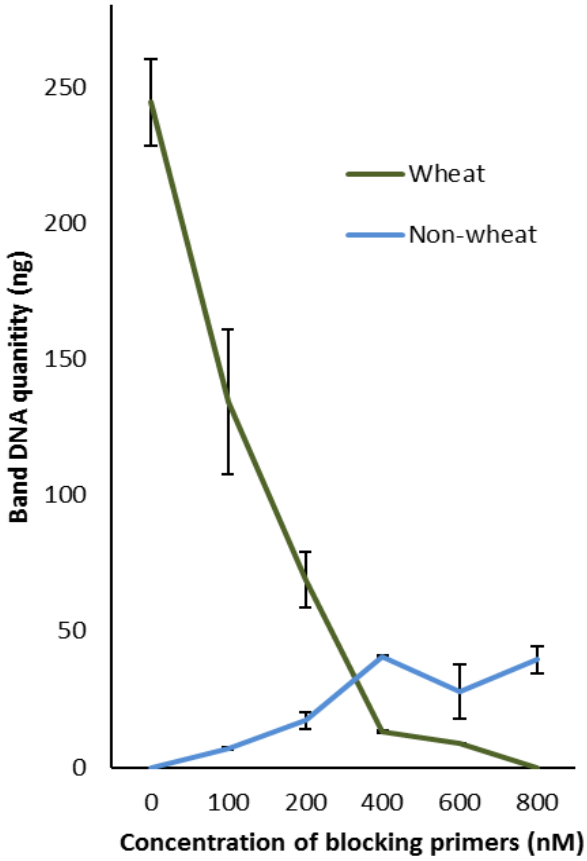

Figure 6.4 Response of mean DNA quantities, for bands shown in Figure 6.3, to increasing concentrations of NEBPs. The lines represent the mean amplified wheat ITS DNA band quantities and the summed DNA band quantities of the remaining bands (excluding primer dimers) separately. Error bars show the standard error for the duplicate samples.

The qPCR fluorescence curves in Figure 6.5 showed that the increase in NEBP concentration in the ITS-PCR of wheat mDNA delayed the exponential increase of fluorescence, indicating a suppression of PCR amplicon formation. This was demonstrated by the steepest portions (exponential DNA amplification) of the mean curves of fluorescence versus cycle number occurring at increasingly high cycle numbers, as NEBP concentrations increased.

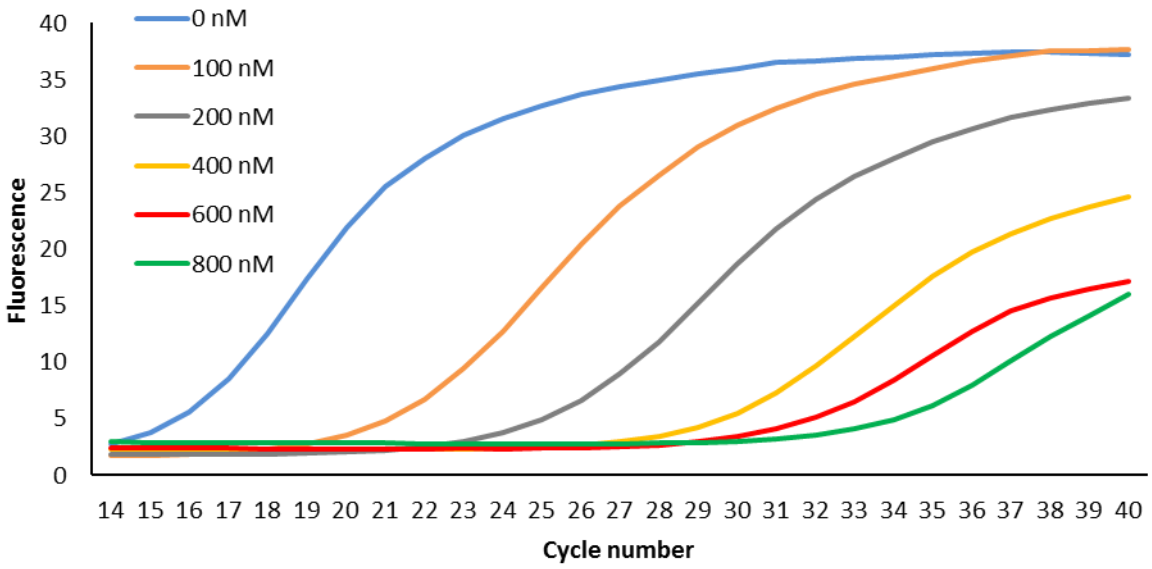

Figure 6.5 Plot of real-time PCR progression, showing mean sample fluorescence per cycle, with ITS5 and ITS4 primers and increasing concentrations of NEBPs. The legend indicates the concentration of both of the NEBPs in each pair of sample replicates.

A plot of C_T values against the increasing NEBP concentration (Figure 6.6) showed that the C_T values increased as NEBP concentrations increased, but at a decreasing rate. Extrapolation from the quadratic equation of the fitted line showed that the C_T reached a maximum of 33.24 at the NEBP concentration of 780 nM, after which the C_T started decreasing.

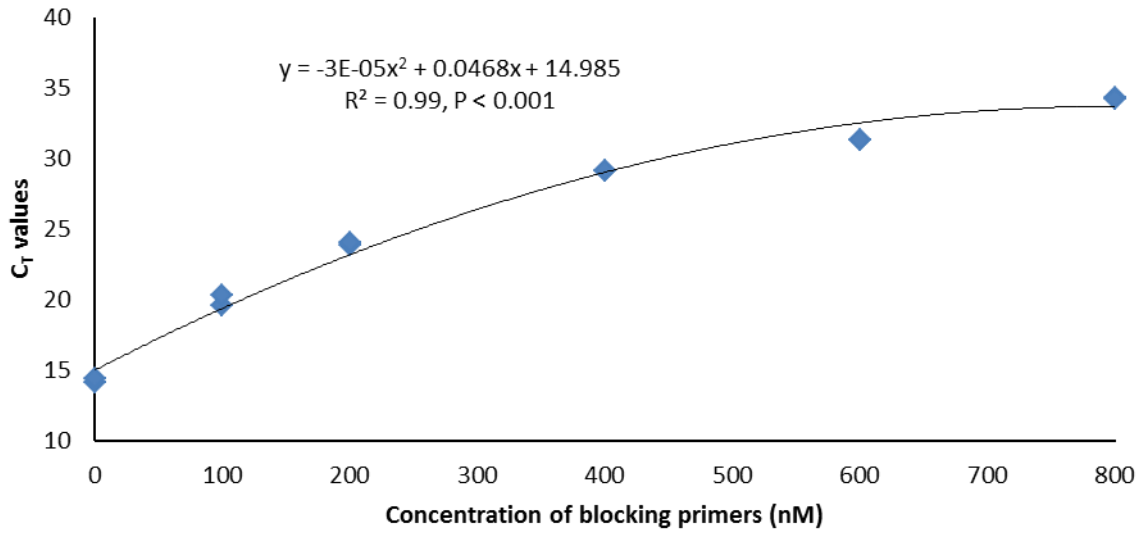

Figure 6.6 Quadratic response of C_T values to NEBP concentration for ITS-PCR with markers showing the duplicate C_T values for each NEBP concentration.

6.4.3 Wheat and *Puccinia* qPCR

In Figure 6.7 the wheat-specific qPCR amplifying wheat ITS DNA from 10^{-5} dilutions of PCR products from the ITS-PCR showed that the use of increasing concentrations of NEBPs had inhibited the amplification of wheat ITS DNA sequences. This was demonstrated by the delay in exponential increase in fluorescence as NEBP concentrations increased.

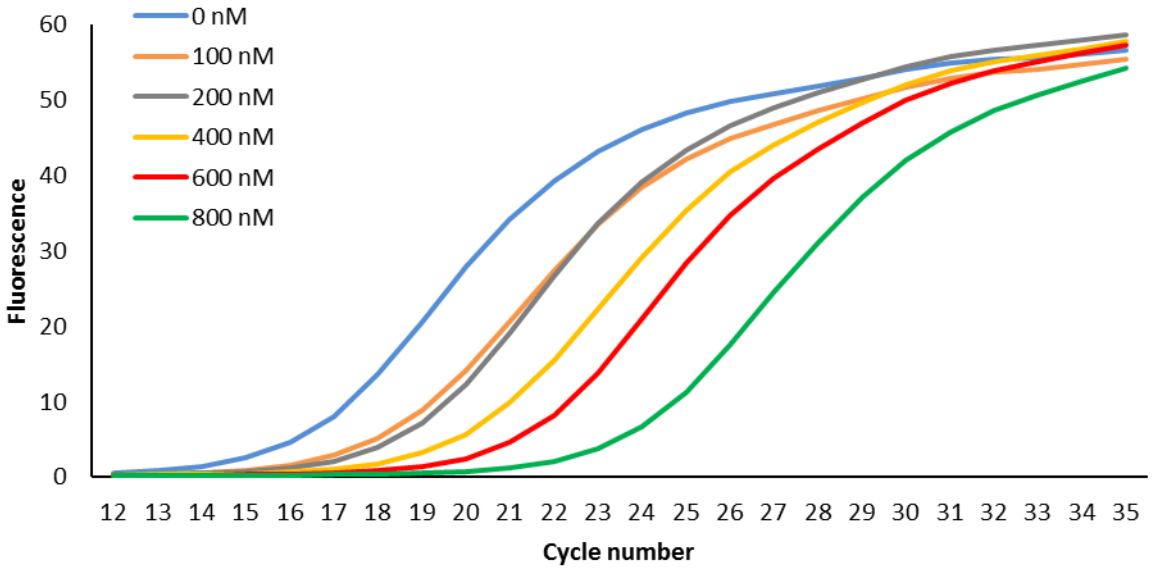

Figure 6.7 Mean fluorescence against cycle number for wheat-specific qPCR amplification of templates from 10^{-5} dilutions of replicate samples shown in Figure 6.5. The legend indicates the concentration of each NEBP in each pair of sample replicates.

In Figure 6.8 the plot of the C_T values against NEBP concentrations for the wheat-specific qPCR showed that there was a strong positive correlation ($R^2 = 0.90$) between increasing C_T values and increased NEBP concentration, demonstrating that the NEBPs caused a significant reduction in the amount of wheat ITS sequences that were produced during ITS-PCR with the universal eukaryotic primers.

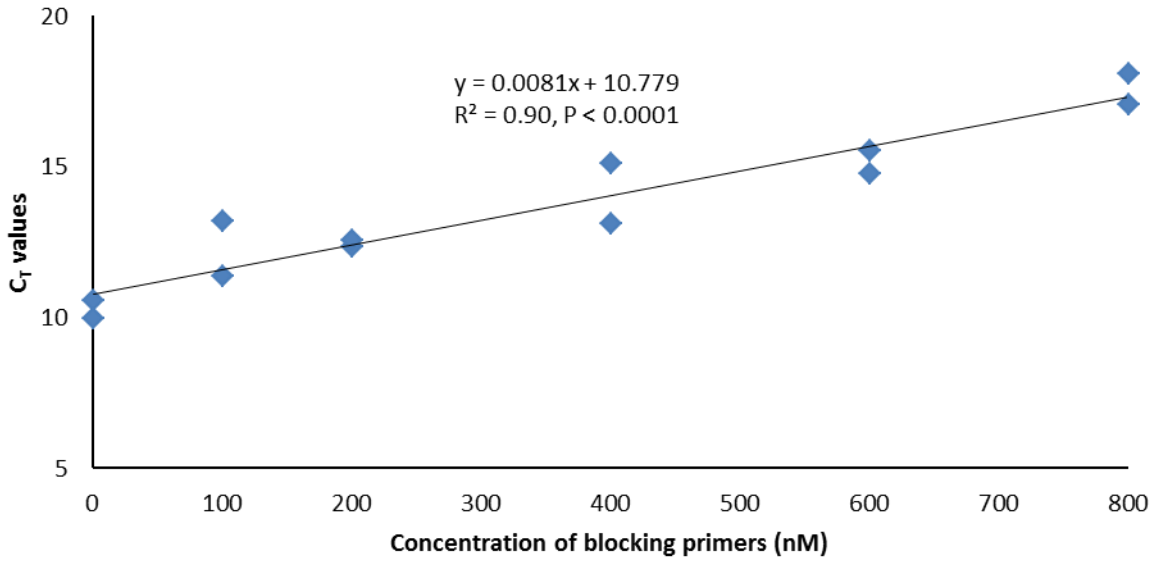

Figure 6.8 Linear response of C_T values to increasing NEBP concentrations for wheat-specific PCR in samples shown in Figure 6.7, with markers showing the duplicate C_T values at each NEBP concentration.

In Figure 6.9 the curve of mean fluorescence against cycle numbers for *Puccinia*-specific qPCR of the 10^{-4}diluted PCR product showed an initial increase in *Puccinia* amplicon production, with exponential amplification occurring at the lowest number of cycles for PCR products at 400 nM NEBP. The sample produced without the use of NEBPs (0 nM) had the latest amplification take-off. This showed that the NEBPs increased the production of *Puccinia* amplicons up to a specific concentration, after which it started declining; however, there were always more *Puccinia* target sequences produced in the presence of NEBPs than in the sample produced without the use of the NEBPs.

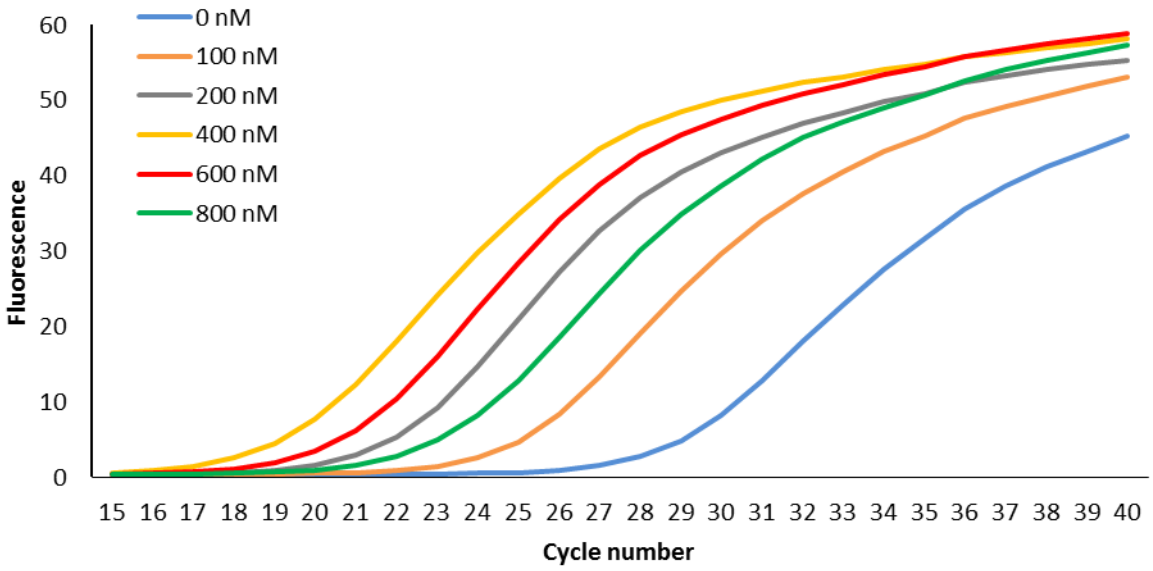

Figure 6.9 Mean fluorescence against cycle number curve plots for *Puccinia*-specific qPCR amplification of templates from 10^{-4} dilutions of the samples shown in Figure 5. The legend indicates the concentration of each of the NEBPs per sample.

Figure 6.10 for qPCR of *Puccinia* in the 10^{-4} diluted PCR products showed a strong correlation with the increasing concentrations of NEBPs ($R^2 = 0.93$). This demonstrated that increasing concentrations of NEBPs caused a lowering in C_T value, until a point after which the C_T value gradually increased. The maximum C_T response of 13.94 occurred at a 466.25 nM concentration of NEBP.

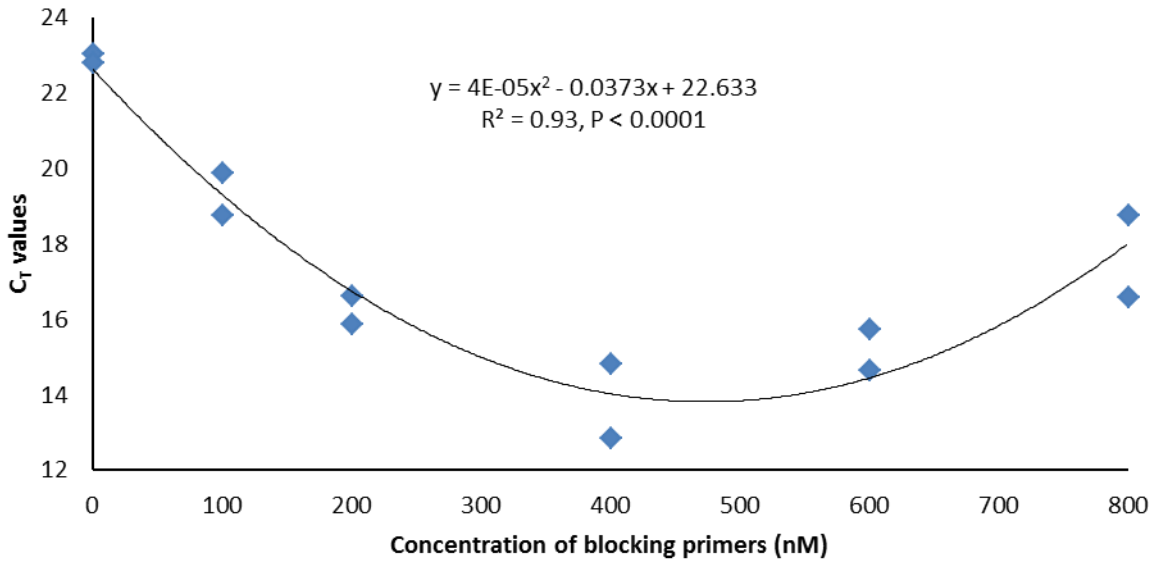

Figure 6.10 Response of C_T values to NEBP concentration for *Puccinia*-specific PCR of samples shown in Figure 6.9, with markers showing the duplicate C_T values at each NEBP concentration.

Agarose gels of the wheat-specific and *Puccinia*-specific PCR confirmed the presence of single bands of appropriate size (~145 bp and ~140 bp respectively, data not shown).

6.5 Discussion

The purpose of this work was to enhance the detection of pathogens, such as *Puccinia* sp., as well as other fungal endophytes that could be amplified from wheat mDNA, with NEBPs used in conjunction with universal eukaryotic primers. This approach can reveal characteristics of the fungal endophyte community composition during infection by a pathogen such as *Puccinia*. The use of universal primers would result in reduced bias in PCR amplification of target organisms, therefore providing a more accurate representation of the eukaryotic microbial community composition within the wheat tissues. The NEBPs would also reduce the amplification of host plant DNA, allowing for deeper sequence reads and resulting in more sensitive detection of relevant organisms during future NGS analysis.

The NEBPs designed in this study matched a wide variety of plant sequences, which indicates that these NEBPs could also be used in PCR-based studies that describe the eukaryotic microbial community composition within other host plants, especially grasses. The NEBPs

designed in this work straddled an interface between regions of conservation (at the ribosomal sequence ends) and hypervariability (within the ITS1 and ITS2 sequence regions). This was a particularly useful characteristic for the design of NEBPs employed to reduce bias in culture-independent PCR analysis of the fungal pathogen and endophyte community composition because the sequence conservation at the priming site accommodates universal extending primers, while the hypervariable region further downstream allows for highly specific annealing of the NEBP to the host plant sequence, thereby blocking its amplification. The few Genbank matches to purported fungal sequences were due to erroneous taxonomic assignment of submitted sequences, which is a recognized problem (Nilsson *et al.*, 2006).

The effect of the NEBPs was clear in the agarose gel of the wheat mDNA-derived ITS products (Figure 6.3) as the intensity of the ~700 bp band representing wheat diminished rapidly while other bands started appearing with varying intensity as the NEBP concentrations increased. This was because the NEBPs were successfully inhibiting the amplification of the wheat ITS sequences by the universal eukaryotic primers. The extendable universal primers were, therefore, annealing to the targeted *Puccinia* and fungal (or non-fungal eukaryotic e.g. Oomycete) endophyte sequences that were available instead. However, as NEBP concentrations increased, they were starting to anneal to the target non-host sequences as well, as evidenced by the formation and suppression of different bands at higher NEBP concentrations, i.e., the formation of band F6 up to 400 nM NEBP, while at 600 nM and higher, the formation of F5 took place instead (Figure 6.3). This occurred because the high concentrations of NEBPs and the large difference between the T_m of the NEBPs and the amplifying primers ITS5 and ITS4 (16°C and 19°C respectively) allowed the NEBP TaITS5blk to start annealing non-specifically to the conserved ~30 bp region of non-host sequences, downstream from the ITS5 annealing region where it is complementary to both wheat and endophyte sequences (Figure 6.1).

Further supporting this argument, certain fungal sequences that were previously detected in the wheat mDNA samples (Burgdorf *et al.*, 2016) that were used in this study had mismatches (e.g. *Cladosporium* and *Alternaria*, Figure 6.1) within the conserved region, which would bind to the NEBPs less efficiently, therefore allowing for a greater probability of

annealing with amplifying primers than those with fewer mismatches to the NEBPs. This explains how the formation or suppression of different non-host sequences (e.g. band F5 versus F6, Figure 6.3) took place as NEBP concentrations increased. The TaITS4blk NEBP (at the ITS4 priming site, Figure 6.2) would have made a lower contribution to this effect than the TaITS5blk NEBP (at the ITS5 priming site, Figure 6.1) since the mismatches were spread more evenly across the NEBP, therefore reducing the level of non-specific binding. It is worth noting that in the case of the TaITS5blk primer, the conserved region corresponds to that of the ITS1 primer (White *et al.*, 1990), which lies between the two mismatches seen in the *Cladosporium* and *Alternaria* sequences. The ITS1 primer may be a better option to use in conjunction with the TaITS5blk NEBP, as it would reduce the size of the conserved area and the length of the more variable region downstream could be extended in the design of an alternative NEBP, lessening non-specific binding of NEBPs to non-host sequences. However, the ITS1/ITS4 primer pair had high self-complementarity, and the ITS1 primer had three G's and one C at the 3' end, both of which have been considered to be sub-optimal primer characteristics (Chen *et al.*, 2002), therefore this combination was not used in this study.

The graphical representation of the quantified agarose gel bands for the PCR products of wheat ITS sequences and the sum of all other measured PCR products (Figure 6.4) exhibited an inverse correlation between the wheat and target product formation, due to the NEBPs effectively binding to the wheat sequences and suppressing their amplification, allowing for the increased formation of alternative products. However, the efficacy tapered off after 400 nM for the same reasons explained previously, whereby the NEBPs started inhibiting target non-host sequence amplification as well. The sum quantity of non-wheat bands remained substantially lower than the single wheat band found in uninhibited PCR of wheat mDNA with ITS5 and ITS4 primers (Figure 6.4) because the non-wheat target DNA template concentration was low relative to that of wheat ITS sequences, so higher cycle numbers would be required to increase band intensities.

The delay in take-off of fluorescence in the mean cycle curves for the ITS-PCR from wheat mDNA with increasing NEBP concentrations (Figure 6.5) demonstrated how the exponential increase in fluorescence was delayed because the increasing NEBP concentrations were

inhibiting the PCR by preventing the amplification of wheat ITS sequences. In the absence of NEBPs and when their concentration was low, the bulk of amplification was initially due to amplification of wheat ITS sequences, as seen in Figure 6.4, but as the amplification of wheat sequences was increasingly inhibited by the increasing NEBP concentration, exponential amplification was delayed to later cycles. This rate of increase in C_T values eventually diminished as non-host sequence amplification and primer-dimer formation started increasing, therefore reducing the relative delay in exponential amplification. The deceleration in the response rate of the C_T to increasing NEBP at high levels suggests that the overall inhibition of amplification by the NEBPs started becoming less effective as NEBP concentrations increased. This was because other products were still forming, even though the amplification of wheat sequences was inhibited. After 780 nM concentration of NEBP the C_T values started to decrease, i.e., fluorescence due to PCR amplification started to increase, because of rising fluorescence from the increasing production of primer dimers, as can be seen in agarose gel (Figure 6.3). Primer dimers are a result of interactions between primers that are at high concentrations and develop at later PCR cycles (Brownie *et al.*, 1997), so because the NEBPs were inhibiting amplification and consequently inhibiting the incorporation of the extendable primers, this effect became more pronounced as NEBP concentration increased.

The mean fluorescence curves for PCR amplification, using wheat-specific primers, of wheat ITS sequences from 10^{-5} dilutions of the ITS-PCR products, at increasing NEBP concentrations (Figure 6.7), showed that the production of wheat ITS sequences was being suppressed by increased levels of NEBP. The plot of C_T values against NEBP concentration (Figure 6.8) was strongly linear, indicating that wheat sequence amplification would continue to be suppressed as NEBP concentrations increased. This supports the previous assertion that the diminishing rate at which C_T values increased for PCR with universal primers (Figure 6.6) was not due to the increase in wheat sequences being amplified as cycle numbers increase, but rather due to increased amplification of target *Puccinia* and fungal endophyte sequences, as well as (at higher NEBP concentrations) increasing primer dimer production.

The exponential DNA amplification of *Puccinia* ITS sequences, revealed by the earlier take-off of fluorescence, occurred at earlier PCR cycles as NEBP concentrations increased up to 400 nM (Figure 6.9); the subsequent reversal of the trend (seen clearly in Figure 6.10) corresponded to the fading of the band represented by *Puccinia* (F3 in Figure 6.3) and the band representing wheat ITS amplicons. The reversal occurred because the increased NEBP concentrations started having an inhibitory effect on *Puccinia* sequence amplification by non-specific binding of NEBPs to *Puccinia* amplicons, as discussed previously. It was evident from the parabolic response of the C_T values to NEBP concentrations that an optimum concentration of NEBP exists. Vestheim and Jarman (2008) determined that there was an optimum ratio between NEBP and extending primers, with ratios as high as 20:1 for NEBP to primer. However, it is also necessary to consider the quantity of potential priming sites, regardless of this ratio, since any unblocked, host plant priming sites could be primed for extension if there were insufficient NEBPs. It is possible that optimum NEBP concentration can be estimated using qPCR, whereby the NEBP concentration should at least match the estimated concentration of non-target annealing sites.

In culture-independent PCR-based microbial community composition studies, sequence depth is preferable to sample replication (Smith & Peay, 2014); therefore, lower sample numbers can be analyzed with NGS studies as compared to the use of traditional clone libraries. Combined with barcode tagging which allows for the pooling of different samples (Frank, 2009), this potentially results in a substantial reduction of analysis costs when using NGS. Any methods that can reduce the number of redundant sequences and increase the production of target sequences will complement these factors since they increase the relative quantity of relevant data. In this sense, the application of NEBPs is particularly suited to studies where target DNA quantities are relatively low when compared to the host, especially since non-target DNA can often represent a significant portion of the NGS data. For example, even with the use of fungi-specific primers, Karlsson *et al*. (2014) found that 3.5% of the detected sequences their NGS study of wheat fungal endophytes belonged to the host plant. Similarly, also with the use of fungi-specific primers, Schmidt *et al*. (2013) observed that almost 20% of their usable sequences amplified from soil were of plant origin. Culture-independent PCR-based studies of fungal plant pathogens and endophytes will

always be influenced by the presence of host DNA. Along with the reduction of PCR amplification bias with the use of universal primers, reduction of this non-target DNA 'noise' can be achieved by using NEBPs.

Further work that needs to be done is a comparative study using fungal ITS specific primers versus universal primers with NEBPs on an artificial fungal pathogen and endophyte community mixed with wheat mDNA, to see which sets produce amplification products that most closely reflect the original community composition, as has been done in other primer design studies (Manter and Vivanco, 2007, Ihrmark *et al.*, 2012). A comparison of the efficacy of using only the forward or reverse NEBPs described in this work would also provide information on NEBP design improvement since these NEBPs differed in the distribution of mismatches (Figures 6.1 and 6.2). The important effects of such mismatches have already been characterized for extendable primers (Stadhouders *et al.*, 2010).

It is possible that the use of NEBPs could displace nested PCR, which has been used to enhance detection of target sequences in mixed templates (Landa *et al.*, 2013). Furthermore, using universal primers with NEBPs may aid the detection and characterization of cryptic or non-culturable organisms, such as the newly described *Cryptomycota* M.D.M. Jones & T.A. Richards (Lazarus and James, 2015), that could form part of the plant endosphere community.

The use of NEBPs in combination with primers that have a broad target range offers a highly relevant solution to some of the challenges that face culture-independent PCR-based plant pathogen detection and fungal endophyte community composition studies since target DNA is such a minor component of mDNA extracted from the host plant. This work showed that the use of these NEBPs enhances target sequence amplification, which may reveal greater numbers of organisms that associate with plants or describe new organisms altogether. Their use can, therefore, complement and enhance the power of PCR-based NGS studies that aim to elucidate on fungal endophyte composition during infection by *Puccinia*. This could assist in the development of alternative pest management strategies for the control of this serious threat to wheat production.

6.6 References

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Thesis overview

Introduction

The hypothesis that inspired this investigation of fungal wheat endophytes was that wheat cultivar phenotypic traits influence the composition of the fungal wheat endophyte communities that reside within them. It was postulated that selected endophytes exert epigenetic influence on the host plant that would be expressed as cultivar traits, such as disease resistance or increased yield. The work of this thesis was a study on the culture-independent detection of endophytes for the comparison of the fungal endophyte community composition of different cultivars of wheat, and of the effect of certain agronomic conditions on fungal wheat endophyte populations.

Chapter 1-The literature review

This review serves as a reference point for further studies on wheat endophytes. The review was chiefly related to fungal endophyte diversity, potential functions, and association with wheat. The review encompassed the definition of endophytes; fungal endophyte diversity and classification; fungal endophyte evolution; methods and technologies for studying fungal endophytes from both a culture-dependent and culture-independent perspective; interactions between fungal endophytes and plants, specifically in relation to their colonization of plant tissues and the consequences thereof; and the biological control potential of fungal endophytes. However, one area that was not discussed was the diversity and function of bacterial endophytes, in general, and of wheat. This was beyond the scope of the work here, but the hypotheses and questions raised during this work could equally be tested in the context of bacterial endophytes, or better still, for both fungal and bacterial endophytes combined.

The focus on fungal endophytes probably echoes a traditional perception by plant pathologists who see fungi (and fungus-like organisms) as the dominant participants in plant symbioses and disease (Ainsworth, 1981) because bacterial diseases of plants are considered less commercially significant than fungal diseases and more difficult to treat (Vidhyasekaran,

2004). Therefore, fungi are considered to have a more significant relationship with healthy plants as well. Considering aspects such as cytokinin production (Holland, 1997), and nitrogen fixation by bacteria (White *et al.*, 2012) and their impact on plant survival, let alone health, this view is not adequate, and bacterial endophytes of plants require consideration. However, puzzles are completed one piece at a time, which is why this work focused only on the fungal endophytes of wheat. A single, broader review of the literature available on all the microbes that form endophytes with wheat will emerge with the exploration of the interactions between different endophyte groups. As such, the interactions between endophytic fungi and bacteria and their hosts are beginning to attract attention (van Overbeek and Saikkonen, 2016).

Microbial endophytes offer a trove of novel genetic resources that can be harnessed for crop protection and enhancement. Plant breeding and crop management strategies that consider the gene-environment (G X E) interactions, such as the work performed by Paterson *et al.* (2003), could also consider the microbial interactions that can affect plant growth and health. These interactions could explain some of the difficulties in predicting G X E interactions that were reported by Chapman *et al.* (2000). El-Soda *et al.* (2014) include 'epigenesis' as an underlying model for G X E interactions; therefore, the epigenetic effects of fungal endophytes need to be explored further.

In crop production, microbes can vary within and between host plants, and within the environment, because of agronomic conditions (Burgdorf *et al.*, 2016). Further exploration of the functions and potential of endophytes in the 'agro-biome' will undoubtedly emerge to increase food production. Such work will describe the effects and interactions between successive crops, soil management, and endophytes, as has been done to examine important agronomic microbes such as the arbuscular mycorrhizal fungi (Brígido *et al.*, 2014) and nitrogen-fixing rhizobia (Souza *et al.*, 2013).

Chapter 2- Plant surface decontamination for PCR detection of endophytes

This was the first experimental chapter of this thesis and this study was published in the Brazilian Journal of Microbiology in 2014. A technical aspect of culture-independent PCR-based endophyte studies, namely, how to distinguish between endophytic organisms and those that are not inhabitants of the internal plant tissue was addressed and the findings were presented.

The term 'surface sterilization' is probably better applied to the concept of killing contaminating organisms, while 'surface decontamination' is a more appropriate term that implies the removal of any traces of these organisms. In the latter context, the experiment was designed to test modified surface sterilization procedures used previously in culture-independent studies, specifically the physical and chemical removal of plant surface contaminants. Their performance was tested in terms of surface decontamination as detected by PCR and visualized on agarose and denaturation gradient gel electrophoresis (DGGE) gels. After applying *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen as a test organism to wheat leaf surfaces, it was evident that the yeast could live on the wheat surface and was subsequently used in the assessment of surface decontamination. *S. cerevisiae* was not detected in the controls and has not been reported as a wheat endophyte. The *S. cerevisiae* produced a PCR band that was distinct from other endophytes and epiphytes in both the agarose and DGGE gels, which was vital to being able to visualize the treatment effects. After the surface sterilization treatments, PCR-DGGE profiles of fungal community composition showed clear differences between treatments and variability in the consistencies of the treatments. Gel analyses, including non-metric multidimensional scaling (nMDS) of DGGE profiles, successfully characterized these differences between methods and intra-treatment variability. This revealed that the way the plant surface is decontaminated has a discernible effect on the PCR-detected community composition. The simple technique of PCR-amplification of DNA from surface-applied non-endophytic microbes, which produce distinctive PCR product bands, can be a standard protocol by which surface decontamination methods can be evaluated in the future.

Future work evaluating surface decontamination methods could incorporate qPCR, with organism-specific primers, instead of standard PCR, to accurately quantify the removal of the applied organisms as a measure of the effectiveness of the surface decontamination techniques. These techniques could include variations in the chemical and physical removal or degradation of DNA to determine the most effective surface decontamination approach that is suitable for the plant species being studied. *S. cerevisiae*, used in baking, is resilient and ubiquitously available and can, therefore, serve as a standard organism for benchmarking other organisms. Other microbes can be evaluated as epiphytic organisms to test for intra-organism variation, on the condition that they are not commonly found as endophytes, or at least not, detected in untreated controls of the plant being studied. Other microbes for testing should include biofilm producing fungi and bacteria to determine the extent to which biofilm may shield epiphytes from surface decontamination treatments. There are also the differences in adsorption to plant surfaces amongst microbes to consider.

During this research, some experimentation was performed on the effects of surface sterilization on the roots and stems of wheat. The results showed similar outcomes to those seen for leaves. While this approach was successful on leaves, it is acknowledged that the surface of roots and leaves are quite different, so alternative methods of both treatment and the evaluation thereof may need to be considered. The relationship between microbes that exist on the surface of leaves and stems differ from those that inhabit the root surface, which is inhabited by many fungi that are essential for plant growth. Consider, for example, mycorrhizal fungi which penetrate the root tissue (Allen, 1991); therefore, alternative treatment and assay methods may be more appropriate for root endophyte studies.

Technological advances in both molecular biology and microscopy have provided several new ways for microbes to be visualized within plant tissues. Microbes that have been modified to include green fluorescent protein (GFP) could be employed to observe and measure the presence of endophytic and epiphytic fungi (or bacteria). Such organisms have been used in fungal pathogen (Lagopodi *et al.*, 2002) and endophyte studies (Sasan and Bidochka, 2012). This approach would be appropriate for root-associated microbe studies because it would indicate the degree of colonization of exterior and interior tissues. Confocal

microscopy with fluorescent *in situ* hybridization (FISH) could also be utilized in this way, as employed by Takeuchi and Frank (2001) in the examination of the microbial decontamination of lettuce leaves. Confocal microscopy of these FISH probes would also reveal the regions of colonization of either applied or autochthonous fungi, and their subsequent removal.

Confocal microscopy with FISH and the use of GFP-modified organism are more costly and laborious techniques than the protocol developed in this work. The increased research investment into the issue of surface decontamination inevitably raises the question of whether it is necessary to distinguish between epiphytes and endophytes, since the exhaustive investigation of how effective a surface decontamination procedure could seem excessive when the most important factor is whether the organisms that are being detected are interacting with the plant or protecting it in some way, or not. It is therefore also necessary to establish how epiphytic microbes affect the plant relative to endophytes. Work by Osono (2008) revealed the that the endophytic colonization was synchronized with leaf development while the epiphyte community composition was undifferentiated during host-leaf aging. This is one example suggesting that the relationship between endophyte and host may be of greater significance than that of microbial epiphytes, under typical circumstances where the epiphyte is defined according to the same conditions of having no negative effect on the host plant (for example, the covering of leaves by sooty mould, inhibiting photosynthesis, has a substantially negative effect on a plant). Other factors to consider, in comparing the relative significance of interactions, are that the water-repelling properties of a leaf influences microbial presence on the leaf surface and that the waxy cuticle tends to discourage microbial growth (Barthlott and Neinhuis, 1997), so many of the epiphytic microbes referred to by Lindow and Brandl (2003) could be incidental contaminants that are not necessarily growing. It was, therefore, assumed that there is a greater intimacy between endophytes and their hosts, which was why it was deemed necessary to remove any epiphytic microbes because they were not considered to interact significantly with the host plant. It would, however, be interesting to explore the ideas proposed by Leben (1965), who speculated on the effect that epiphytic microbes may have on plant pathogens; and Lindow

and Brandl (2003), who saw the study of the microbial ecology of leaf surfaces as being of increasing interest.

Chapter 3- DGGE versus TTGE

Denaturing electrophoresis is a technique that is likely to remain in use for some time (Izard, 2015). In the third chapter, two variants of the DGE technique were compared to establish which one would be most suitable for comparing endophyte community composition in terms of data quality, cost, and labour, with the materials and equipment that was available. The most commonly applied method, according to literature searches of published research, was denaturing gradient gel electrophoresis (DGGE), which uses a chemical gradient to denature DNA as it migrates down an acrylamide gel. This was compared to the denaturation of DNA by increasing the temperature of the tank buffer as the DNA migrated through an acrylamide gel. This variation of DGE is referred to as temporal temperature gradient electrophoresis (TTGE). Both methods were performed on the same equipment and the only differences were in the addition of denaturing chemicals to the gels and changes in the temperature during electrophoresis. The cost and time required to prepare gels for each were recorded. Identical samples were run on each of the two kinds of gels and band profiles and band quality was compared. Under the tested conditions, it was evident that DGGE produced bands that seemed more numerous and clearer. Profile correlations between methods were moderately to strongly positive, decreasing with increasing gel profile complexity. Band quality and number tended to be higher for DGGE but the difference was not always significant. The TTGE gels were slightly less costly to produce, but not so much as to overlook other performance criteria. The most significant difference was in preparation time where TTGE was much quicker to prepare. This was a major factor that initially motivated the research.

When screening large sample numbers, the preparation time can be a significant factor, yet it was shown that relatively few studies make use of this version of DGE. Inspection of some of the studies that did make use of TTGE often displayed gels that were clearly not as well resolved as can be seen on DGGE gels. An example of this can be seen in the TTGE gels produced by Nieguitsila *et al.*(2007) compared to those produced by Götz *et al.*(2006).

However, this view could be regarded as subjective. In some instances, TTGE seemed to produce perfectly adequate gels.

The aim of this chapter was to establish a means of objectively comparing the methods and then use that information to make a judgment on whether the benefits of the TTGE outweighed the disadvantages, if any. While a great number of variables could have been tested, we decided to make only the minimum changes in the materials when comparing the two methods. To this end, a conversion method was developed to compare denaturing conditions with varying denaturant and temperature. This can be of use when employing TTGE *in lieu* of DDGE for screening purposes or vice versa.

A method of comparing the techniques was successfully developed. This has not been described before and it appears that generally methods were simply assessed as to whether they produced visible band profiles that could be analysed further. Peak height and width are values commonly determined with image capture systems, in addition to the general profile properties such as band number and position. It was reasoned that the greater the peak height/width ratio was, the better the quality of a band is, i.e., a high peak with a narrow base is more crisply resolved and distinguished from neighbouring bands. This can provide a general quality control method, to assist with decision-making in whether to accept a band or not for all types of DGE gels. This is important, because the presence or absence of a band can be a subjective decision, or left to the settings on the image capture system, which may not always be reasonable, e.g., setting pixel height or density thresholds may include dark smears that do not represent clearly resolved bands. This can result in poor data quality or biased data that is subsequently processed by various statistical methods that fail to detect meaningful differences due to excessive background noise on the gels. Testing for statistically significant differences in band number and peak height/width allows for a decision to be made on the suitability of the DGE method that is used. In this work, in all cases, the DGGE had at least one significantly better characteristic than TTGE; and band numbers and height/width ratios tended to be higher for DGGE. This led to the conclusion that DGGE was producing better data for analysis.

The results of this work suggested that DGGE is a better method in terms of data quality, explaining its popularity in scientific literature compared to other variants of DGE. It is a robust, reliable and established method that can be used successfully for microbial community profiling. This may discourage the use of TTGE in the future, but more factors could still be tested to see if TTGE could be optimized to produce gels of sufficient or equal quality compared to that of DGGE. For example, Yoshino *et al.* (1991) found that temperature sweep gel electrophoresis (TSGE), a synonym for TTGE, performed as well as temperature gradient gel electrophoresis (TGGE) in point mutation detection. They noted that a very high ratio of acrylamide to bisacrylamide was crucial for producing acceptable results. This was not a factor that was explored in depth in this work. Considering the major reduction in preparation time and the significant reduction in cost, it is worth testing their recommendation further and providing a final verdict on the utility of TTGE.

Chapter 4- Comparison of fungal endophyte profiles for different wheat cultivars

In this study, we explored the hypothesis that wheat cultivar phenotypic traits influence the endophytic colonization of the wheat plant. The development of DGGE gels required the initial PCR amplification of fungal internal transcribed spacer (ITS) DNA sequences from metagenomic wheat DNA. This was performed on a quantitative PCR (qPCR) system to measure target DNA amounts as an indicator of fungal endophyte biomass. The qPCR was followed by high-resolution melt (HRM) analysis. The HRM profiles were shown to produce the same conclusions as found by DGGE analysis, in that there were no apparent differences in fungal profiles among wheat cultivars, although profiles differed significantly between tissue types. This HRM analysis of community profiles has recently emerged as a method to quickly and reliably compare microbial community profiles (Kim and Lee, 2014). The benefit is that there no further DNA processing, i.e., gel analysis, is required, other than PCR, saving a great deal of time and effort and allowing for greater numbers of samples to be evaluated, increasing the likelihood of detecting significant changes in community composition.

While DGGE and the HRM analysis failed to detect a cultivar effect on fungal endophyte community composition, the qPCR detected a significant cultivar-organ interaction on the amount of target DNA found in the various tissues. This is an important finding because much of the past work has focused on changes or differences in endophyte community composition, without quantitative comparisons of biomass.

Previously discovered interactions in culture-based studies were not necessarily in terms of composition, but possibly in the frequency of isolating the most abundant colonizer with limited sampling depth, i.e., this may be a quantitative issue in terms of certain plants favouring more colonization of certain tissues, rather than the types of fungi that colonize them. Host plants will accommodate the microbes that are available to them, unless they are vertically transmitted (Christian *et al.*, 2016). If the available microbes are opportunistic endophytes, with no long-established evolutionary symbiosis with the host (such as found with *Epichlöe* fungi and *Festuca* grasses), there will not necessarily be a selective exclusion of other fungi, but rather a greater or lesser interaction with those available, varying across different organs and influenced by the cultivar, as indicated in this study. For future work, it would be important to consider biomass as well as community composition when looking for cultivar effects on the phytobiome.

It would also be worth having an idea of the relative amounts of endophytes living within plants. Raw qPCR data showed that the threshold cycles for amplification occurred at very high cycle numbers. This hinted that the quantities of fungal genomic DNA were extremely low in the metagenomic wheat DNA template solution, suggesting that actual fungal biomass in plant tissue could be quite low. Future work on these quantities would provide some insight into the metabolic significance of these organisms and the energetic impact they have on the host. Just as ecosystems with higher species richness are seen as healthy, we would also be interested in determining how endophyte biomass relates to crop performance in environments where species richness is constrained by agronomic practices. Furthermore, the fact that endophyte community composition depends on the microbes that are locally available to colonize the host was clearly demonstrated by the strong presence of *Olpidium brassicae* sequences detected in wheat leaves, stems, and roots. This

organism has not been commonly reported as an endophyte, although it is a known fungal root pathogen of several broad-leaf crops. The association of *O. brassicae* with wheat was probably the result of the environmental conditions under which the wheat was growing. How this organism affects wheat relative to other endophytes may be worth exploring. It may be that certain opportunistic endophytes, while not overtly pathogenic, may reduce yields or disease resistance, instead of conferring any benefit. The definition of an endophyte as an organism found within a plant without causing disease symptoms could be reviewed in this light. Organisms should be evaluated using a procedure analogous to the Koch's postulate procedure, to show that they confer benefits before they can be categorized as an endophyte. This will be challenging for organisms that defy axenic culture, but plant performance with and without these organisms could classify organisms as endophytes, or place them along a spectrum ranging from plant pathogens to endophytes, in general, and for specific host plants.

The individual testing of endophytes on host plants could have further implications. The testing of individually isolated endophytes on different host cultivars would also be a sensible approach to test cultivar effects on endophyte selection. The degree of colonization could be measured by qPCR, culture-based methods, or microscopically. If a particular cultivar had a higher affinity for colonization by a specific endophytic fungus this would indicate that the plant genome, at cultivar level, determined the endophyte community composition. If that particular endophyte possessed plant growth promotion or protective properties, then these could be considered as epigenetic traits of a particular cultivar. This would encourage breeding for increased cultivar affinity for beneficial microbes, which could be introduced to the environment in which the crop is grown, or inoculated at planting. This approach deconstructs the original hypothesis, putting it into effect, as opposed to searching for evidence thereof in trials where many other factors may exist. Overall, the opportunistic association of plants with available microbes bodes well for the biological control and growth-promoting potential of endophytes.

Chapter 5- NGS analysis of fungicide effects on endophytes

Throughout the research performed for this thesis, observations were made regarding methodological variations that could influence experimental outcomes. These included

sample treatment, as described in Chapter 2; post-PCR DNA analysis, as seen in Chapter 3 and the possible effects of fungicides on fungal endophyte community composition that were considered in Chapter 4. It was suspected that fungicides limited the number of fungi available to associate with the host plants in the field, masking the selective associations by the host. Additionally, there was also a concern that the use of fungal-specific primers would not allow the detection of potential non-fungal endophytes such as Oomycetes (which are prevalent in soil but not commonly reported as endophytes). Consequently, the question arose whether Oomycetes were not reported because they were absent as endophytes, or because they were present but not detected by the method used. Therefore, in this study we i) investigated the effect of fungicides (which could create an open niche for non-fungal endophytes) on wheat endophytes and ii) expanded the range of NGS detection of wheat endophytes, to potentially include the detection of previously unreported eukaryotic organisms, by using universal primers.

The ITS5 and ITS4 primers described by White *et al.* (1990), commonly used in the identification of fungi and in culture-independent analyses of soil microbiota (Slabbert *et al.*, 2010) were used. While there were several base pair mismatches to the corresponding wheat ITS priming region, it was anticipated that wheat sequences would be amplified. To this end, it was presumed that next generation sequencing, with its substantial depth of sequencing, would detect most systemically significant organisms. This data was expected to show what the effect of the fungicide was on the main endophytic organisms, as well as whether there are non-fungal eukaryotic endophytes forming a significant component of the endophyte community.

It was found that most detected sequences belonged to wheat with sufficient fungal sequences detected for further analysis. The fungicides did not dramatically change the types and quantity of fungi detected, and the predominant fungi were members of the Dikarya. Although the most abundant fungal species detected in this work differed somewhat from that of the Swedish study of cereal phyllosphere fungi by Sapkota *et al.* (2015), it was interesting to note that they also detected pathogenic fungi in the healthy leaves of both fungicide-treated and untreated samples, representing substantial portions of

the fungal community. In this study the chytrids (seen in Chapter 4) were absent, indicating that wheat associates with what is available, and not always with what would be expected, as exemplified by the detection of *Sclerotinia*/*Botrytis* and *Pleurotus* spp.

The sequence analysis revealed the ambiguous taxonomic identification of sequence clusters on databases used by NGS data analysis pipelines. Even the curated database used to identify the sequence clusters contained non-type material and in some instances teleomorphic and anamorphic sequences of the same organism were represented separately. Such ambiguity hinders OTU richness estimates because sequence clusters are occasionally assigned to different species names which are in fact anamorphic. When sample data files are analysed individually the OTUs are assigned identities before comparisons between samples are made. Consequently, the downstream problems of taxonomic ambiguity are amplified and will negatively affect the interpretation of the experimental results. To overcome OTU assignment errors based on multiple species name identity, in this work all sequence data for all samples used in the experiment was merged, clustered, identified and then used as the reference database for each individual sample. This ensured that at an OTU level the clusters were assigned to the same OTU and taxonomic identity in all samples.

Overall, irrespective of a relatively low number of detected species the most numerous organisms were detected and their relative abundance was determined from read numbers. Dikarya were found to be the dominant organisms present in both fungicide-treated and untreated wheat. This meant that the application of fungicides was not providing a niche for alternative non-fungal taxa to occupy. The detection of the Dikarya as the dominant organisms alluded to the reason why many other studies of fungal endophytes focus on this group of organisms in their selection of taxon-specific primers.

The primers universal ITS primers, developed by White *et al*. (1990), that were used in this study, had several mismatches to wheat. However, because there were no terminal 3' mismatches to wheat DNA annealing sites, wheat DNA amplification was not greatly inhibited. For the same reason, alternative primers that have been subsequently been

developed for fungal community analysis, for example, those proposed by Bokulich and Mills (2013) would also amplify wheat. Therefore, such Dikarya-specific primers are still unable to overcome the problem of host DNA amplification, which is abundant in the mDNA used as template in endophyte studies. From this, it is evident that endophytes studies have a unique challenge compared with other environmental studies, i.e., the overwhelming presence of contaminating host DNA that can be co-amplified.

Chapter 6- Blocking primers for endophyte studies

The conflict between taxonomic range versus the taxonomic specificity of primers used in fungal community composition studies was addressed in the final experimental chapter. The abundance of host DNA in the mDNA template deters the use of universal primers that target the conserved regions among eukaryotic taxa.

Some strategies have been proposed to reduce the presence of non-target DNA, including sample enrichment to increase microbial DNA while reducing plant DNA. For example, in a study of the bacterial composition of stem bark of a plant with potential pharmacological use, DNA was extracted from the supernatants of large quantities of homogenized bark, as compared to the DNA extraction from a few grams of bark (Wang *et al.*, 2008). This is not viable with softer tissues such as wheat leaves, where the homogenization would release a great deal of host DNA, compared to bark. Pressure cycling technology (PCT) has been to extract DNA from wheat samples and found to increase the detection of root pathogens (Okubara *et al.*, 2007), probably because the PCT approach to lysis was less likely to damage DNA, so rarer sequences could be detected. PCT has resulted in the detection of higher numbers of unique bacterial T-RFLP fragments compared with bead beating, but there was no difference in fungal T-RFLP numbers suggesting that PCT possesses innate extraction biases as well (Bruner *et al.*, (2015). However, regardless of any potential benefits, both methods do not eliminate host DNA, therefore the problem of contaminating host DNA is not resolved. An alternative to consider could include subtractive hybridization, whereby endophyte free host DNA is used as a subtractive template to immobilise host DNA in an mDNA solution. Shotgun metagenomic sequencing of mDNA with data filtering was discussed in a personal communication with Oxford Nanopore, who suggested that their

MinION single molecule desktop PC USB stick sequencer could be set to reject sequences identified as wheat, sequencing only non-wheat fragments. This may be a way in which to physically filter and sequence the DNA in the sequencer itself, providing large numbers of mostly microbial DNA sequences without any PCR bias. These are potential methods that could be explored and adapted to endophyte studies.

In the meantime, it is unlikely that PCR-based NGS approaches to community composition analysis of plant pathogens and endophytes will discontinue in the immediate future. The use of non-extendable blocking primers (NEBPs) to mask the presence of host DNA is a simple and effective way to prevent host sequence amplification. In this study, it was shown that increasing concentrations of NEBPs led to a corresponding decrease in host DNA sequence amplification and an increase in the detection of fungal pathogen and endophyte sequences. The increase in fungal sequence amplification occurred up to an optimum NEBP concentration, after which it was increasingly inhibited and primer dimers began to form, demonstrating that optimum NEBP concentrations can be determined for the maximum detection of non-host sequences by universal primers. The use of such primers reduces biases caused by primer specificity.

The implementation of NEBPs significantly benefits PCR-based NGS studies of plant-associated microbes that attempt to describe the effect of endophyte composition on disease development Endophytic organisms that exhibit disease reducing properties could then be isolated, cultured and applied to crops to measure their performance. This is a useful tool for elucidating on general endophyte diversity using PCR-base culture-independent studies for application in the pursuit of targeted biological control research.

Conclusion

Throughout this work, challenges of studying environmental microorganisms were encountered and engaged with, in the context of endophytes within a host plant. Methods for culture-independent endophyte composition analysis have been changing dramatically, for example, methods such as clone library development are being usurped by NGS. Such culture-independent studies can provide far greater depth and quantity of information in this regard compared to culture-based studies. However, this knowledge is irrelevant

without having a prescribed purpose. It is, therefore, important to recognize the applications and value of the two approaches: culture-dependent studies can still be valuable in experiments where useful products can be tested; whereas culture-independent methods produce knowledge which creates a framework from which such applications can develop. In this context, the work performed in this produced new knowledge to strengthen experimental scientific research into harnessing endophytes for crop production.

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