Molecular response of wheat to *Bipolaris sorokiniana* under nitrogen stress

Saddam Hussein Abbas Baba

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بسم الله الرحمن الرحيم

"سَنُرِيهِمۡ ءَايَٰتِنَا فِي ٱلۡأٓفَاقِ وَفِيٓ أَنفُسِهِمۡ حَتَّىٰ يَتَبَيَّنَ لَهُمۡ أَنَّهُ ٱلۡحَقُّۗ أَوَلَمۡ يَكۡفِ بِرَبِّكَ أَنَّهُۥ عَلَىٰ كُلِّ شَيۡءٖ شَهِيد"

"We (God) will show them Our Signs in the universe, and in their own selves, until it becomes manifest to them that this (the Qur'ân) is the truth. Is it not sufficient in regard to your Lord that He is a Witness over all things?" (53)

Holy Quran - القران الكريم

Fussilat - سورة فصلت

Declaration

This thesis is submitted to Newcastle University for the degree requirements of Doctor of Philosophy in Biology. The research detailed within was performed during the period of 2015-2019, and was conducted in Newcastle University laboratories under the supervision of Prof Angharad Gatehouse, Dr Ethan Hack and Dr Martin Edwards.

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Saddam Hussein Abbas BABA

May 2019

Certificate of Approval

I confirm that, to the best of my knowledge, this thesis is from the student's own work and effort, and all other sources of information used have been acknowledged. This thesis has been submitted with my approval for the PhD degree.

Professor Angharad Gatehouse, Dr Ethan Hack and Dr Martin Edwards

May 2019

Dedication

I would like to dedicate this modest effort to the martyrs of Iraq who irrigated the motherland with their innocent blood and became a symbol of defiance and steadfastness. As well as for my loving children Zahraa, Zainab and Hussein who brighten my life. A special feeling of gratitude to my beloved mother, late father, wife, sisters and brothers who have supported me throughout the entire doctorate programme.

Saddam Hussein Abbas BABA

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Abstract

Spot blotch caused by *Bipolaris sorokiniana* (*Cochliobolus sativus*) is a serious disease of wheat grown in warm climates. The impact of plant diseases is influenced by plant nutrition, and it has previously been reported that nutrient stress affects spot blotch severity. The investigation of gene expression in response to stress is an excellent approach to crop improvement. The interaction between nitrogen supply, spot blotch disease severity and the regulation of WRKY TF defence gene expression in Iraqi commercial wheat and *Brachypodium* was investigated. Nitrogen levels significantly affected the growth of wheat cultivars Rashid (susceptible to spot blotch) and Latifia (resistant to spot blotch) and susceptibility to *B. sorokiniana* in a dose-dependent manner under growth room conditions. Levels of the internal transcribed spacer of *B. sorokiniana* ribosomal RNA, measured with three primer pairs, increased with increasing pathogen load. Plant growth with reduced nitrogen (0.75 mM, 3.75 mM) resulted in the up-regulation of all seven *TaWRKY* genes investigated and in the up-regulation of *PR1* in both Rashid and Latifia compared to when grown under optimal (7.5 mM) conditions; in contrast, the RuBisCO large subunit (*rbcL*) gene was down-regulated. Similar trends were observed in *Brachypodium* for the corresponding WRKY TF genes. The response to infection in the more resistant cultivar Latifia was earlier than in susceptible Rashid, with up-regulation of WRKY TF genes occurring by 24 hours post infection (hpi) compared to 72 h for Rashid. Measurement of chitin content using fluorescence microscopy not only showed the ability to detect spot blotch in the early stages during the biotrophic phase but also demonstrated that it would produce an absolute measure of the fungal load, thus providing a reliable method to measure the development of disease and spot blotch resistance. For all three fungal genes transcription was >50% in the infected susceptible Rashid compared to infected resistant Latifia after 72 hpi. TILLING lines (with mutations in *TaWRKY19* and *TaWRKY68a*) showed different development patterns and defence responses to *B. sorokiniana* compared to the wild-type (Cadenza cultivar). Microscopic analysis of the chitin content of the *W68aQ245* mutant showed a greater visualisation of the pathogen abundance and spread correlating with high levels of damage in the infected leaves under nitrogen stress in a dose-dependent manner (from 0.75 mM to 3.75 mM and 7.5 mM), in contrast to the wild-type. Interestingly, in older plants (25 days old) pre-inoculation with *Micromonospora luteifusca* showed an enhanced defence response in Rashid and Cordaile, which was significantly greater than in Latifia following infection by *B. sorokiniana* as verified by disease severity, number of spores and chitin content. Surprisingly expression of WRKY TFs was significantly higher in Latifia and Cordiale than Rashid in response to spot blotch infection in the presence of *Micromonospora*. The *rbcL* gene showed significant down-regulation of expression post spot blotch challenge in both the presence and absence of the *Micromonospora* inoculation in Rashid. However, in Latifia and Cordiale, *rbcL* was only downregulated in the absence of the bacterium post spot blotch challenge. This study shows that there is a complex pattern of changes in the expression of genes encoding WRKY transcription factors in response to nitrate stress and infection by *B. sorokiniana*. There remains a great deal of potential in the use of WRKY TFs to better understand how wheat responds to nitrogen stress and *B. sorokiniana* infection, and how the two might be linked. Continued investigation into the function of WRKY TFs will provide a basis via which we can understand, interpret and potentially manipulate this link in a way that ensures wheat production is improved and ongoing global food security increased.

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Abbreviations

ANOVA	Analysis of variance
AtWRKY TFs	*Arabidopsis thaliana* WRKY transcription factor gene
BdEF1α	*Brachypodium distachyon* elongation factor1α
BdUBC18	*Brachypodium distachyon* ubiquitin C18
bp	Base pairs
Bs28S rRNA	*Bipolaris sorokiniana* 28S ribosomal ribonucleic acid
CCI	Chlorophyll content index
CI	Confidence intervals
Cq	Threshold cycles
dpi, hpi	Days, hours post infection
ETI	Effector-triggered immunity
g	Centrifugal force
GS	Zadoks growth stage
HR	Hypersensitive response
JA	Jasmonic acid
MAPK	Mitogen-activated protein kinase
PAMP	Pathogen-associated molecular pattern
PCoA	Principal coordinate analysis
PR gene	Pathogenesis-related protein gene
PTI	PAMP-triggered immunity
r	Correlation coefficient
R gene	Resistance gene
R^2	Coefficient of determination
rbcL	Gene for large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)
rpm	Revolutions per minute
SA	Salicylic acid

SAR	Systemic acquired resistance
SEM	Standard error of mean
Ta28S rRNA	*Triticum aestivum* 28S ribosomal ribonucleic acid
TaeIF4A	*Triticum aestivum* eukaryotic initiation factor-4A
TILLING	Targeting Induced Local Lesions in Genomes
Tris	Tris (hydroxymethyl) aminomethane
Tukey	Tukey post-hoc test
Tween 20	Polyoxyethylene (20) sorbitan monolaurate
WGA	Wheat germ agglutinin (lectin from *Triticum aestivum*) - fluorescein isothiocyanate conjugate.
Xgwm	Microsatellite Loci
YECSA	Yeast extract corn starch agar
ΔCq	Cq (target gene) - Cq (endogenous control)
ΔΔCq	ΔCq (treatment condition) - ΔCq (reference condition)

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1 General Introduction

1.1 Challenges of food production

Agricultural cultivation of cereal crops is an important practice and contributes to the dietary staples of the majority of people worldwide. Wheat, rice and maize provide 60% of human food and they have become the most abundant plants on earth (FAO, 2018). However, these crops are face challenges; therefore, there is a requirement to increase global food production to meet the growing population over the next few decades (Tilman *et al.*, 2002). The UN (2017) report showed that the world population is projected to reach 9.8 billion in 2050, which is 35% higher than the current population (7.2 billion). This suggests that agricultural production must double in order to meet the needs of this rapidly growing population (Tilman *et al.*, 2011; USCB, 2015).

One of the strenuous challenges over the next fifty years will be to continue to produce essential resources sustainably, whilst attempting to negate the detrimental effects of previous endeavours. This is true among the full range of industries, but perhaps most notably in sustainable energy production in the wake of a century of dependence on fossil fuels, and in sustainable food production after years of reliance on developing new agrochemicals and agricultural expansion, resulting in a dramatic increase in our ecological footprint into global ecological deficit (GFPN, 2018). These challenges are especially pertinent as some of the largest populations in the world move towards more developed economies, and in doing so increase both energy and agricultural demands significantly. Climate change can also alter the distribution and severity of pests and diseases in crops and livestock, with a potential impact on food production and animal welfare. Approximately one-third of the world's food produced for human consumption is lost, both at an early stage of the supply chain through pests, diseases, post-harvest loss, or later in retail and consumption. The extent of such losses affects how much we may need to produce in the future (Global Food Security, 2018).

It is critical to focus on increasing productivity by improving the tolerance of crops to abiotic stresses (heat, cold, drought, salinity, and nutrient) and biotic stresses (insects, diseases, weeds), which can reduce yield in wheat by over 50%, and by growing plants with optimal soil fertility (Wang *et al.*, 2003). Globally, an average of 35% of crop yields are lost to pre-harvest pests (Figure 1.1), and more than 50% by abiotic stress (Ashraf *et al.*, 2012). In some developing countries, pre-harvest losses can reach 70% (Popp and Hantos, 2011). In terms of loss to biological factors, wheat differs from other crops in that the majority of yield loss occurs as a direct result of pathogen attack. In most other crop species weeds, animals and pathogens all

contribute equally to the estimated loss of yield based on a meta-analysis from the Crop Protection Compendium (Oerke, 2006).

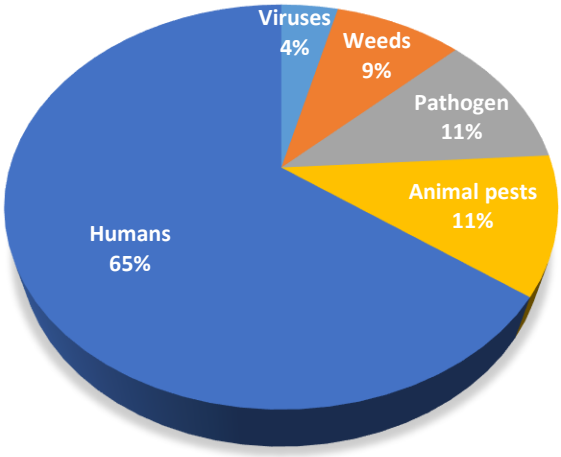


Figure 1.1. The world agricultural cake. Adapted from Oerke (2006) and Popp and Hantos (2011).

In the last five decades, there has been great success in increasing crop yield in the UK and other very productive regions through genotypic improvement, mechanisation of farming, and by the application of large amounts of nitrogen fertiliser up to 280 kg N ha^{-1} (DEFRA, 2010) and other agrochemicals. However, this appears not to be a sustainable agricultural practice due to its adverse effect on the environment. The use of fertiliser and other agrochemicals has shown that they can do more damage than good, with the adverse effects of fertilisers and pesticides leading to several human diseases, polluting our air, land and water (Sharma and Singhvi, 2017). Further to this, the presence of abiotic stress can have the effect of reducing or enhancing susceptibility to biotic pests and pathogens (Atkinson and Urwin, 2012). An additional challenge is that there are trade-offs between soil fertility and the effects of stress. Notably, low fertility favours some plant diseases, and high fertility favours others (Loyce *et al.*, 2008). I will discuss biotic and abiotic stress in the next sections.

There is an effort by different researchers to increase crop yield and its resistance to stress. One of the most important approaches to enhance crop production is through conventional breeding, which allows for small year on year improvements in key characteristics, such as yield or disease tolerance, by crossing elite lines. Whilst this approach is a key component of increasing yield and sustaining food security, it has some significant limitations. Thus, there is a need to focus on developing entirely novel approaches to the problems of crop production. Fundamental to this, we must improve our understanding of the mechanisms which plants employ and their interactions with the environment to ensure large yields and resistance to pests and pathogens.

If we can understand how plants have evolved to fill grain, be tolerant to stress and resistant to pests, we can employ this understanding in a variety of approaches to ensure production of food meets the demands of the next fifty years. Conventional breeding programmes combined with the potential which could be unlocked with pre-breeding research have a vital role to play in this, but it is only part of an overall solution. Whilst pesticides have provided a layer of defence against crop loss due to pathogens, it is becoming increasingly apparent that the intensity of selection pressure that this puts on pathogenic species means resistance mechanisms develop quickly.

1.2 Role of wheat in global food security

Wheat (*Triticum aestivum* L.) is an important grain crop in the world with over 757 million tonnes produced annually (Figure 1.2). It has been used as a source of nutrient for more than five thousand years (Peng *et al.*, 2011). More 215 million hectares of wheat are cultured annually (STATISTA, 2018). As agriculture developed, polyploid wheat varieties took the first stage in cultivation. Wheat cultivation played a significant role in establishing and sustaining ancient civilisations in serving as a source of food for both rich and poor, supporting cities for the first time and a providing a resource that led to the rise of the great civilisations of Babylon, Assyria and Egypt (Curtis and Halford, 2014). It is now grown more widely than any other crop, and has many uses has been developed, such as various types of bread, biscuits, cakes, pasta, noodles and couscous (Curtis and Halford, 2014). Wheat is a major source of calories (accounting for up to 20% of our calorie intake) in low- and middle-income countries. It accounts for global trade with a net worth around 50 billion US dollars annually (Peña-Bautista *et al.*, 2014).

However, wheat is threatened by both biotic and abiotic factors that reduce its optimal yields (Ashraf *et al.*, 2012). Water, heat waves, drought and subsequent wildfires cause significant losses in crop production. For example, Russia's wheat production fell by 33% in 2010 (FAOSTAT, 2014). In terms of losses due to biological factors, wheat differs from other crops in that the majority of yield loss occurs as a direct result of pathogen attack. Approximately 43% of wheat loss attributable to biotic factors is as a result of pathogens. This is the equivalent of over 100 million tonnes of global wheat loss annually to disease (Oerke, 2006; Popp and Hantos, 2011).

Farmers in developing countries lose a significant portion (20–70%) of the potential yield because of biotic and abiotic stresses (Datta, 2004). Cultivation of wheat with improved yield, tolerance to both biotic and abiotic factors will be the ideal approach. This will require basic

knowledge of how wheat responds to stress and interacts with the microbial community, as well as how it responds to the pathogenic attack. Such factors will play a significant role in increasing crop productivity and hence improving food security. As such, it is imperious that we understand wheat-pathogen interactions in an attempt to minimise yield losses. By understanding the mechanisms by which plants defend themselves against pathogens, we can reduce these losses and significantly improve wheat production without sole reliance on agrochemicals.

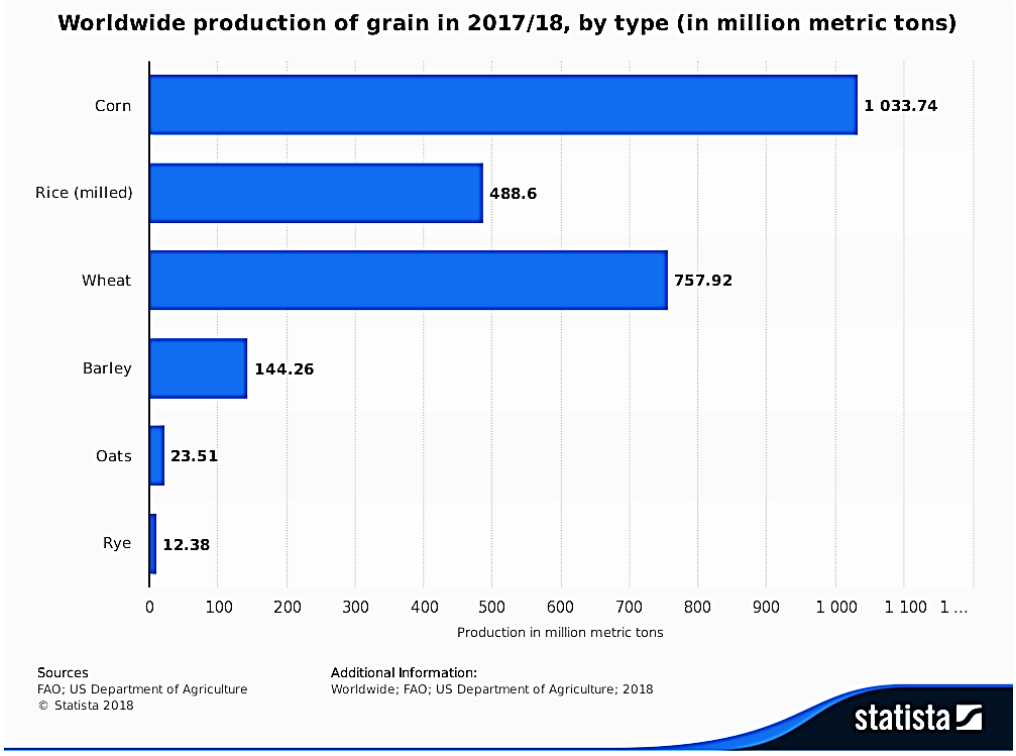


Figure 1.2. Worldwide production of grain in 2017/2018, (million metric tons). Source (STATISTA, 2018).

1.3 Genetic state of wheat

Common bread wheat, *Triticum aestivum*, is an allohexaploid (AABBDD) that arose from two wide hybridisation events. The first of these was between two diploid ancestral species carrying the A (*Triticum urartu*) and B (an unknown species) genomes and occurred 0.5–3.0 million years ago. Chromosome doubling formed wild tetraploid wheat (*T. turgidum* ssp. dicoccoides, AABB). This species was domesticated to cultivated emmer (*T. turgidum* ssp. dicoccum, AABB) (Dubcovsky and Dvorak, 2007). The second hybridisation took place about 9000 years ago between cultivated emmer and another wild species, diploid goatgrass (*Aegilops tauschii*, DD) to form allohexaploid common wheat (Peng *et al.*, 2011). The different ecotypes of common wheat (such as winter wheat and spring wheat) adapt well to a wide range of climates. Hexaploid wheat has one of the most complex genomes known to science. The three component

genomes – wheat A, B, and D, each comprising seven chromosomes – share many regions of high similarity, with six copies of each chromosome, enormous numbers of near-identical sequences scattered throughout, and overall haploid size of more than 15 billion bases (Zimin *et al.*, 2017).

The combination between the vast genome size and the hexaploid nature of wheat makes many standard genetics techniques impractical. A common method to investigate genes is by using "knock-out" lines, that is individuals in which the gene is removed, not expressed or otherwise non-functional. To obtain plants homozygous for a knockout in a diploid plant is not a significant challenge. For example, in the model plant *Arabidopsis thaliana*, methods such as insertional mutagenesis provide relatively simple mechanisms to obtain plants which do not express certain genes (Alonso and Stepanova, 2003). Furthermore, the model grass *Brachypodium distachyon* is becoming an excellent model system for studying the basic biology underlying characters relevant to the use of grasses as food, forage and energy crops. The next section discusses Brachypodium in detail. The relatively poor annotation of the wheat genome also makes genetic and bioinformatic approaches more challenging than in a diploid plant. Coding sequences for genes may not be fully determined, and many genes for which the genomic or coding sequences are known may not have functions attributed to them. This makes genetic studies in wheat more challenging and time-consuming than in species with simpler genetics. However, there is need to study wheat directly in order to understand disease tolerance in this important crop species. In particular, this project focuses on *Triticum aestivum* (wheat) and its response to pathogen stress (spot blotch- *Bipolaris sorokiniana*) as biotic stress; those are grown in reducing nutrient input on the optimum level (nitrogen) as abiotic stress.

1.4 Brachypodium as a potential model system for cereal crops

Brachypodium distachyon is a monocot, temperate wild grass species. Its simple growth requirements, morphological, genomic, and molecular genetic characteristics make it an important model system for grass biology (Hong *et al.*, 2008). The Brachypodium genome is one of the smallest of grasses, approximately 272 Mb (Mega base pair), it is diploid (2n = 10), and its genes are highly related to wheat genes with a better syntenic relationship than rice (Griffiths *et al.*, 2006; International Brachypodium Initiative, 2010). Model systems can significantly advance research by allowing scientists to investigate complex processes that are not simple to study in non-model organisms. Although the dicot Arabidopsis *(Arabidopsis thaliana*) is widely considered to be an eminent model plant, and has been one of the exciting

model plants used for almost 30 years, Brachypodium is genetically and morphologically the nearest model plant to wheat (International Brachypodium Initiative, 2010).

The evolutionary relationships between Brachypodium, sorghum, rice and wheat were assessed by measuring the mean synonymous substitution rates (Ks) of orthologous gene pairs. It was estimated that Brachypodium diverged from wheat 32–39 million years (Myr) ago, rice 40–53 Myr ago, and sorghum diverged 45–60 Myr ago (Figure 1.3 **a**) were estimated. There is an internal genome duplication in Brachypodium. The mean synonymous substitution rates (Ks) of orthologous duplicated gene pairs suggest that the duplication took place 56–72 Myr ago, before the diversification of the grasses. Comparison of the diploid Brachypodium and hexaploid wheat genomes has identified 5,003 ESTs (expressed sequence tags) that define orthologous gene relationships mapped to wheat deletion bins (Figure 1.3 **b**).

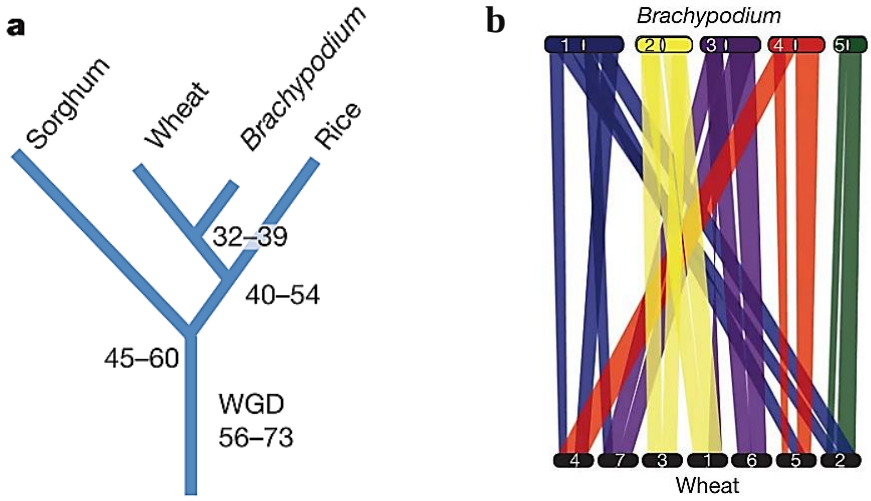


Figure 1.3. Brachypodium genome evolution and synteny between grass subfamilies.
Whole-genome duplication, were used to define the divergence times of Brachypodium, rice, sorghum and wheat (**a**). Orthologous gene relationships between Brachypodium and hexaploid bread wheat (**b**) (International Brachypodium Initiative, 2010).

Brachypodium also has a short growth cycle; it can be completed for less than four months. Other advantages include self-fertility, the availability of several diploid accessions, and intrinsic resistance to various biotic and abiotic stress conditions (Draper *et al.*, 2001). It is interesting to plant pathologists in research areas including decoding the molecular mechanisms determining the outcomes of host-pathogen interactions, which may provide a clear understanding for plant-based approaches for disease management. Wheat has a large genome and inefficient transformation systems, so that there are massive challenges to research on host-pathogen interactions. Thus, establishing *Brachypodium distachyon* as a model system for cereal crop diseases is highly attractive.

Our understanding of wheat defences against *B. sorokiniana*, especially at the molecular level, is lacking as well. In part, this is because wheat has a large and complex genome. Chapter 3 of this study investigated the host status of *B. distachyon* and its interaction with *B. sorokiniana* as a new model pathosystem for studying its pathogenicity.

1.5 Role of microorganisms in plant defence

Microorganisms aid significantly in healthy crop growth and defence against pathogen attack. Among the numerous microorganisms found to be of economic importance to plants are actinobacteria (gram-positive), which have demonstrated the ability to enhance plant defence against pathogens, by inhibiting their growth and reproduction (Hirsch and Valdes, 2010; Verma *et al.*, 2011). There is increasing evidence of the harmful effects of chemical pesticides on the environment, so that more environmentally sustainable and safe methods for crop protection are needed. Formulating bioinoculants with both growth and defence promoting effects on plants is a major goal in modern agriculture. The actinobacterium *Micromonospora* has been reported to have antifungal properties (Turner *et al.*, 2013; Martínez-Hidalgo *et al*., 2014; Martin-Rodriguez *et al.*, 2015). The genus *Micromonospora* belongs to the family *Micromonosporaceae*. The bacteria in this family, which currently contains 32 genera, are "Gram-positive, aerobic, filamentous, spore-producing and mesophilic microorganisms". Many of them have orange to red colonies because they produce carotenoid pigments (Turner *et al.*, 2013). *Micromonospora* species are commonly found in nitrogen-fixing nodules of legumes and actinorhizal plants, including *Micromonospora coriarie*, *Micromonospora lupine* (Trujillo *et al.*, 2007), *Micromonospora saelicesensis* (Trujillo *et al.*, 2006) and *Micromonospora pisi* (Garcia *et al*., 2010). Three novel actinobacterial strains GUI2T, GUI42 and CR21, represent a novel *Micromonospora* species, named *Micromonospora luteifusca*. The type strain is GUI2T (Carro *et al.*, 2016). Scientific classification of the *Micromonospora luteifusca* strain is presented in (Table 1.1).

Exposure to non-pathogenic microorganisms may increase resistance to future pathogen attack through the development of induced systemic resistance (ISR). For example, rhizobia in the root zone stimulate the formation of root nodules, in addition to initiating a signalling cascade throughout the plant. As a result of this signalling cascade, jasmonic acid (JA) and ethylene are produced, thus stimulating plant defence mechanisms active against attack from pathogens. The defensive response is thus stronger and faster response than that caused by other defence-related mechanisms such as salicylic acid (SA) or pathogenesis related (PR) proteins, which occur after actual pathogen attack (Taiz and Zeiger, 2010). This defensive strategy has advantages to the

plant as it produces the defence response needed, making more resources available for plant growth and yield, as predicted by the growth versus defence hypothesis (Huot *et al.*, 2014).

Chapter 7 of this study investigates the potential of *Micromonospora luteifusca* as a biocontrol agent, testing its antifungal properties and its ability to stimulate wheat defence against *Bipolaris sorokiniana.*

Table 1.1. Scientific classification of *Micromonospora luteifusca* strain

Scientific classification	
Kingdom	*Bacteria*
Phylum	Actinobacteria
Order	Actinomycetales
Family	Micromonosporaceae
Genus	*Micromonospora*
Species	*Luteifusca*

1.6 Environmental stress and plant response

Plants are displayed to various and complex types of interactions involving numerous environmental factors. These factors affect several plant mechanisms at the molecular, morphological, and physiological-biochemical levels (Wang *et al*., 2003). In the course of evolution in nature, plants have developed particular mechanisms allowing them to adapt and survive under harsh conditions. When the plants exposed to biotic and abiotic stress this results in induction of a dysfunction in plant metabolism suggesting physiological costs (Massad *et al*., 2012), and thus leading to a reduction in fitness and ultimately in productivity by altering plant metabolic homeostasis and modifying source-sink relationships (Shao *et al*., 2008; Albacete *et al*., 2014).

Stress causes many changes in plant metabolism, affecting growth and development, in some cases leading to low yield and even death. If the stress is moderate and short-term, the injury may be temporary, and the plant may recover when the challenge is removed. Previous studies have shown that some plants acclimatise to avoid stress but in some extreme cases the plant cannot survive; such plants are considered to be susceptible to stress (Huot *et al.*, 2014; Wasternack, 2017) as described in (Figure 1.4). The results of new researches of abiotic and biotic stress tolerance mechanisms would provide a solid grounding for knowledge will help in the application of stress-responsive determinants and engineering plants with enhanced tolerance to stress (Hasanuzzaman *et al.*, 2012).

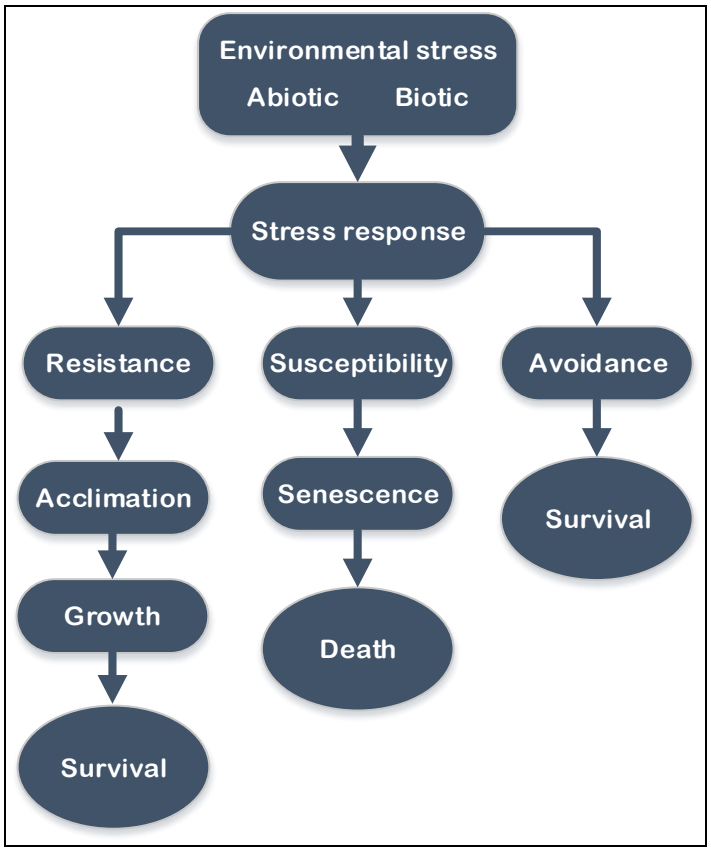


Figure 1.4. Schematic diagram outlining the response of plants to environmental stress. Adapted from Hopkins and Huner (2009).

1.7 Biotic stress (plants and microbial communities interaction)

Biotic stress is defined as "stress that is caused in plants due to damage instigated by other living organisms, including fungi, bacteria, viruses, parasites, weeds, insects, and other native or cultivated plants" (Newton *et al.*, 2011). Therefore, biotic stress is an additional challenge placing strong pressure on plants (Mordecai, 2011).

Complex interactions with different microbial communities influence all plants. Plant interactions may be detrimental (causing damage or disease to the host plant), as in the interaction with pathogenic bacteria or fungi, oomycetes, nematodes, or parasitic root plants. In all these plant interactions, chemical communication between the plant and the other organism is essential. Soil microorganisms are well known to be able to affect plant health and nutrition (Lareen *et al.*, 2016). Direct interactions may range from parasitic to mutualistic symbioses. There can also be indirect effects. Microbes have important roles in nutrient cycling and nutrient acquisition (Bulgarelli *et al.*, 2013).

1.7.1 *Bipolaris sorokiniana*- disease cycle and reproduction

Bipolaris sorokiniana (Sacc.) Shoemaker (hemibiotrophic fungal pathogen; former teleomorph name *Cochliobolus sativus*) is characterised by thick-walled, elliptical conidia (60-120 μm × 12-20 μm) with 5 to 9 cells (Shoemaker, 1959). Genetically, when combined gene analysis of rDNA internal transcribed spacer (ITS), the gene encoding the large subunit of ribosomal RNA (LSU), glyceraldehyde 3 phosphate dehydrogenase(GPDH) and translation elongation factor 1α (EF1α) shows *B. sorokiniana* and *C. sativus* represent the two same taxon (Manamgoda *et al.*, 2012). Table 1.2 shows the scientific classification of *B. sorokiniana*.

Table 1.2. Scientific classification of *Bipolaris sorokiniana*

Scientific classification	
Kingdom	Fungi
Division	Ascomycota
Class	Dothideomycetes
Order	Pleosporales
Family	Pleosporaceae
Genus	*Bipolaris*
Species	*Sorokiniana*

It reproduces through asexual means mainly through conidia, although the occurrence of sexuality and a parasexual cycle have been described (Zillinsky, 1983). Disease symptoms are to some extent dependent upon on environmental conditions and route of entry; they include seedling blight, node cankers and spot blotch on wheat leaves, as well as common root rot conditions (Zillinsky, 1983).

Like many other fungal leaf pathogens, *B. sorokiniana* may be transmitted on seeds (Pandey *et al.*, 2005), with the primary infection through the seeds, but also via other infected crop residues and other hosts. Conidia that are dormant in the soil or plant debris are probably the major source of primary infection (Lanoiselet *et al.*, 2005). Infected seeds, when sown are a source of spores to other newly grown crops (Reis, 1990), and infection can reduce seedling emergence by up to 38% (Clark and Wallen, 1969; Manamgoda *et al.*, 2011). Airborne conidia are a secondary source of infection, contributing to increased disease severity. The conidia contain nutrients to formation of appressoria, but the fungus requires additional nutrients from healthy plant tissues in order to penetrate successfully (Manamgoda *et al.*, 2011). Dark brown lesions

characterise the main disease symptoms that can occur on the coleoptiles, crowns, culms and roots (Wiese, 1987). Lesions on the leaves appear as small, dark brown flecks 1–2 mm long, which in susceptible cultivars quickly expand into oval to elongated blotches, light brown to dark brown (Wiese, 1987; Cook and Vaseth, 1991). Lesions may reach several centimetres before coalescing, reducing photosynthesis and finally causing leaf death. Leaf symptoms are most apparent after heading and occur most often on the lower leaves (Al-Sadi, 2016). Ideal conditions for *Bipolaris sorokiniana* development on the leaves are a relative humidity of near 95 percent with an average temperature above 17°C in the coldest month and extended periods (more than 12 to 18 hours) of leaf wetness caused by rainfall, irrigation, fog or dew (Chowdhury *et al.*, 2013).

The life cycle of *Bipolaris sorokiniana* is represented below (Figure 1.5) and shows the development of spot blotch pathogen during different developmental stages. Infection for spot blotch is initiated by adhesion of the conidia to the leaf surface, followed by their germination and formation of germ tubes (Acharya *et al.*, 2011). Within eight hours, the germ tubes swell sufficiently to produce an appressorium, from which infecting hyphae develop (Jansson and Akesson, 2003).

Then, 12 h is enough to hyphae penetrate the host's cuticle (Sahu *et al.*, 2016) and multiply rapidly, extending and spreading into the intercellular space within the mesophyll tissue of the leaf (Acharya *et al.*, 2011). And produce a new generation of conidia within 48 h, which are carried on conidiophores that are 100–150 × 6–8 μm long. Forms of these conidia are olive-brown, oblong, tapered towards the ends and have a prominent basal scar. They measure 60–120 × 15–20 μm in size and have three to nine thick septa. Conidia produced is possible in several cycles in the crop season, which causes secondary infections involving dispersal of conidia through dew and rain (Acharya *et al.*, 2011) by airborne conidia of *B. sorokiniana* (Duveiller *et al.*, 2005).

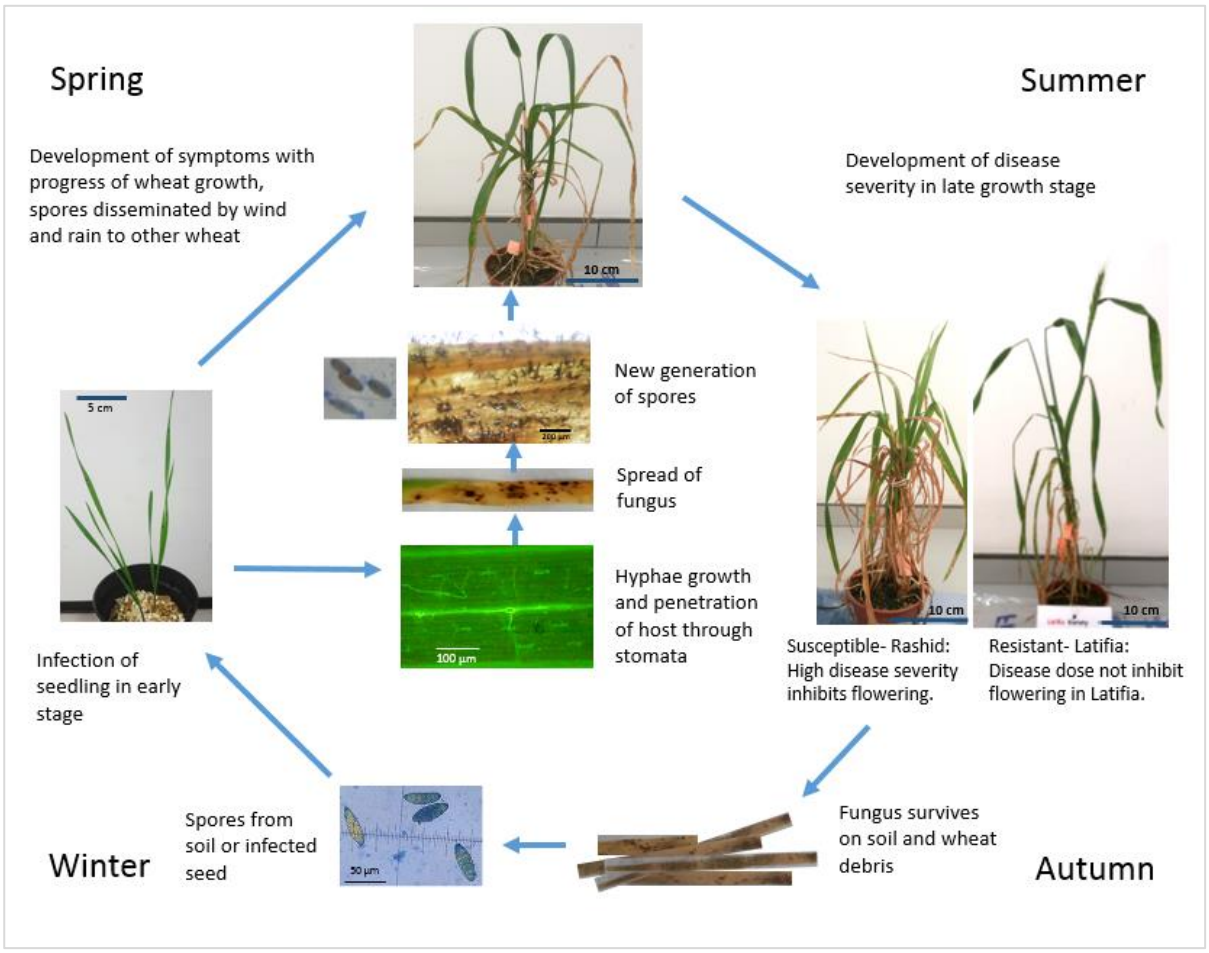


Figure 1.5. The life cycle of *Bipolaris sorokiniana* on susceptible (Rashid) and resistant (Latifia) cultivars. Adapted from Manamgoda *et al.* (2011). Photographs by S.H.A. Baba.

1.7.2 Worldwide distribution of spot blotch

Bipolaris sorokiniana is a serious pathogen in wheat grown in warm regions with high humidity (Chowdhury *et al.*, 2013). Globally, spot blotch has been reported to affect 25 million hectares of wheat, which is 12% of the entire wheat growing area (Duveiller and Sharma, 2009). It is ranked number one among wheat diseases in rice-wheat cropping systems in India (Jaiswal *et al.*, 2007). Average yield loss due to spot blotch under epidemic conditions can cause losses as high as 100%. Last five decades, spot blotch has been a severe constraint in wheat production in several regions of the world, particularly in India, South and Southeast Asia, Latin America, USA, Canada and other countries as recorded in (Table.1.3) (Joshi *et al.*, 2007; Gupta *et al.*, 2018). Saari (1998) reported that spot blotch losses exceed 20% in South Asia, where it is considered a limiting factor to the production of wheat and may even lead to complete crop loss. Wheat production in these environments is precarious if no effective methods of protection are available such as the use of disease resistant cultivars or commercial fungicide treatment. Temperature is a critical factor, which plays a vital role coupled with high humidity; a temperature range of 18 °C to 32 °C favours the growth of *B. sorokiniana*. In the Indo-Gangetic

Plains of India, high relative humidity arising from high levels of soil moisture and fog leads to long periods of leaf wetness on leaf blades, promoting establishment and multiplication of the pathogen even in the absence of rainfall (Chowdhury *et al.*, 2013).

Table.1.3. Worldwide distribution of *Bipolaris sorokiniana*. Adapted from Gupta *et al.* (2018).

Continent	**Country**
Africa	Kenya, Malawi, South Africa, Sudan, Tanzania, Zimbabwe
Asia	Bangladesh, China, India, Indonesia, South Korea, Nepal, Pakistan, Taiwan, Thailand
Australasia	Australia, New Zealand, Papua New Guinea
Europe	Austria, Belgium, Czech Republic, Denmark, Germany, Greece, Hungary, Italy, Ukraine, Turkey, Poland, Russian Federation, United Kingdom
North America	Canada, USA
South America	Argentina, Brazil

B. sorokiniana is an important wheat pathogen in Turkey (Eken and Demirci, 1998). In Europe, it causes yield losses in wheat northwest Italy, mostly due to root rot (Rossi *et al*., 1995). *Bipolaris* disease of barley in Ukraine has reached catastrophic proportions that sometimes affect 75% of crops (Kriuchkova, 2017).

To avoid the harmful effects of spot blotch in wheat production, the use of chemical control such as fungicide shows significant benefits, but it is not environmentally sustainable. A study in Nebraska, United States has demonstrated that the application of fungicide has a substantial effect by decreasing the rate of spot blotch infection from 42% to 27% in wheat fields (Wegulo *et al.*, 2009). In Nepal, a field study of foliar fungicide application in wheat has shown increases in grain yield over the control of 38% and 83% with grain infection of 39% and 70% in control plots reduced to 16 and 24% in 2002 and 2003, respectively (Sharma-Poudyal *et al.*, 2005). Diseased plot yields were 43.2% lower than fungicide-protected plot yields (Villareal *et al.*, 1995). However, the use of cultivars resistant to pathogen attack is completely acceptable, because it is environmentally sustainable. Yield losses are variable, but resistant cultivars are essential in fields with low nitrogen inputs and under late-sown conditions (Duveiller and Sharma, 2009). Therefore, the present study aimed to investigate *B. sorokiniana* resistance in wheat cultivars and breeding lines and the possibility of improved spot blotch control with a better understanding of cultivar resistance by using physiological and molecular approaches.

To optimise wheat production by reducing damage and loss as a consequence of *B. sorokiniana*, we need to understand the relationship between plant and fungus and how to avoid the conditions that help to increase pathogen impact on plants, such as nitrogen availability.

1.8 Plant Immunity

Plants have various abilities to resist diseases caused by pathogens via stimulation of a wide variety of immune responses that reduce the growth and spread of invading pathogens. Pathogens can be found in the environment (soil, air and water) and include bacteria, fungi and viruses. These organisms can cause infection with different intensity and severity. Therefore, for plants to grow and reproduce, they need to protect themselves against these pathogens.

Fungi are heterotrophs and obtain their carbon and energy from other organisms (Carris, 2012). Plant pathogens generally fall within two feeding types: biotrophs, which feed on living tissue, and necrotrophs, which feed on the dead tissue (Tel-Aviv, 2012; Weete, 2012). Hemibiotrophs is the third group, which shows both forms of nutrient use, from an early biotrophic phase to necrotrophy latterly. Plant defences against biotrophic fungal pathogens involve the prevention of fungal penetration and programmed cell death (PCD). The plant enhances its resistance to penetration of the cell wall in two ways. The first is to prevent spore germination and inhibit the formation of the haustorium by penetration resistance. The second is to avoid nutrient supply to the fungus in the penetrated cell by inducing cell death (Gebrie, 2016). There are differences in the biotrophic or necrotrophic phase that diverge significantly among hemibiotrophic pathogens. Pathogens are classified by differences in immune responses due to the different methods of nutrient uptake (Maloy and Murray, 2001). Therefore, plant defence responses are diverse depending upon the pathogen in question and its feeding mechanisms (Glazebrook, 2005).

Plants have defence methods in addition to physical barriers, including production of toxic and inhibitory substances, such as phenolics, phytoalexins and hydrolytic enzymes targeting pathogen cell walls (Daayf *et al.*, 2012; Rovenich *et al.*, 2014). Whilst these defence mechanisms may be able to reduce pathogen attack they are often unable to prevent it completely. However, plants have a repertoire of inducible defences that enable them to respond to further pathogen attack (Spoel and Dong, 2012).

1.9 Pathogen Recognition in Plants

Plants have evolved different types of adaptive mechanisms that enhance basal immunity as protective measures against various microbial pathogens and animal pests. Plant defence

responses can occur in two basic forms: the first form is pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) and the second is effector-triggered immunity (ETI). Necrotrophs can be host-specific or broad-host-range pathogens and their effectors can be target specific or have activity against a wide range of species (Mengiste, 2012). However, in turn, biotrophic fungi have several mechanisms to defend their effectors from plant receptor molecules, resulting in a reduction of defence signalling molecule like salicylic acid (Gebrie, 2016).

Generally, PTI and ETI trigger similar defence responses. Both involve the production of reactive oxygen species and defence gene induction, but ETI is much faster and plant responses are stronger (Wei *et al.*, 2013). ETI has often been associated with localised cell death termed the hypersensitive response (HR) that functions to restrict further spread of microbial attack (Kiraly *et al.*, 2007; Dodds and Rathjen, 2010). ETI gives plants the ability to sense effects of pathogens on vulnerable processes in the host, whilst PTI mainly distinguishes self and non-self. By guarding against pathogen effects, ETI is an efficient defence system for more progressive infections (van der Hoorn and Jones, 2004; Malinovsky *et al.*, 2014), whereas PTI is important for non-host resistance and basal immunity.

On the surface of the host cell, plants carry receptor proteins that recognise conserved components of the microbial surface called pathogen-associated molecular patterns for fungus (PAMPs), although their presence in non-pathogenic organisms makes the term Microbe-Associated Molecular Patterns (MAMPs) are more accurate (Ausubel, 2005). Pattern-recognition receptors (PRRs) recognise the PAMPs/ MAMPs, and a signalling cascade is induced. This cascade proceeds via the Mitogen-Activated Protein Kinase (MAPK) pathway (Meng and Zhang, 2013). This step alone indicates the complexity of the reactions; in the Arabidopsis genome, 20 MAPKs have been identified, with 10 MAPK kinases and 60 MAPK kinase kinases putatively identified (Ichimura *et al.*, 2002). Via these and other proteins, the signal is transduced to the nucleus. Here, extensive transcriptional reprogramming facilitated by an array of transcription factor families (Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Alves *et al.*, 2013; Buscaill and Rivas, 2014) and chromatin remodelling (Ma *et al.*, 2011) confers resistance. This PAMP perception initiates PAMP-triggered immunity (PTI in A-line, Figure 1.6).

The PTI response is the plant's first active response to microbial attack. This immunity is termed PAMP-, pathogen- or pattern- triggered Immunity (PTI), and is reviewed in detail by Bigeard *et al.* (2015).

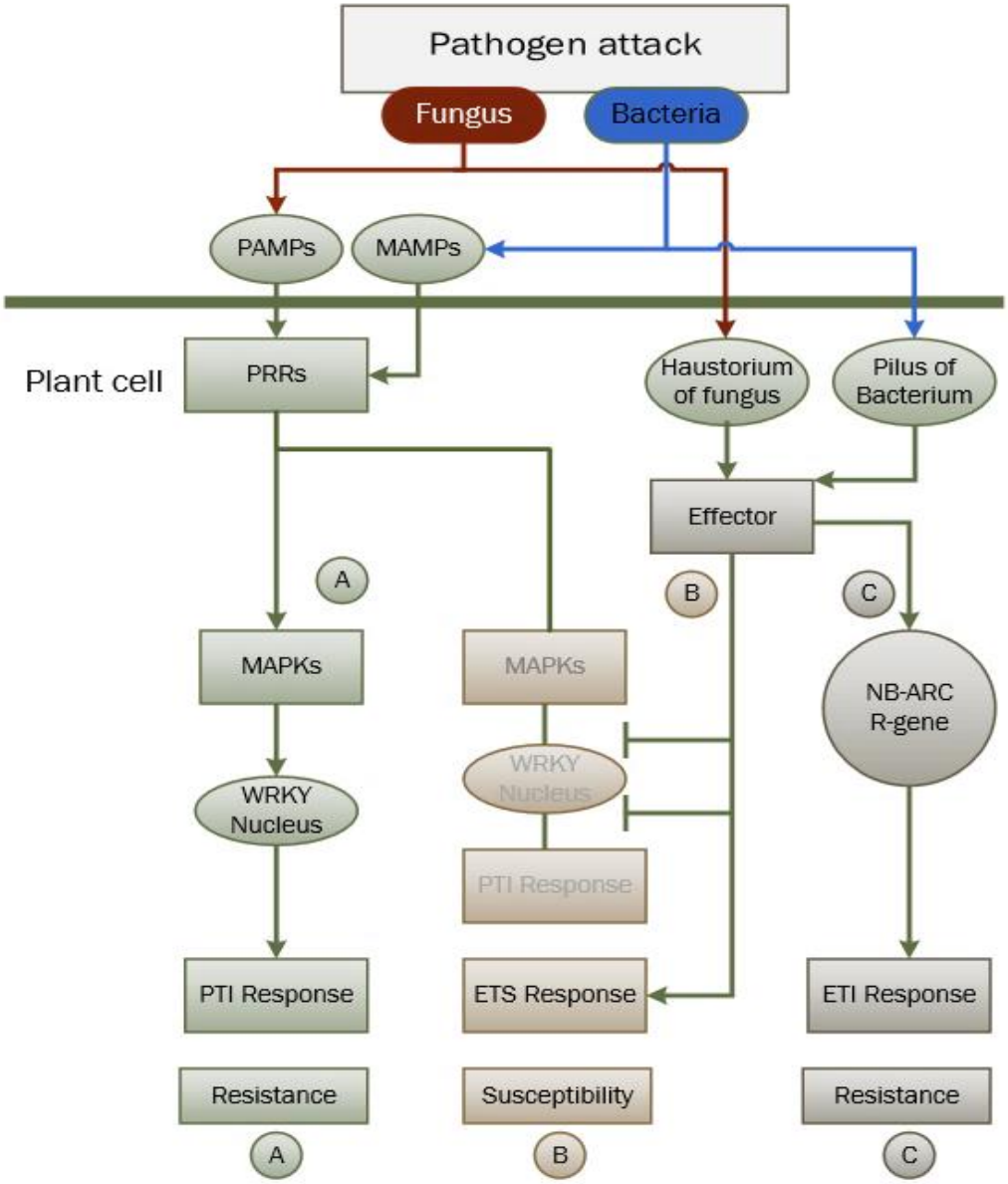


Figure 1.6. Model for plant immunity. Pathogens release pathogen-associated molecular patterns (PAMPs and MAMPs) that are perceived by host pattern recognition receptors (PRRs). Recognition initiates signalling via mitogen-activated protein kinases (MAPKs) thereby inducing PAMP or MAMP-triggered immunity (PTI, indicated by A line). Host-targeted effectors are able to suppress PTI leading to effector-triggered susceptibility (ETS, indicated by B line). Some effectors are recognised by effector-triggered immunity (ETI, indicated by C line) via plant resistance proteins of the NBS-LRR class. Adapted from Chisholm *et al.* (2006) and Dodds and Rathjen (2010).

Only very few, often highly specialised, microbes are able to suppress PTI via the secretion of effector molecules into the host cell that interferes with immune signalling and suppress the plant's response, leading to effector-triggered susceptibility (ETS) (B-line, Figure 1.6). Evolution of the ability to detect these effectors leads to the second immune phase, called effector-triggered immunity (ETI), involving plant resistance (R) proteins commonly of the nucleotide binding NBS-LRR class (C-line, Figure 1.6) (Jones and Dangl, 2006). R gene-activated ETI involves a complex defence program. Major components are production of

reactive oxygen species (ROS) associated with rapid cell programmed death (HR), salicylic acid (SA) production, and induction of expression of many host genes including those coding for pathogenesis-related (PR) proteins (Jones and Dangl, 2006; Oliver and Solomon, 2010).

Another type of resistance is systemic acquired resistance (SAR), in which defence proteins accumulate systemically in uninfected tissues. SAR can provide long-lasting defence against a broad range of pathogens, and has some similarities to induced systemic resistance (ISR) (Gao *et al.*, 2015). When we are able to understand the mechanisms by which a plant may improve its resistance to the fungus, it may be possible to assist wheat in the ongoing arms race that occurs in the evolution of plants and pathogens. Factors which have been shown a role to increase the expression of defence-related genes have been shown to down-regulate those associated with growth (Davidsson *et al.*, 2017). Therefore, in understanding the context of this research, it is necessary to understand what is already known about the plant defence system, and which genes may be essential in developing our understanding of this generally, and specifically in the case of resistance to spot blotch.

1.10 Abiotic stress

Abiotic stress has a significant impact on plant growth and consequently is responsible for severe losses in the field. The various environmental stresses such as high winds, extreme temperatures, soil nutrient imbalance, soil salinity, drought and flood affect the production and cultivation of crops. The resulting growth reductions represent the most significant constraints for crop production worldwide reducing yield by as much as 70%. Such factors will play a significant role in increasing crop productivity and hence improving food security. Furthermore, many crops perform only at 30% of their genetic potential under abiotic stress conditions (Wang *et al.*, 2003). Most plants grown under field conditions are often exposed to various types of abiotic stresses. Such stresses commonly result in accelerated production of reactive oxygen species (ROS), which leads to increase of oxidative stress. In nature, plants may never be completely free from abiotic challenges (Hasanuzzaman *et al.*, 2012). The reliance of food production on the use of mineral nitrogen fertilisers is of concern because their manufacture and use is associated with the use of high levels of energy and greenhouse gas emissions (e.g. CO_2 and N_2O), as well as eutrophication of freshwater and marine ecosystems (Shahbaz and Ashraf, 2013; Tetard-Jones *et al.*, 2013). This is a specific apprehension in agriculture where stress-related changes reduce productivity and can lead to extreme economic loss to the farmers.

Current policies encourage increased agricultural production at the expense of ecosystem services. Interestingly, many studies indicate that fertiliser-use efficiency could be significantly improved by improving the match between nutrient inputs and crop requirements (Matson *et al*., 1998), but more investments in research on nutrient management and promotion of such practices are needed. There is also potential for significant increases in fertiliser efficiency in small-scale intensive rice cropping systems in Asia (Tilman *et al*., 2002).

1.11 Nitrogen availability and plant response

Aside from the many extensively studied side effects that large inputs of nitrogen can have (Byrnes, 1990; Tilman *et al.*, 2002; Masclaux-Daubresse *et al.*, 2010), there is increasing evidence that this input may have an adverse effect within the plant itself (Tetard-Jones *et al.*, 2013). Requirements for mineral elements (Especially N) change during the growth and development of the plant. As such, all plants need a substantial amount of nitrogen in order to grow and seed production and yield are correlated with nitrogen input. Nitrogen fertilisation is considered to be required to achieve high yield in wheat; increasing sustainability through a reduction in nitrogen input is not commercially credible due to reducing yield (Hinzman *et al.*, 1986; DEFRA, 2010).

In plants, nitrogen is required for the synthesis of amino acids, proteins, nucleic acids, chlorophyll, and enzymes required for photosynthesis (Binkley *et al.*, 2000). Thus, the photosynthetic capacity of leaves is related to their N content (Evans, 1989). Photosynthesis is essential for plants to convert light energy into chemical energy in the form of carbohydrate for growth and defence. However, there are trade-offs between growth and defence which have profound implications for agricultural productivity. The molecular mechanisms underlying these trade-offs are not fully understood but are thought to be mediated via hormone cross-talk. As deficiencies in defence capabilities can result in pathogen-induced decimation of the plant population, a balance must be achieved between growth and defence to optimise plant fitness. The principle of the Growth vs Defence hypothesis is relatively simple: a plant has a limited amount of energy available to it, which can be used either in growth of the plant, or towards ensuring it is well protected in the case of any expected by attack by pest or pathogen (Huot *et al.*, 2014).

As stated previously, nitrogen use globally is approximately 200 million tonnes of fertiliser per year (FAO, 2015). In the UK nitrogen fertiliser application is typically 160–280 $kg\ N\ ha^{-1}$ (EFMA, 2007; DEFRA, 2010). Therefore, there is an essential need for either a reduction in input of mineral fertilisers or a complete replacement with alternative fertilisers (Tétard-Jones

et al., 2013). Nitrogen fertiliser is one of the most expensive inputs, but, yield responses can be large. For the profitable production of wheat, it is important to assessment N requirements in relation to both crop performance and the intended market. The source of nitrogen is not critical for wheat (Figure 1.7) (HGCA, 2009), provided other nutrients are sufficient and lodging, serious weeds and diseases are controlled. The increase of nitrogen input to 100 kg/ha in soil might enhance grain yield to 0.2 – 0.3 t/ha, but the cost of additional nitrogen equates to only additional 0.5 t/ha of grain. The gap between actual yield and attainable yield can be closed by optimising growing conditions, eliminating the production-limiting effect of the resource in minimum supply (Bos *et al.*, 2013).

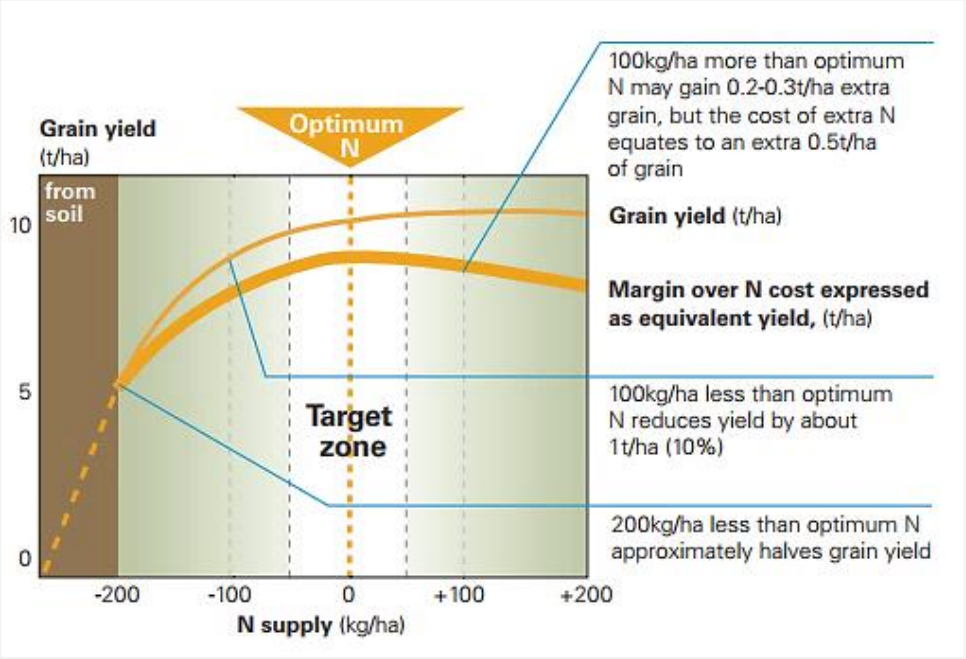


Figure 1.7. Wheat response to nitrogen supply. Source (HGCA, 2009).

Nitrogen availability has been extensively studied in relation to the severity of plant diseases for many years, particularly the undesirable side-effect of nitrogen fertilisation. It is believed that plants are better defended against pathogens when they are grown under conditions of low N availability because there is an increase in the synthesis of defence-related compounds (Hoffland *et al.*, 2000). High nitrogen concentrations extremely increase the proportion of disease symptoms, which is often attributed to the specific forms of nitrogen available to the pathogen (Lin *et al.*, 2016). Although N is indispensable for plant growth and influences disease development, reports of effects of N on disease development can be contradictory, and the causes of these inconsistencies are poorly understood (Hoffland *et al.*, 2000). Also, there is a lack of thorough studies of how N supply affects disease resistance and biocontrol agents' activity (Tziros *et al.*, 2006).

It has been reported that high soil nitrogen can enhance the development of fungal pathogens that cause wheat leaf blotch diseases (Simon *et al.*, 2003; Loyce *et al.*, 2008). High crop densities and high canopy densities resulting from N fertilisation can create a favourable microclimate for stripe rust development (Devadas *et al.*, 2014). The effect of N on stripe rust may also be related to increases in N content of host tissue (Savary *et al.*, 1995). However, the mechanisms leading to these nutrient-induced changes in disease development are not known. If we can understand the mechanisms which reduce spot blotch severity when wheat is grown under reduced nitrogen conditions, it may be possible to engineer wheat such that resistance to the fungus is improved while at the same time producing plants of commercially viable productivity.

1.12 WRKY transcription factor genes and their role in plant defence

1.12.1 WRKY transcription factors

WRKY transcription factors (TF) are one of the largest families of transcriptional regulators find in plants. They are key parts of signalling networks in many plant processes (Rushton *et al.*, 2010). They are characterised by a highly conserved 60 amino acid for WRKY domain, which contains a conserved amino acid sequence motif of WRKYGQK at the N-terminus and a C_2H_2 or C_2HC zinc finger-like motif at the C-terminus (Eulgem *et al.*, 2000). The WRKYGQK motif may have slight modifications such as WRKYGEK or WRKYGKK (Wang *et al.*, 2013). The WRKY factors demonstrate high binding affinity to DNA cis-acting element named the W box (C/T) TGAC (T/C) (Mingyu *et al.*, 2012) or may bind the WK box (TTGAC (C/T) (Yang *et al.*, 2009; Rushton *et al.*, 2010). This permits signal transduction to regulate the expression of genes that increase plant tolerance to stress (Yang *et al.*, 2009). The WRKY domain binds the W box in the promoter of the target gene to modulate transcription to regulate gene expression. This sequence does not show specificity towards individual WRKY proteins, so that it is not possible to find targets of specific WRKY proteins by looking at the promoter regions of defence related genes. The exact mechanisms which confer specificity are unclear, although it is known that bases immediately outside the W-box play a role in this (Ciolkowski *et al.*, 2008). WRKY proteins are divided into three groups based on the number of WRKY domains and type of zinc finger motif. The first group has two WRKY domains. Groups II and III have a single WRKY domain but different types of zinc finger motif (Yang *et al.*, 2009). Groups I and II share a C_2H_2 zinc finger motif ($C–X_{4–5}–C–X_{22–23}–H–X_1–H$), whereas group III contains a C_2-HC-type ($C–X_7–C–X_{23}–H–X_1–C$) motif (Eulgem *et al.*, 2000).

The number of WRKY genes in each plant species is different. The primitive green algae *C. reinhardtii* contains just one WRKY gene, while the model plant *Arabidopsis thaliana* contains 76 in the GenBank database (www.ncbi.nlm.nih.gov/genbank). The total number of WRKY genes found in *Brachypodium distachyon* is 86, in maize (*Zea mays*) is 124, in rice *(Oryza sativa*) is 126 and in *Sorghum bicolor* is 93 (Wen *et al.*, 2014). The species which contains the most confirmed members of this family is soybean (*Glycine max*), with 133 WRKY genes (Yin *et al.* 2013). In barley 94 WRKY genes have been identified (Liu *et al.*, 2014). Due to the incomplete annotation of the wheat genome, it is not known precisely how many WRKY genes there are in wheat; only 96 sequences are recorded in the GenBank database. However, predictive models suggest more may be present by *Okay et al.* (2014) used a Hidden Markov Model to predict up to 160 WRKY genes may be present in wheat, and more recently 171 wheat WRKY genes have been identified from whole genome sequencing (Ning *et al.*, 2017; Gao *et al.*, 2018).

1.12.2 Role of WRKY transcription factors in tolerance/defence

The involvement of WRKY proteins in the response to both biotic and abiotic stressors in different plant species has been well documented (Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Niu *et al.*, 2012; Zhu *et al.*, 2013; Wen *et al.*, 2014; Zhang *et al.*, 2015). WRKY transcription factors have been shown to be variously expressed in response to a wide range of plant stressors, and a broad range of species. Abiotic stresses, including drought, heat, cold and salinity, cause the up or down- regulation of WRKY genes in a range of species (Qin *et al.*, 2013; Wang *et al.*, 2013; Okay *et al.*, 2014). Changes in expression of WRKY genes are also seen in response to biotic stresses, including in response to bacterial infections such as *Agrobacterium* (Ding *et al.*, 2014), and fungal diseases such as rice blast and powdery mildew (Mangelsen *et al.*, 2008).

Some downstream targets of WRKY proteins have been classified as “defence-related genes” for over a decade based on their ability to affect the susceptibility of Arabidopsis to a variety of pathogens (Eulgem and Somssich, 2007; Jiang *et al.*, 2015); recently, their precise downstream targets have begun to be elucidated in model plants (Tripathi *et al.*, 2014; Birkenbihl *et al.*, 2017). Transcription factors with specific DNA-binding domains (DBD) combine with specific DNA sequences to activate or suppress transcription of downstream genes. Using transcription factors to improve plants' ability to withstand biotic and abiotic challenges is a promising strategy due to their ability to regulate sets of genes (Chi *et al.*, 2013). Downstream targets have included ethylene response factors (ERF), jasmonic acid (JA) and salicylic acid (SA) mediated signalling components and other WRKY transcription factors,

which supports the hypothesis of their involvement in molecular defence responses and the ability to crosstalk to one another. The WRKY family plays an crucial role in regulating the biofuel, biosynthesis of important pharmaceutical, aromatherapy, and industrial components, making them of interest beyond plant defence responses (Schluttenhofer and Yuan, 2015). They are also involved in several developmental and physiological processes such as embryogenesis, seed coat and trichome development, anthocyanin biosynthesis, and hormone signalling (Rushton *et al.*, 2010).

Studies have suggested that WRKY transcription factors are central components of many aspects of the innate immune system of the plant, including PAMP-triggered immunity (PTI) and MAMP-triggered immunity (MTI), effector-triggered immunity (ETI), basal defence (BD) and systemic acquired resistance (SAR) (Eulgem and Somssich, 2007; Rushton *et al.*, 2010).

Because of the lack of understanding of the roles of WRKY proteins in wheat, Arabidopsis has been intensively used as a model plant through comparisons between wheat *TaWRKY* and Arabidopsis *AtWRKY* genes (Appendix A). The Arabidopsis *AtWRKY* proteins are much better characterised, including some functional descriptions of their roles in biotic and abiotic defence. There are complete numbered sequences for 66 of the proposed 74 WRKY proteins with experimental validation of their existence in Arabidopsis among 683. Whilst *Brachypodium* has 84 entries, but all are predicted proteins without functional annotation or even complete nomenclature by using GeneBank records.

The roles that specific WRKY proteins play at the molecular level remains to be elucidated, but their capacity for coordinating a wide range of stress responses is evident. In Arabidopsis, *WRKY70* acts at a convergence point determining the balance between salicylic acid (SA) and jasmonic acid (JA) dependent defence pathways as well as being required for R gene-mediated resistance (Li *et al.*, 2016). SA mediated signalling is usually active in response to biotrophic pathogens, whereas JA mediated signalling is closely associated with necrotrophic pathogen attack, and the two act antagonistically to one another (Tamaoki *et al.*, 2013). Although this link appears to be less clear cut in monocotyledonous plants, there is a clear potential for WRKY proteins to act as crucial signalling molecules in mediating the plant defence response to pathogens via these pathways. *AtWRKY3* and *AtWRKY4* induce resistance to necrotrophic pathogens, whilst overexpression of *AtWRKY4* caused increased susceptibility to the biotrophic bacterium *Pseudomonas syringae* and suppressed pathogen-induced *PR1* gene expression. This finding demonstrates that a single WRKY protein can negatively and positively regulate the plant's response to various pathogens (Lai *et al.*, 2008). WRKY TF genes are also involved in

other stress responses such as nutrient stress, dark treatment and UV radiation (Devaiah *et al.*, 2007). *AtWRKY75*, *AtWRKY6* and *AtWRKY42* transcription factors influence the ability of Arabidopsis to take up phosphate (Pi). *AtWRKY75* was induced strongly during Pi deficiency, and suppression of its expression in mutant plants increased susceptibility to Pi stress and decreased Pi uptake (Devaiah *et al.*, 2007).

When the seedlings of the monocot *Brachypodium distachyon* were sprayed with either *Fusarium graminearum* or two different strains of *Magnaporthe grisea*, there was a rapid up-regulation of at least 15 *BdWRKY* genes and there was greater correlation between promoter cis-elements and phytohormone-induced *BdWRKY* gene expression (Wen *et al.*, 2014). Furthermore, 60% and 80% of *BdWRKY* genes were up-regulated under heat and cold stress conditions, respectively, and almost 50% of the *BdWRKY* genes were down-regulated under three or more stress conditions. Treatment with ABA down-regulated most *BdWRKY* genes, and expression of three and eight *BdWRKY* genes increased in response to MeJA and SA treatments, respectively (Jiang *et al.*, 2017).

In wheat, silencing of *TaWRKY49* led to enhanced HTSP (high-temperature seedling-plant) resistance to *Puccinia striiformis*. In contrast, silencing of *TaWRKY62* reduced this type of resistance. These results suggest that *TaWRKY49* has negative regulatory role and *TaWRKY62* has a positive regulatory role in HTSP resistance (Wang *et al*., 2017). The function of *TaWRKY68* appears to differ during different plant developmental stages and it is possible that it functions as a hub gene in wheat in response to *Agrobacterium tumefaciens* and *Blumeria graminis* infection (Ding *et al.*, 2014). Furthermore, there is evidence to suggest that manipulation of *TaWRKY2* and *TaWRKY19* in wheat should improve their performance in various abiotic stress conditions (Niu *et al.*, 2012). Drought stress in wheat caused differential expression of *TaWRKY16*, *TaWRKY24*, *TaWRKY59*, *TaWRKY61* and *TaWRKY82* genes (Okay *et al.*, 2014).

The nature of the regulatory processes that WRKY proteins control remains a challenging question. Gene expression studies may help to provide answers. Because many WRKY genes are themselves transcriptionally regulated, their expression patterns under specific conditions might help to define the regulatory functions of their products. However, to fully understand their biological roles, it will be necessary to identify the target genes whose expression is affected by infection and reduced nitrogen interactions.

1.13 Research Rationale:

Bipolaris sorokiniana is a serious hemibiotrophic pathogen of wheat growing in warm and humid conditions (Chowdhury *et al.*, 2013). Abiotic stresses, especially nitrogen (N) deficiency is a major limiting factor for plant growth, development, and agricultural productivity (HGCA, 2009). Wheat is one of the major cereals that have been used for human food and livestock feed for more than 5000 years (Peng *et al.*, 2011). It is believed that plants are better defended against pathogens when they are grown under conditions of low N availability because there is an increase in the synthesis of defence-related compounds (Hoffland *et al.*, 2000). For example, Loyce *et al.* (2008) demonstrated a link between nitrogen input and Septoria blotch disease in the field. Studies have suggested that WRKY transcription factors are central to plant defence in model systems. To better understand the molecular response of wheat to pathogen attack the differential expression of WRKY genes was investigated over time after infection. TILLING lines with mutations in selected WRKY genes were used to demonstrate 'proof of concept'. TILLING lines also find application in crop improvement, as the mutants identified by TILLING can be readily utilised in conventional breeding programs since they are non-transgenic and the novel variations can be inherited stably (Henikoff *et al.*, 2004; Uauy *et al.*, 2009). *Micromonospora luteifusca* is an actinobacterial genus which is widely distributed in nature. Some *Micromonospora* strains can promote plant growth (Martínez-Hidalgo *et al.*, 2014; Solans *et al.*, 2015), and *Micromonospora* has been reported to have antifungal activity (Martínez-Hidalgo *et al.*, 2015). The potential of this bacterium to act as a biocontrol agent against *B. sorokiniana* was also studied.

1.14 Hypotheses:

1. The growth of wheat and *Brachypodium* is affected by nitrogen availability.
2. Regulation of disease resistance in wheat by WRKY TFs is affected by nitrogen availability and this differs between resistant and susceptible cultivars
3. TILLING lines in which the WRKYGQK amino acid sequence are mutated confer different levels of resistance under different nitrogen levels in the wheat cultivar Cadenza.
4. *Micromonospora luteifusca* enhances the resistance of wheat to spot blotch.

1.15 The aims of this project:

The over-arching aim of the project is to gain the best understanding of the response of wheat to the pathogen *Bipolaris sorokiniana* in susceptible and resistant Iraqi wheat cultivars and to establish whether there is a link between nitrogen availability and disease symptoms through the investigation of WRKY transcription factors.

The specific objectives are to:

1. Determine how the growth of wheat and Brachypodium is affected by nitrogen availability. For these studies, three different regimes were used representing low, intermediate and optimal nitrogen levels (0.75 mM, 3.75 mM and 7.5 mM nitrate).
2. Identify the effects of different levels of nitrogen and pathogen infection on expression of defence genes in two Iraqi wheat cultivars and *Brachypodium distachyon* as a model system for disease defence, using qRT-PCR.
3. Investigate the effects of *Bipolaris sorokiniana* infection in physiological parameters and expression of WRKY TFs in two Iraqi wheat cultivars over time.
4. Investigate the response of wheat TILLING lines (in which the WRKYGQK amino acid sequence is mutated) to spot blotch infection when grown under different nitrogen levels.
5. Investigate the effect of treatment with *Micromonospora luteifusca* (root bacteria) on subsequent susceptibility of wheat cultivar to spot blotch.

2 Materials and methods

2.1 Plant materials

Seeds of Iraqi wheat cultivars were obtained from the population maintained by the Ayad Al-Maini and Hamza Al-Khafaji lab at the College of Agriculture in Al-Qasim Green University. These wheat cultivars were originally obtained from seed certified in Baghdad/Iraq.

A community standard diploid inbred line of *Brachypodium distachyon* (Bd21), was obtained from Richard Sibout and Sébastien Antelme (INRA Science and Impact, France).

Seeds of Cadenza (wild-type) and TILLING lines were provided by Ed Byrne. Cordiale seed was obtained from KWS (Table 2.1).

Table 2.1. Names and origin of wheat cultivars and Brachypodium used in this study.

	Name	Status	Origin
1	Rashid	Spring	Iraq
2	Latifia	Spring	Iraq
3	Aurok	Spring	Iraq
4	Tamoz3	Spring	Iraq
5	Iraq	Spring	Iraq
6	Cordiale	Winter	UK
7	Cadenza	Winter	UK
8	*Brachypodium distachyon* (Bd21)	-	France

2.2 Seed sterilisation and germination

Seeds were sterilised with 3% calcium hypochlorite (CaClO) for 15 min with shaking, washed three times with distilled water, then seed soaked in distilled water for 10 h and left in the dark at room temperature. Fifteen seeds were placed on two wet filter papers stacked on top of each petri dish. The petri dishes were sealed with Parafilm. For wheat, petri dishes were placed in the controlled temperature room (25 °C) for 48 h. Brachypodium vernalisation was conducted by incubating imbibed seeds at 4 °C for the required amount of time, in darkness (Verelst *et al.*, 2013; Tyler *et al.*, 2014), Seeds were then kept at 25 ^{o}C for 72 h for germination.

2.3 Plant growth conditions

Germinated seeds were transferred to silver sand or soil (John Innes No. 2 soil-based compost). They were grown in the growth room with 25/20 °C day/night temperature and 16 h light/8 h

darkness photoperiod, above 60% relative humidity and photon flux density of 200 µmol $m^{-2}s^{-1}$). Plants were provided with nutrients via modified Hoagland's solution. Nitrogen control plants grown with the standard level (7.5 mM-NO_3^-) were supplied with 2.5 mM $Ca(NO_3)_2.4H_2O$, 2.5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$, 23 µM H_3BO_3, 4.6 µM $MnCl_2.4H_2O$, 0.38 µM $ZnSO_4.7H_2O$, 0.10 µM $CuSO_4.5H_2O$, 0.27 µM $NaMoO_4.2H_2O$, 14 µM FeEDTA. Moderately nitrogen stressed plants (50% of standard control, 3.75 mM nitrogen) were supplied with 1.25 mM $Ca(NO_3)_2.4H_2O$, 1.25 mM $CaCl_2$, 1.25 mM KNO_3, 1.25 mM K_2SO_4 with all other nutrients the same as the control. Severely nitrogen stressed plants (10% of optimum, 0.75 mM) were supplied with 0.25 mM $Ca(NO_3)_2.4H_2O$, 2.25 mM $CaCl_2$, 0.25 mM KNO_3, 2.25 mM K_2SO_4 with all other nutrients the same as the optimum control.

2.4 Preparation of spot blotch inoculum

Bipolaris sorokiniana was grown on yeast extract-corn starch agar-YECSA (1.0 g/l KH_2HPO_4, 0.5 g/l $MgSO_4.H_2O$, 4.0 g/l Difco yeast extract, 15.0 g/l corn starch and 20 g/l agar). The YECSA was autoclaved at 121 °C and 15 psi for 20 min, then poured into petri dishes at approximately 50 °C. The cultures were inoculated by a bulk from three weeks old media contained spot blotch conidia, then incubated at 28 °C with fluorescent bulbs emitting 260 to 280 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 12 h photoperiod for 14 days (Fetch and Steffenson, 1999). Conidia were harvested 14 d after incubation by flooding the petri dishes with 10 ml of sterilised distilled water (SDW). The resulting conidial suspension was filtered through two layers of cheesecloth to remove mycelial fragments. Spore concentrations were measured with a haemocytometer with 10× objective magnification.

2.5 Preparation biocontrol bacteria cells

Two bacterial strains were used in this study. *Micromonospora luteifusca* strain CR21 (NCBI accession No: FN659848.1), isolated from the rhizosphere of *Pisum sativum* (Carro *et al.*, 2016), *Micromonospora* was grown in GYM *Streptomyces* medium (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium65.pdf) containing 4.0 g glucose, 4.0 g yeast extract, 10.0 g malt extract and 2.0 g $CaCO_3$. The pH was adjusted to 7.2 then added 18.0 g agar and deionised water up to 1 litre were added. The solution was autoclaved using a standard sterilisation cycle (121 °C and 15 psi for 20 min). After autoclaving, the medium was poured into petri dishes at 50 °C. The cultures were inoculated with *Micromonospora* obtained from cultures grown in GYM, then incubated at 28 °C (Zhao *et al.*, 2017). Cells were harvested after 7 days incubation by flooding the petri dishes with 1 ml of

sterile 0.8% NaCl (Bennett *et al.*, 2018). The resulting cell suspension was diluted until an OD_{600} of 0.8 was obtained. *Pseudomonas aeruginosa* 7NSK2 was provided by Catherine Te´tard-Jones (Newcastle University). Pseudomonas cells were grown aerobically on solid Luria-Bertani medium for 24 h at 30°C. Colonies were harvested from the plates with 3 ml 10 mM magnesium chloride ($MgCl_2$) and gentle agitation with glass beads. The bacterial suspension was washed twice with 10 mM $MgCl_2$ and resuspended at OD_{600} of 1, using 10 mM $MgCl_2$ as a diluent (Tetard-Jones *et al.*, 2007).

2.6 Spot blotch infection

2.6.1 Iraqi and Cordiale wheat cultivars

Five Iraqi and Cordiale (UK) wheat cultivars as shown in (Table 2.1) were grown in 12 cm diameter pots containing John Innes No.2 soil-based compost. The inoculum concentration was adjusted to 6×10^4 conidia ml^{-1} using a haemocytometer. Just before plants inoculation, Tween20 was added to a final concentration of 0.1% as a surfactant. To infect wheat plants, two inoculation methods were used. (i) Plants were sprayed at 36 d after emergence (Feekes growth stage 9 "Ligule of the last leaf"; Zadoks growth stage 39). (ii). The second leaf was brushed (soft bristle brush) at Feekes stage 10.1 "Ligule of last leaf ", Zadoks stage 50 (Zadoks *et al.*, 1974). The inoculated plants were covered by plastic bags for 24 h in the growth room conditions. A complete randomised design (CRD) with three replicates was used to compare treatments statistically. Disease severity was recorded 3, 6 and 9 days after infection based on the size of necrotic and chlorotic lesions observed on infected plants, by using standard area diagrams (Figure 2.1) with a scale of 1 to 100 (Domiciano *et al.*, 2014).

2.6.2 Infection of Rashid and Latifia wheat cultivars

Two Iraqi wheat cultivars (Rashid and Latifia) were selected from the previous experiment based on susceptibility to spot blotch. Seedlings of each genotype were grown in 10 cm diameter black plastic pots, with three different levels of nitrogen in Hoagland's solution (7.5 mM, 3.75 mM, and 0.75 mM-NO_3^-). Plants were grown under growth room conditions in sterilised silver sand that autoclaved using a standard sterilisation cycle (121°C and 15 psi for 20 min). The pots were placed in the container containing nutrient solution.

Plants were treated with 3×10^4 *Bipolaris sorokiniana* conidia ml^{-1}. Tween 20 was added to 0.1% as surfactant just before inoculation. The conidial suspension was painted with a soft bristle brush on wheat leaves (one leaf per plant) at 4 leaf stage (Zadoks growth stage 14) (Zadoks *et*

al., 1974). Control treatment with sterilised deionised water with Tween 20 was used for comparison.

The experiment used a factorial experiment design for two factors (two wheat cultivars and three levels of nitrogen) in complete randomised design (CRD) with six replicates for inoculated plants and four replicates for control.

Disease severity was recorded in infected Rashid and Latifia wheat plants at 4 days after infection by using standard area diagrams with a scale of 1 to 100 (Domiciano *et al.*, 2014) see Figure 2.1.

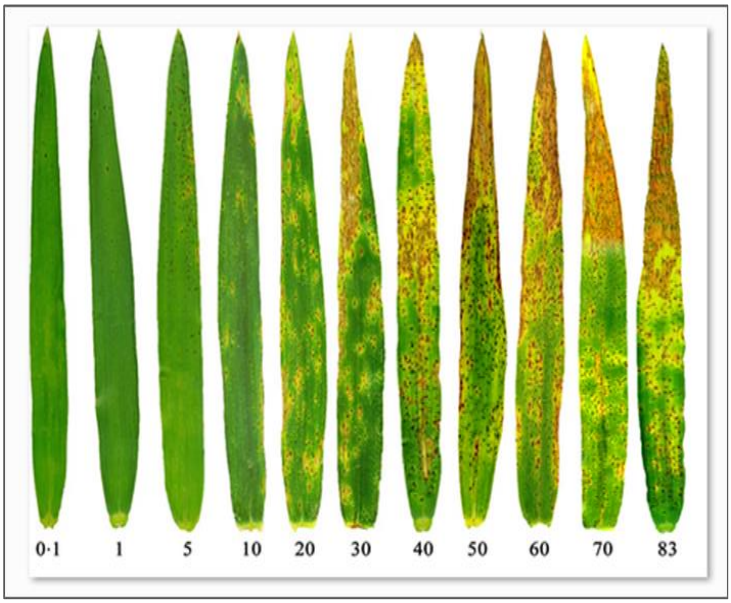


Figure 2.1. Standard area diagrams to assess spot blotch severity on wheat leaves. The numbers represent the percentage (%) of leaf area showing symptoms (necrosis and chlorosis) of spot blotch. source (Domiciano *et al.*, 2014).

To measure sporulation, 10 cm leaf length for four leaves per treatment were harvested four days after infection and incubated in petri dishes (15×1.5 cm) containing wet filter paper and sealed by parafilm to obtain high humidity (Figure 2.2). Then, each leaf was placed in a 50 ml Falcon tube containing 5 ml SDW. The tube was mixed with a vortex mixer for 10 min and centrifuged for 10 min at 5000×*g*. Then the supernatant was discarded and the spore pellet was resuspended in 1 ml SDW. The concentration of spores was measured using a haemocytometer.

Leaf area index measured by the equation:

Leaf area index (LAI) = Leaf length × Max. Leaf width× β

β=0.75 (Chanda and Singh, 2002; Aldesuquy *et al.*, 2014).

Chlorophyll content index (CCI) was measured by OPTI-SCIENCES- CCM-200 machine as the mean of three readings for each leaf.

Shoot height was measured for plants from the sand surface level to the top of the last leaf (cm). Also, number of leaves and number of tillers were measured in this study for each plant.

2.6.3 *Brachypodium distachyon* infection

After three days of incubation in petri dishes, germinated seeds of Brachypodium were transferred to silver sand with one seedling per 9 cm diameter black plastic pot, with three different levels of nitrogen (0.75 mM, 3.75 mM and 7.5 mM NO_3^- in Hoagland's solution).

Plants were treated with 3×10^4 conidia ml^{-1}. Tween 20 was added to 0.1% as surfactant just before inoculation. The conidial suspension and control treatment (Tween 20) were sprayed on all Brachypodium leaves at 27 days old. The inoculated and control plants were covered by plastic bags with deionised water sprayed three times during 24 h in the growth room conditions. Disease severity was recorded at four days post-infection, using standard area diagrams as described in section 2.6.2 (Figure 2.1). The experiment used a complete randomised design-CRD (two factors: three levels of nitrogen and two infection states) with nine replicates for each treatment.

2.6.4 Time course of infection

Seeds of two Iraqi wheat cultivars (Rashid and Latifia) were sterilised, germinated and transferred to 9 cm plastic pots containing sterilised silver sand (autoclaved using a standard sterilisation cycle, 121 °C and 15 psi for 20 min) and placed in a container containing Hoagland's solution with 7.5 mM nitrate. Plants grown in the growth room were treated with a conidial suspension containing 3×10^4 conidia ml^{-1}. Tween 20 was added to 0.1% as surfactant just before inoculation. The conidial suspension was painted with a soft bristle brush (one leaf per plant) at the four-leaf stage, Zadoks: stage 14 (Zadoks *et al.*, 1974). Furthermore, control treatment (sterilised water with Tween 20) was used for comparison. The inoculated and control plants were covered by plastic bags and were sprayed with sterilised distilled water every three hours after infection for 24 h in a growth chamber at 25/20 °C day/night under photoperiod (16 h light/8 h night).

To measure numbers of spores produced over time (0 h, 24 h, 48 h, 72 h and 96 h after infection), 10 cm lengths of inoculated leaves were placed in petri dishes containing wet filter paper, as explained in session 2.6.2. For the 0 h point, 10 cm lengths of leaves were placed in the petri dishes containing wet filter paper directly after infection.

Chlorophyll content index was measured at 0 h, 24 h, 48 h, 72 h and 96 h after infection, as described in the section 2.6.2.

Microscopic analysis of samples was carried out and chitin content was measured at 0 h, 24 h, 48 h, 72 h and 96 h after infection, as described in sections 2.21 and 2.20, respectively.

The experiment used a complete randomised design-CRD for two factors (two wheat cultivars and time after infection – 0 h, 24 h, 48 h, 72 h and 96 h). Results were analysed via ANOVA with Tukey tests ($P < 0.05$) used for comparing treatments statistically.

2.6.5 Infection of wheat TILLING lines

Seeds of Cadenza (wild-type) and TILLING lines (details given in Chapter 6) were kindly provided by Ed Byrne, KW Seeds (Ed.Byrne@kws.com). Seeds were sterilised, germinated and transferred to 10 cm black plastic pots containing silver sand. Seedlings were grown with three different levels of nitrogen in Hoagland's liquid medium (0.75 mM, 3.75 mM and 7.5 mM NO_3^-) with six replicates for each treatment in the growth room conditions as described in section 2.3.

Four leaf stage of wheat (Zadoks: stage 14) (Zadoks *et al.*, 1974) were brushed with 3×10^3 *Bipolaris sorokiniana* conidia ml^{-1}, Tween 20 was added to 0.1% as surfactant just before inoculation. After four days (three weeks old plants) the following were measured:

Disease severity was recorded in infected leaves by using standard area diagrams with a scale of 1 to 100 (Domiciano *et al.*, 2014) Figure 2.1.

Numbers of spores were measured in incubated leaves as explained in section 2.6.2 (see Figure 2.2).

Chitin content was measured as described in section 2.20.

Microscopic analysis of infected leaves was carried out as described in section 2.21.

The experiment used two factors (four wheats and three levels of nitrogen) in a complete randomised design-CRD. Results were analysed via ANOVA with Tukey tests ($P < 0.05$) used for comparing treatments statistically.

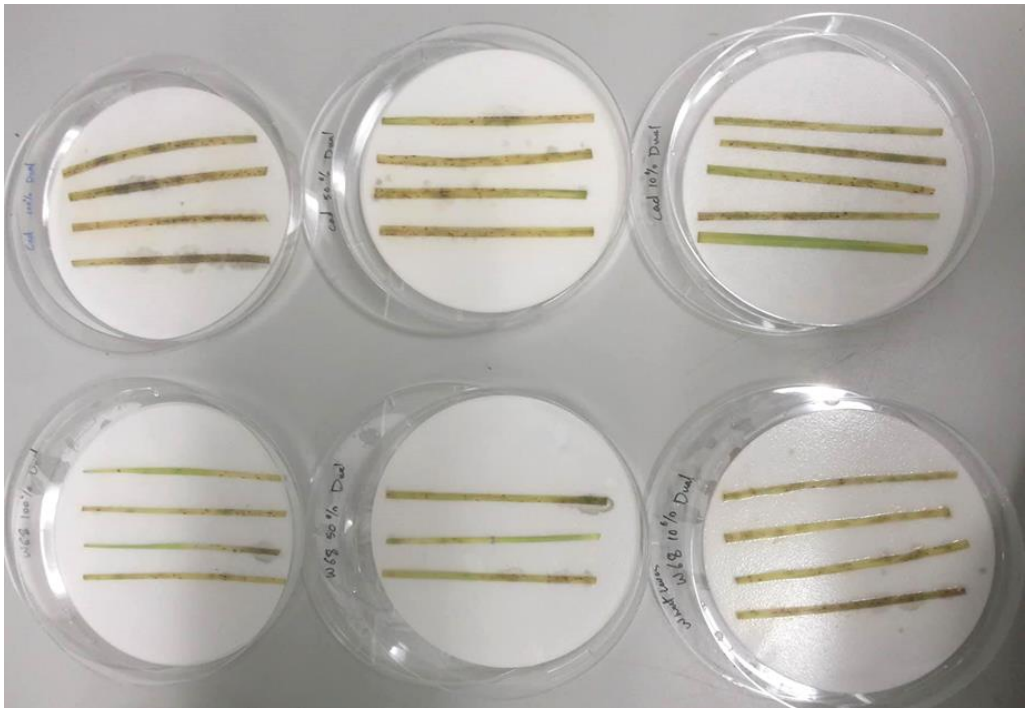


Figure 2.2. Infected leaves incubated in high humidity for four days after four days post-infection on the plants to count numbers of spores in Cadenza and *WRKY68a* mutant.

2.6.6 Wheat inoculation with *Micromonospora* and spot blotch infection

Seeds of three wheat cultivars (Rashid and Latifia from Iraq and Cordiale from KWS –UK-Ltd.) were germinated in sterile conditions. Seedlings were transferred to 10 cm pots containing a moistened 1:1 (v:v) mixture of sand and standard size (2-5 mm) Perlite that had been autoclaved (using a standard sterilisation cycle, 121 °C and 15 psi for 20 min) three times (with 24 h in between). The pots were placed in a container containing sterilised modified Hoagland's solution under controlled growth room conditions as previously described in section 2.6.2. For inoculation of plants with *Micromonospora luteifusca*, an aqueous suspension of bacteria in 0.8% NaCl solution was prepared from cultures grown in GYM *Streptomyces* medium to OD_{600} 0.8. Roots of plants were treated by inoculation with one ml suspension twice: first, the day that seedling roots were placed in the sand; second, twenty day old seedling roots (24 h before inoculation with *Bipolaris sorokiniana*).

For infection of plants with the fungi, a suspension of 3×10^4 *B. sorokiniana* conidia/ml in SDW was prepared. Tween 20 was added to 0.1% as surfactant just before inoculation. The conidial suspension was painted with a soft bristle brush onto two-second leaves of 21 d old plants (Zadoks growth stage 20, tillering begins) (Zadoks *et al.*, 1974).

Disease severity was recorded four days after infection (25 days old) as described in section 2.6.2 (Figure 2.1).

The number of spores was measured at 25 days old four days post-infection. Infected leaves were incubated in plastic trays, which contained wet filter paper, and sealed in plastic bags to obtain high humidity. Then, 10 cm from each leaf was placed in a 15 × 1.5 cm Falcon tube containing 5 ml SDW, and the number of spores for each replicate (leaf) was measured as described in section 2.6.2.

Chitin content was measured as described in section 2.20.

Microscopic analysis of samples was carried out as described in section 2.21.

The experiment used factorial experiments for two factors (three cultivars and four treatements) in complete randomised design-CRD with six replicates/treat.

2.7 RNA Extraction

Approximately 20 mg frozen leaf tissue (lyophilised tissue) was placed in microcentrifuge tube 1.5 ml containing 0.2 mm diameter beads and the frozen material was disrupted in a mixer mill (Qiagen- TissueLyser II) for 2 minutes at 30 Hz. Total RNA was isolated from 20 mg powdered frozen leaf tissue by homogenisation in 1 ml TRIzol (Invitrogen) and held at room temperature for five minutes. A 200 µl aliquot of chloroform was added to each extract with shaking for 15 seconds, and then incubated at room temperature for 3 minutes, and the resulting mixture was centrifuged (15 min 12000×*g* at 4 ^{o}C). The aqueous layer was transferred to a clean tube, RNA was precipitated by the addition of an equal volume of 70% ethanol with a vortex to mix well for few seconds, the sample was transferred to the spin cartridge tube, and centrifuged for 15 s at 12,000×*g*. PureLink RNA Mini Kit (Life Technologies) was used to extract RNA; it is suitable for extracting both plant and fungal RNA from tissue samples. The pellets of RNA, first, washed with 350 µl buffer I, centrifugation at (15 sec, 12,000×*g*), then add 80 µl DNase mixture (PureLink DNase Set - Thermo Fisher Scientific) that used to provide rapid and efficient removal of DNA from RNA, then it washed with 350 µl buffer I for one time centrifugation at (15 sec, 12,000×*g*), and washed twice with 500 µl buffer II, centrifugation at (15 sec, 12,000×*g*). Once more centrifuge the Spin Cartridge at 12,000×*g* for two minutes to dry the membrane with attached the RNA. Discard the collection Tube and insert the Spin Cartridge into a Recovery Tube 1.5 ml. Add 50 µl RNase–Free Water or sterilised water to the centre of the Spin Cartridge. Incubate at room temperature for 1 minute. Centrifuge the Spin Cartridge for 2 min at ≥12,000×*g* at room temperature to elute the RNA from the membrane into the Recovery tube. Finally, it has created 50 µl purified total RNA and the concentration

of RNA was determined using the Thermo Scientific 'NanoDrop® ND-1000' spectrophotometer, then purified total RNA stored in -80°C for future work.

2.8 Genomic DNA extraction from pathogen

DNA was extracted from a culture of *B. sorokiniana* grown on YECSA after 14 days using CTAB (hexadecyltrimethylammonium bromide) method as described previously (Aada, 2013) and in Appendix C. DNA was stored at -20 °C for future work.

2.9 Genomic DNA extraction from plants

DNA was isolated using TRIzol reagent (Invitrogen) from 20 mg powdered frozen leaf tissue (lyophilised tissue) by homogenisation or from 80-100 mg fresh leaves tissues which were ground to a fine powder in liquid nitrogen with a chilled mortar and pestle, according to the manufacturer's instructions (see Appendix D). DNA was stored at -20 °C for future work.

2.10 Genomic DNA extraction from bacteria

DNA was extracted from a culture of *Micomonospora luteifusca* by taking 1 ml from bacteria culture, centrifuge for high speed for 3 minutes. Pellets were washed twice with sterilised water with centrifugation after each wash and supernatant was discarded, then the mixture was added (5 µl 100 mM NaCl, 0.5 µl (10 mM Tris-HCl pH 8), 1 µl proteinase K (0.4 mg/ml) and up to 50 µl by sterilized water after mixture mixed gently) on pellets of bacteria. The homogenate sample was incubated at 55 °C (heat block) for 1 hour with three times mixing by inverting the tube, then mixing with a vortex mixer for 10 s. Samples were placed in a heat block at 85 °C for 5 min, vortexed for 10 s and centrifuged at 13000×*g* for 5 min, then 30-40 µl from the supernatant was transferred. DNA was stored at -20 °C for future work.

2.11 Complementary DNA (cDNA) Synthesis

The first strand cDNA was synthesised from 1 µg total RNA using a reverse transcriptase kit (SensiFAST^{TM} cDNA Synthesis Kit- BIOLINE). The master mix was prepared on the ice with 4 µl TranAmp buffer, 1 µl Reverse transcriptase, 1 µg total RNA and up to 20 µl by RNase–Free Water or sterilised water, then mixture mixed gently and centrifuge for few seconds. This was heated by thermal cycler following 25 °C for 10 minutes, 42 °C for 15 minutes then 85 °C for 5 minutes and 4 °C hold. The cDNA was stored at -20 °C for long-term storage. Use 10 µM from cDNA synthesis product to real-time qPCR.

Three controls were prepared: Non-template control (NTC), RNA from uninfected plant leaf (wheat or Brachypodium) only (PL) and minus Reverse Transcriptase (RT).

2.12 Polymerase Chain Reaction (PCR)

2.12.1 Primers

The primers used in this study were designed using Primer3plus software, targeting an 80 to 200 bp amplicon, R^2 value 0.98–0.99 and no primer-dimer or secondary structures. Primers were also designed to amplify side to the annotated 3/ end of the transcripts, to encompass all known splice variants, and at least one primer of a pair was designed to cover an exon-exon junction (transcript-specific) if possible. Information on all primers used is listed in the appendix B1, B2, B3 and B4. All primers were ordered from Sigma Life Science and used at a concentration of 10 pmol/ml. cDNA of infected and uninfected leaves and fungal genomic DNA were serially diluted to 1:10, 1:20, 1:40, 1:80 and 1:160 of the original concentration. A standard qPCR reaction was then run for each of the primer pairs using the qPCR method described in the section 2.15, testing each of the dilutions by plotting the threshold cycles (Cq) against the logarithm concentration of the fungal and plant DNA and RNA. Primers that are amplifying product efficiently will display an increase of 1 Cq unit when the cDNA and genomic DNA concentration has halved. Calculation of primer efficiencies using five-fold dilution of pooled complementary DNA (cDNA) for all primers gave coefficient of determination value $R^2 > 0.98$ and 96 – 110% efficiency (E) values.

2.12.2 Taq Polymerase PCR

50 µl PCR (polymerase chain reaction) reactions at a final volume contain 25 µl of 2X PCR Master Mix (Thermo Scientific Taq DNA polymerase), 0.3 mM forward primer, 0.3 mM reverse primer, 0.1 µg template cDNA up to 50 µM by sterile distilled water were run in a heat cycler (BioRad). A variation of the following standard conditions: 95 °C for 3 minutes, then 30 cycles of 95 °C 30 seconds, annealing temperature* for 30 seconds, 72 °C 1 minute per 1 kb (kilobase pair) of the expected product, followed by 10 minutes at 72 °C and hold 4 °C.

*Annealing temperature depended on melting temperature of primers. New primers were tested by a gradient PCR with a range of annealing temperatures.

The PCR products were run on an agarose gel (1-2 %). Single bands were visualised by ethidium bromide staining under UV.

2.13 Gel Electrophoresis

Gels between 1-2 % agarose (Melford, Ipswich, UK) were made, depending on the size of the product to be visualised, Agarose was melted in 1X TAE buffer, and was allowed to cool to approximately 50 °C before the addition of ethidium bromide at a final concentration of 0.5 µl/ml (Fischer Scientific, Waltham, USA). Electrophoresis was carried out at 80-100 V in a tank of 1X TAE. 10 µl of each sample with add 2 µl from 5X DNA Loading Buffer Blue (Bioline) were run, along with 5 µl of either 50 bp or 1 kb hyper ladder (Bioline). DNA was visualised under UV (BIO-RAD).

2.14 DNA sequencing

All single read sequencing of PCR products and gDNA was carried out by GATC Biotech and DBS Genomic in Department of Biosciences, Durham University.

2.15 Quantitative Real-Time PCR (qPCR)

All Quantitative Real-time PCRs (qRT-PCR) was performed in a 50 µl volume containing 25µl 2X SYBR green (2X SensiFAST™ SYBR® No-ROX Kit Master Mix (Bioline), with primers concentration, 0.2 mM forward and 0.2 mM reverse primers. 1 µl diluted (1:10 v/v) first-strand cDNA (10 ng). The reaction up to 50 µl by sterilised water. PCR conditions (Rotor-Gene Q QIAGEN) set up with an initial denaturation step (95 °C/2 min) followed by 40 cycles of 95 °C/5 sec, 60 °C/10 s, 72 °C/20 sec. A melting curve analysis was performed over the range 60–95 °C at 1 °C intervals. Three technical replicates were used for each three biological replicates and were normalised against reference genes by using the ΔΔCq method. For normalising, the endogenous control genes were used, for wheat *TaWRKY* genes used *TaEF1α* (Elongation Factor 1 subunit α), *TaeIF4A* (translation initiation factor 4A), *Ta28S rRNA* and *Ta18S rRNA* (ribosomal rRNA), for Brachypodium *BdWRKY*s used *BdEF1α* and *BdUb18* (ubiquitin 18) and for *Bipolaris sorokinana* used (*Bs28S rRNA*, *BsGPD* (glyceraldehyde-3-phosphate dehydrogenase) and *Bs18S rRNA*) genes. The large-chain gene (*rbcL*: Ribulose-1,5-bisphosphate carboxylase/oxygenase -RuBisCO) is encoded by the chloroplast DNA in plants was used. In addition to pathogenesis-related protein 1 (*PR1*) which accumulates after infection with a pathogen (See Appendices B1, B2, B3 and B4). Livak and Schmittgen (Livak and Schmittgen, 2001) method used to find values of ΔCq were calculated by first normalising Cq values to the endogenous control, and subsequently calculating ΔΔCq values using the ΔCq value of 7.5 mM nitrate as a compared level for wheat genes, either 0.75 mM nitrate as a compared level for fungal genes. Fold difference in gene expression level was calculated using

the 2^ (−ΔΔCq) formula (Livak and Schmittgen, 2001). Fold difference in gene expression was calculated based on the comparative threshold cycle method. Data were presented as average of three biological replicates with three technical replicates each replicate. The error bar of the mean" refers to the Confident intervals estimates of the mean expressions, which depend to ΔΔCq and Fold change in critical t value in freedom degree is eight at 95%, 99%, 99.9%. The smaller error bar and not cut x-axis is significant as described in section 2.17.

2.16 Quantitative Real-Time PCR (qPCR) data analysis

2.16.1 Internal control gene – geNorm analysis

The geNorm analysis is based on the principle that the experimental conditions should not affect the expression ratio of two adequate reference genes (Vandesompele *et al.*, 2002). Details of the principles are given in Appendix G.

2.16.2 Best housekeeping analysis

Best Keeper is based on the principle that proper reference genes should display similar expression patterns and should be highly correlated. A simple statistical analysis is very important to ensure that all candidates did not show extreme variation when tested on all of the different treatments required for this experiment. For a gene to be a suitable candidate (housekeeping gene) for normalisation, it must be expressed to a highly similar level in all of the tissue samples under different treatments being investigated. A number of statistical parameters used to verify the stability of potential normalisation genes by way of pairwise testing (Silver *et al.*, 2006).

2.16.3 Analysis of raw Cq values

A simple statistical analysis is very important to ensure that all candidates did not show extreme variation when tested on all of the different treatments required for this experiment. For a gene to be a suitable candidate (housekeeping gene) for normalisation, it must be expressed to a highly similar level in all of the tissue samples under different treatments being investigated. Standard deviation to raw Cq values suggested by De Spiegelaere *et al.* (2015) is unacceptable for normalisation with a standard deviation of more than 1.5.

2.17 The confidence intervals of the difference between fold change means

The confidence interval is based upon the SEM value (Equation 1). The limits are constructed as differences between the mean and the SEM multiplied by a percentile of a t-distribution. The confidence level is set by the confidence level associated with the critical value t; typically a

95%, 99%, 99.9% confidence level with degrees of freedom (d.f. =8), as in Equation 2. FC is Fold change to treated samples displayed relative to non-treated samples (standard level). Error bars indicate 95%, 99%, 99.9% confidence interval estimates of the mean expressions, the smaller error bar and not cut x-axis is significant (Ganger *et al.*, 2017).

$$SEM = \frac{SD}{\sqrt{N}} \qquad (1)$$

$$CI = \left(FC - t * \frac{SD}{\sqrt{N}}\right) to \left(FC + t * \frac{SD}{\sqrt{N}}\right) \qquad (2)$$

https://www.sigmaaldrich.com/technical-documents/articles/biology/data-analysis.html

2.18 Identification and phylogenetic analysis of WRKY superfamily in bread wheat

To identify wheat WRKY proteins, the amino acid sequences in Plant Transcription Factor Database v3.0 (PlantTFDB, http://planttfdb.cbi.pku.edu.cn/index.php) (Jin *et al.*, 2014) and in GenBank (release 199.0) (http://www.ncbi.nlm.nih.gov/protein) were used. *TaWRKY* sequences were aligned by ClustalW using the program MEGA7 (http://www.megasoftware.net) (Tamura *et al.*, 2013). The unrooted phylogenetic tree in different cereals was constructed by the Neighbour-Joining method with a bootstrap test (1,000 replicates) using MEGA7 (Figure 2.3) to obtain the synteny between WRKY TF genes in wheat with nearest WRKY TF genes in the other crops. (Ta; Wheat *Triticum aestivum*, Bd; Brachypodium *Brachypodium distachyon*, Hv; Barley *Hordeum vulgare* Os; Rice *Oryza sativa*, Zm; Maize *Zea mays* and Sb; Sorghum *Sorghum bicolor*). Furthermore, a neighbour-joining cladogram of the WRKY domains of Arabidopsis and wheat WRKY gene used in this study (see Appendix A) was constructed. BLASTX (DNA vs protein) search for *T. aestivum* in PlantTFDB was used with default parameters to identify the transcripts encoding for WRKY motif. Sequences were translated using the ExPASy portal (http://web.expasy.org/translate), Appendix B 5 1 and B 5 2.

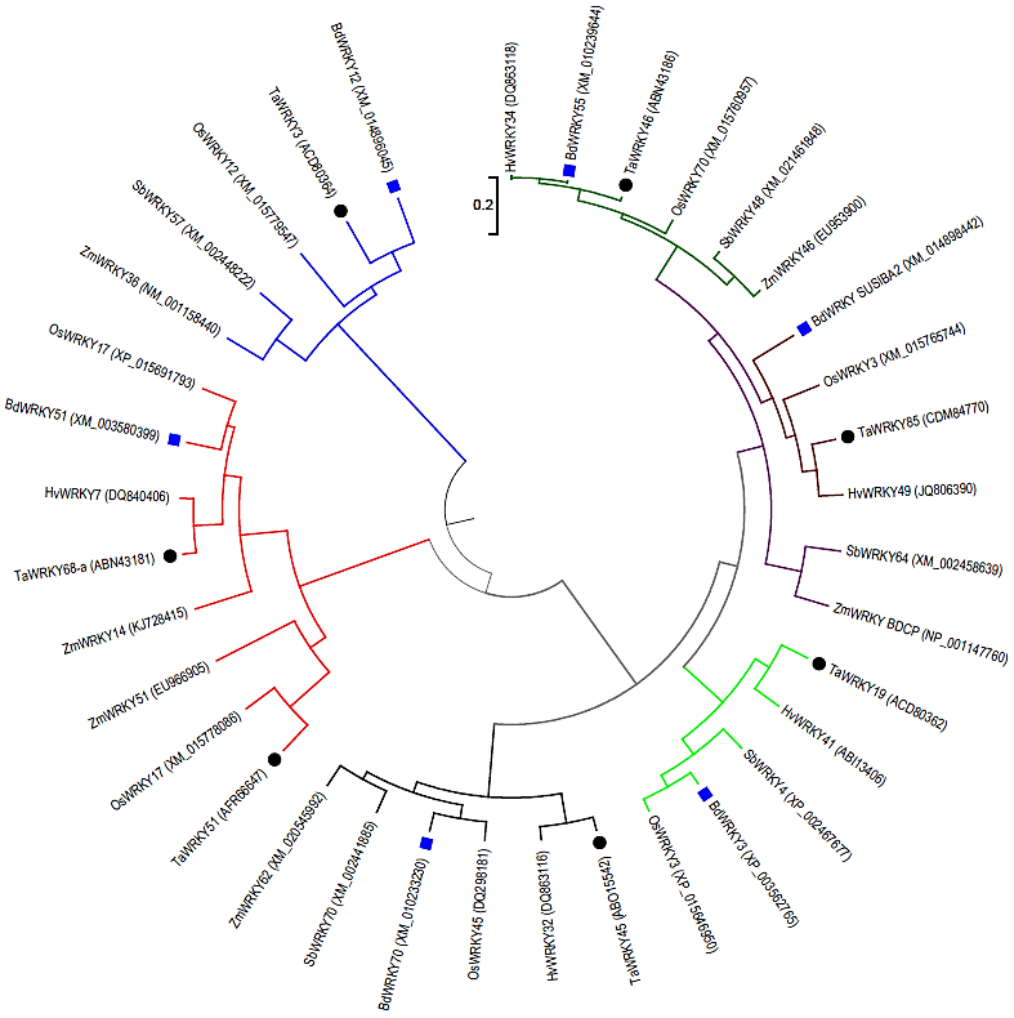


Figure 2.3. Phylogenetic tree: the synteny between WRKY TF genes in wheat with nearest WRKY TF genes in the other crops. (Black circle= Ta; Wheat, blue square=Bd; Brachypodium, Hv; Barley, Os; Rice, Zm; Maize and Sb; Sorghum) produced by MEGA7 software, scale bar (0.2) is marked.

2.19 Genetic diversity data analysis

Genetic diversity of 21 wheat cultivars were tested (6 Iraqi, 6 UK and 7 Syrian and one for Chinese and French wheat cultivar), see Table 2.2. Only 15 microsatellite markers (Xgwm 3, 18, 95, 155, 190, 261, 325, 357, 389, 408, 458, 513, 577, 631, 680), in addition to Taglgap as reference gene have successfully amplified from 21 microsatellite markers. Whereas, xgwm 46, 437 and 619 with two reference genes Taglut and Secalin were failed. The primers amplifying the respective microsatellite loci (Xgwm) were used by Roder *et al.* (2002). Primers that showed a single band of PCR products from most of the wheat chromosomes were used. This number of markers was sufficient to distinguish between most of the 21 varieties. For closely related varieties, more markers are required for detecting differences because only limited regions of the genome differ in their genomic composition.

Table 2.2. Name, code, origin and growth status of bread wheat tested by microsatellite analysis.

No.	Sample	Sample	Variety	Type
1.	QANDAHARI-A	21	Syrian	spring
2.	QANDAHARI-B1	22	Syrian	spring
3.	QANDAHARI-B2	23	Syrian	spring
4.	BRIJI_ABIAD	24	Syrian	spring
5.	BRIJI_AHMAR	25	Syrian	spring
6.	SALMUNI	26	Syrian	spring
7.	SUWEED	27	Syrian	spring
8.	RASHEED	Rashid	Iraqi	spring
9.	LATIFIA	Latifia	Iraqi	spring
10.	TAMOZZ	Tamoz3	Iraqi	spring
11.	AUROK	Aurok	Iraqi	spring
12.	IRAQ	Iraq	Iraqi	spring
13.	EBA99	EbA99	Iraqi	spring
14.	PARAGON	30	British	spring
15.	ROBIGUS	31	British	winter
16.	CHINESESPRING	32	Chinese	spring
17.	RIALTO	33	British	winter
18.	SOISSONS	34	French	winter
19.	CONSORT	35	British	winter
20.	CORDIALE	Cordiale	British	winter
21.	CADENZA	Cadenza	British	spring

DNA extraction from wheat leaves carried out as described in session 2.9. Taq polymerase PCR ran used as described in section 2.12, by adding three fluorescent dyes (FAM, HAM and HEX) in PCR reaction to distinguish among results of different samples during the same run in sequencing machine, then send to sequencing as is described in section 2.14. Genetic diversity of 21 different wheat cultivars in this study were computed with GenALEx version 6.5 software (Peakall and Smouse, 2012) by Principal Coordinate Analysis (PCoA). PCoA involves a mathematical procedure that able to transform a much number of correlated variables into a small number of uncorrelated variables is called principal components of data set. Firstly, the principal component may account for as much of the variability in the data as possible, and then each succeeding component can account for as much of the remaining variability as possible. In more words, it is possible to discover or to reduce the dimensionality of the data set, also, improving the ability to identify new meaningful underlying variables.

2.20 Quantification of fungal biomass by chitin measurement

The procedure of Ayliffe *et al*. (2013) was followed in this study. Infected and control (uninfected) leaves were harvested, weighed, cut into 2 cm lengths, and put into 15 ml Falcon tubes. 1 M KOH containing 0.1% (vol/vol) Silwet L-77 was added to each tube to cover the tissue. Tissues were autoclaved (121 °C and 15 psi for 20 min). The KOH solution was gently discarded and replaced with 15 ml of 50 mM TEA (Tris-acetate-EDTA buffer), pH 7.5. This solution was then replaced with another 10 ml of TAE and the tissue left for 30 min, then the buffer was replaced with 3 ml of TAE solution for each 700 mg of plant tissue. The plant tissue was macerated either by sonication or with a Polytron blender to produce a fine, uniform tissue suspension. For leaves of wheat at 18 or 25 days old approximately 1 min of sonication or blending generally produced a uniform suspension. Tissues with more fibres were macerated more. A sample (usually 800 µl) of each suspension was added to an Eppendorf centrifuge tube containing 40 µl of a 1 mg ml^{-1} solution of wheat germ agglutinin WGA conjugate (Sigma Aldrich) dissolved in sterilised distilled water (SDW).

The ends of pipette tips were cut before samples were pipetted and homogenates were agitated regularly to ensure that the sample collected was uniform. The sample and stain solution were mixed briefly by repetitive pipetting and left to stand for at least 30 min at room temperature. Staining samples were kept in the dark. Six replicate samples were used for each tissue preparation. Following staining, samples were centrifuged at 800×*g* for 3 min. The supernatant containing unbound stain was removed by pipetting and the pellet resuspended in 1000 µl of 50 mM TAE. Samples were washed three times in 1000 µl of 50 mM TAE and resuspended in 800 µl of 50 mM TAE. Each sample tube striped to 6 wells for 96-well black microtitre plates for fluorometry for leaf samples, negative control (infected and uninfected samples without WGA), in addition to 50 mM TAE buffer used as another control. Fluorometric measurements were made with 485 nm absorption and 538 nm emission wavelengths and 1.0 second measurement time in a Varioskan Lux multimode microplate reader (Thermo Scientific) (Ayliffe *et al.*, 2013).

2.21 Microscopic analysis of leaves and subsequent chitin quantification

The method for visualisation of internal infection structures in cleared leaf samples was adapted from Ayliffe *et al.* (2013). Leaves were cut into 3 cm pieces and autoclaved in a 15 ml Falcon tubes containing 5 ml of 1 M KOH and 0.05% Silwet L-77. Following autoclaving, the KOH solution was gently discarded and replaced with 10 ml of 50 mM TEA (Tris-acetate-EDTA buffer), pH 7.5. This solution was then replaced with another 10 ml of TAE and the tissue left

for 20 min. After 20 min, a leaf piece was gently placed on a glass microscope slide which was put in a petri dish, and 20 µl of a 1 mg ml^{-1} solution of wheat germ agglutinin WGA conjugate (Sigma Aldrich) as directly added to 1 cm of leaf tissue.

Tissue was stained for at least one hour in the dark. After staining, the tissue was washed with 15 ml TAE buffer from a squeeze bottle to rinse off the excess WGA, and the tissue was gently placed on a fresh microscope slide in petri dish. After the tissue was placed on the slide, gently adding 10 ml from 50 mM TAE buffer from a squeeze bottle caused the tissue to unravel before a coverslip was added. Wheat germ agglutinin WGA conjugate-stained tissue was examined with a Leica fluorescence microscope under blue light excitation and 10×objective magnification.

2.22 Statistical analysis

Statistical analyses were performed by using Minitab 17 statistical software. The raw data were subjected to analysis of variance (ANOVA) to determine the effects of nitrogen levels and pathogen inoculation on commercials wheat cultivars. The significant difference between means compared pairwise with Tukey test ($P < 0.05$) was used for comparing treatments statistically. Error bars represent ± 1 standard deviation.

3 Reduced Nitrogen Effects on Wheat and Brachypodium Physiology and Spot Blotch Tolerance

3.1 Abstract

Spot blotch caused by *Bipolaris sorokiniana* (*Cochliobolus sativus*) is a serious disease of wheat grown in warm climates. The impact of plant diseases is influenced by plant nutrition, and it has previously been reported that nutrient stress affects spot blotch severity. The interaction between nutrient supply, spot blotch disease severity in Iraqi wheat and Brachypodium were investigated. The susceptibility of five Iraqi wheat and one UK wheat varieties to spot blotch was tested. Among them, Rashid was the most susceptible and Latifia the most resistant. Seedlings Rashid, Latifia and Brachpodium were grown in sand watered with Hoagland's solution modified to contain 0.75 mM, 3.75 mM or 7.5 mM nitrate. Eighteen-day-old seedlings for wheat and 30 days old for Brachpodium supplied with 0.75 mM nitrate had a significantly small size of plants than those supplied with 3.75 mM or 7.5 mM nitrate. Fourteen-day-old seedlings for wheat and 27 days old for Brachypodium were inoculated with spot blotch. Four days after infection there was a trend to higher disease severity at the higher nitrate levels, particularly for the more susceptible variety Rashid at 7.5 mM nitrate. Quantitative real-time PCR of *B. sorokiniana* ITS RNA in infected Rashid and Latifia wheat cultivars and Brachypodium showed that the RNA abundance increased with increasing spot blotch prevalence associated with increasing nitrogen availability. In contrast, some previous studies, which were mostly with field-grown plants, found that adequate nitrate supply reduces spot blotch severity.

Keywords: WRKY TF genes, Barchypodium, wheat, nitrogen stress, *Bipolaris sorokiniana*, disease severity assessment, genetic diversity, ITS RNA, quantification RT-PCR.

3.2 Introduction

Abiotic stresses, especially nitrogen deficiency are limiting factors for plant growth, development, and agricultural productivity, especially for wheat which is one of the major cereals that have been used for human food and livestock feed (Peng *et al.*, 2011). The purpose of this study was not to establish that nitrogen supply has an effect on the growth of wheat, but to investigate the effect of nitrogen availability on disease severity (Howard *et al.*, 1994; Loyce *et al.*, 2008), and to understand the mechanisms that may arise from their interaction. Phytopathogenic fungi must obtain their nutrients from their hosts. Their ability to gain access to nutrients from living plants distinguishes them from saprophytes. Successful invasion by pathogens depends as much on their ability to exploit plant nutrient sources as on their ability to penetrate plants and evade plant defences (Solomon *et al.*, 2003). The reasons behind the link between nitrogen input and spot blotch tolerance are very important to understand before attempting to manipulate it. The presence of abiotic stress can reduce or increase susceptibility to biotic pests and pathogens (Atkinson and Urwin, 2012). An additional challenge is that there are trade-offs between soil fertility and the effects of stress. Notably, low fertility favours some plant diseases (Sharma and Duveiller, 2004), and high fertility favours others (Loyce *et al.*, 2008). In subsequent investigations, the work needs to be extended to the field to validate the experimental conditions used and determine whether spot blotch severity is affected by nitrogen in the same manner in the field as under growth room conditions with Hoagland's nitrogen supply (Sharma and Duveiller, 2004).

In this study, wheat was grown under three nitrogen regimes: standard (7.5 mM nitrate), moderately reduced (3.75 mM nitrate) and severely reduced (0.75 mM nitrate). Hoagland's solution was used to provide all the nutrients required by the plant, with plants grown in the sand to provide stability in a nutrient-free medium. This enabled precise control over the amount of nitrogen available to the plant more easily than a soil and compost based approach, which would have been less accurate for attempts to quantify the level of available nitrogen and would have precluded development of a quantitative model of spot blotch susceptibility as a result of nitrogen availability. When plants are grown under conditions in which nitrogen is plentiful, such as when fertiliser is applied to crops, rapid growth is possible. Of course, this is preferable in terms of maximising yield. However, most of the plant's finite resources are allocated toward growth, leaving fewer resources available for defence. Therefore, "optimal" growth conditions may leave the plant more susceptible to a pathogen or pest attack than reducing nitrogen availability (Loyce *et al.*, 2008). While this link is hypothesised and has been

observed in specific examples, such as between nitrogen input and fusarium head blight in wheat (Lemmens *et al.*, 2004), it is not universally accepted to describe all growth-defence interactions (de Vries *et al.*, 2017; Wasternack, 2017). Physiological data were collected to reflect the total size and surface area of the plant. It was hypothesised that size and surface of plants would increase with increasing nitrogen availability, indicating that growth conditions became more favourable with increasing nitrogen availability.

3.3 Assessment of fungal development in plant leaves

The possibility of finding more reliable methods for fungal disease assessment is very important for physiological studies, maybe by improving the resistance of plant against the pathogen attack or to control the development and aggressiveness of the pathogen. There are many methods to quantify fungal infection of plants. Of these, three methods are most commonly used: conidia counts, macroscopic categorisation and microscopy-based studies of penetration (Ayliffe *et al.*, 2011; Wessling and Panstruga, 2012), the last two methods will be described in more detail in the next chapters. Disease severity scoring is based on disease symptoms that can be scored by naked eye at later stages of pathogenesis (4 dpi) and ratings assigned based on the severity of disease symptoms based on necrotic and chlorotic lesions of varying sizes observed on infected plants (Domiciano *et al.*, 2014). The use of disease severity scores is quick and suitable for high throughput, relies on equal inoculation densities and can only reveal substantial differences in colonisation that are readily visible to the naked eye. In this study, spot blotch was measured using disease scores and number of spores. However, these two methods face significant issues. Disease scoring was recorded at four days post-infection, while spore counting needed a further four days of incubation at high humidity. Spores are only present externally in the final phases of the disease, and therefore a spore wash assessing the number of conidia being formed by the fungus will only represent disease severity at this final stage (4 dpi in the plant and further four dpi in the high humidity condition).

Previously, researchers in the microbial field have used amplification of *16S rRNA* molecules by RT-PCR to detect active species of bacteria and archaea, and *18S rRNA* molecules for the detection of various fungi. However, *18S rRNA* sequences often do not provide sufficient taxonomic resolution in mixed communities to genus or species level (Anderson and Parkin, 2007). White *et al.* (1990) used primers to amplify the internal transcribed sequences of ribosomal RNA by comparing different flanking sequences in yeasts and rice (*Oryza sativa*). Subsequently, internal transcribed spacer (ITS) sequences have become the most popular tool for species identification of fungal taxa in environmental DNA pools, the result of the highly

variable nature of the rapidly evolving rDNA spacer regions. As a result, this region provides the largest amount of reference database sequence information that is currently available for the molecular identification of fungi (Anderson and Cairney, 2004). The amount of ITS regions is relatively unaffected by the activity of the fungus (Edger *et al.*, 2014). As such, it is a good measure of the total amount of fungal biomass in the plant and therefore relates to disease severity in the infected sample.

Quantitative RT-PCR was used in this study to assess the expression of the internal transcribed spacer region in *Bipolaris sorokiniana* (for example, NCBI accession number., HF934937.1) with three primer pairs amplifying different regions (*BipITS, Coch* and *ITS1-ITS4*). The *BipITS* and *Coch* primers were designed to be specific for *Bipolaris*, but the *ITS1-ITS4* primer pair can amplify sequences from both *B. sorokiniana* (e.g. HF934937.1) and wheat (e.g.FJ609737.1). Therefore, the *BipITS* and *Coch* primers are ideal candidates for quantitatively assessing the amount of pathogen present within an infected sample with *ITS1-ITS4* also reflecting wheat gene expression activity. Similarly. Gardes and Bruns (1993) found that a specially-designed primer pair, ITS1-F/ITS4-B was particularly valuable for detection and analysis of the basidiomycete component in infected tissues. By comparing the expression of *BipITS, ITS1-ITS4* and *Coch* regions in infected plants to that of a standard, an objective and reproducible measure of pathogen load can be obtained from the plant even before symptoms of the infection are present. Consequently, a relative quantitative measure of disease severity can be obtained via this method. Knowing the variation in the response of different wheat cultivars to pathogens is necessary in order to further our understanding of the defensive mechanisms by which plants protect themselves from disease attack. In a parallel study, Brachypodium was used as a model system because mechanistic understanding is difficult in wheat because of its huge genome. Furthermore, Brachypodium as a model system has significantly advanced research, allowing scientists to investigate complex processes that are not simple to study in non-model organisms (Brkljacic *et al.*, 2011). Brachypodium genes are highly similar to wheat genes and it has a higher synteny relationship with wheat than rice (International Brachypodium Initiative, 2010).

The overall objective of this chapter is to investigate how the nutrient regime under which cereals are grown affects their susceptibility to spot blotch disease. Knowledge of such effects should make it possible to manipulate conditions so that plants retain enhanced disease resistance with high or optimum nitrogen input.

3.4 Hypothesis:

1- Different wheat cultivars have different ways to defence to spot blotch infection.
2- Wheat that has resistance to spot blotch disease is less genetic diversity to each other that also has resistance response.
3- The growth of wheat and Brachypodium is different under reducing nitrogen availability.
4- Reduce disease effects by reducing nitrogen input from 7.5 mM to 3.75 mM and nitrogen and 0.75 mM in wheat and Brachypodium.
5. The abundance of spot blotch molecules differs with increasing spot blotch prevalence.

3.5 Aims:

1- Determine which wheat of six wheat cultivars is the most susceptible and which is the most resistant to spot blotch.
2- Identify genetic diversity of different wheat cultivars using microsatellite method.
3- Determine how growth differs with 7.5 mM, 3.75 mM and 0.75 mM nitrogen in wheat and Brachypodium.
4- Determine how wheat and Brachypodium respond to *B. sorokiniana* when nitrogen input is reduced.
5- Compare different methods of scoring disease severity in wheat and Brachypodium.

3.6 Results

3.6.1 Preliminary infection experiments to screen the susceptibility of different wheat cultivars to spot blotch

To identify the wheat cultivars that were most resistant and most susceptible to spot blotch, it was necessary to screen them against spot blotch infection at different growth stages and with different inoculation methods. The susceptibility of five Iraqi wheat cultivars and one UK cultivar to spot blotch disease was measured after infection by two methods. First, plants were sprayed with a spore suspension (6×10^4 *Bipolaris sorokiniana* conidia ml^{-1}) at 36 days old (GS39), second, uppermost leaves were brushed (soft bristle brush) with a spore suspension (6×10^4 conidia ml^{-1}) at 46 days old (GS50) (Zadoks *et al.*, 1974). Mean disease severity in the six wheat cultivars was assessed as % of leaf area affected by spot blotch, based on necrotic and chlorotic lesions of varying sizes observed on the infected leaves of plants. Figure 3.1 shows that disease severity averaged over 3, 6 and 9 days post infection (dpi) differed significantly (ANOVA, Tukey, $P < 0.05$) among the six wheat cultivars in both inoculation

methods. Latifia had the lowest disease severity in both sprayed and brushed inoculation methods; it was significantly different (ANOVA, Tukey, $P < 0.05$) from Rashid and Iraq cultivars.

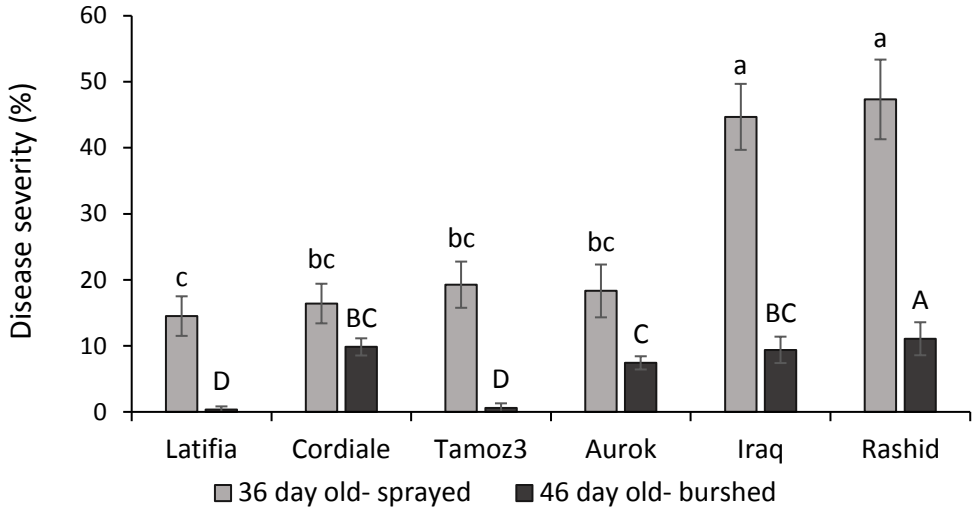


Figure 3.1. Spot blotch disease severity (%) on leaves of six wheat cultivars with two different inoculation methods (sprayed at 36 days old, brushed at 46 days old) at nine dpi.
Different letters among bars represent significant differences for each method (ANOVA, one way, Tukey $P < 0.05$, $n = 9$), error bars represent ± 1 standard deviation.

Disease severity % for all cultivars used in this study showed a significant increase (ANOVA, Tukey, $P < 0.05$) of spot blotch over time after infection from 3, 6 and 9 days after infection with the sprayed method (Figure 3.2 A). With the spray method, there were no significant differences in disease severity among Latifia, Cordiale, Tamoz3 and Aurok at 3 days post-infection (dpi), whereas these cultivars showed significant differences (ANOVA, Tukey, $P < 0.05$) from Rashid and Iraq. Disease severity on Rashid at 6 and 9 dpi was significantly higher than on all other cultivars and at 9 dpi it was significantly higher than on all other cultivars except Iraq. However, disease severity % for Latifia was significantly lower than on all other cultivars at 9 dpi.

With the brush method, disease severity % for the six wheat cultivars increased significantly (ANOVA, Tukey, $P < 0.05$) over time after infection (Figure 3.2 B). Rashid, Iraq and Cordiale showed significantly high disease severity than Latifia and Tamoz3 cultivars, but not significantly different (ANOVA, Tukey, $P > 0.05$) from Aurok cultivar.

From the results above, Rashid wheat cultivar exhibited more severe disease symptoms and supported higher fungal growth than Latifia wheat cultivar after spot blotch infection. Consequently, these two wheat cultivars were selected for the next studies in this project.

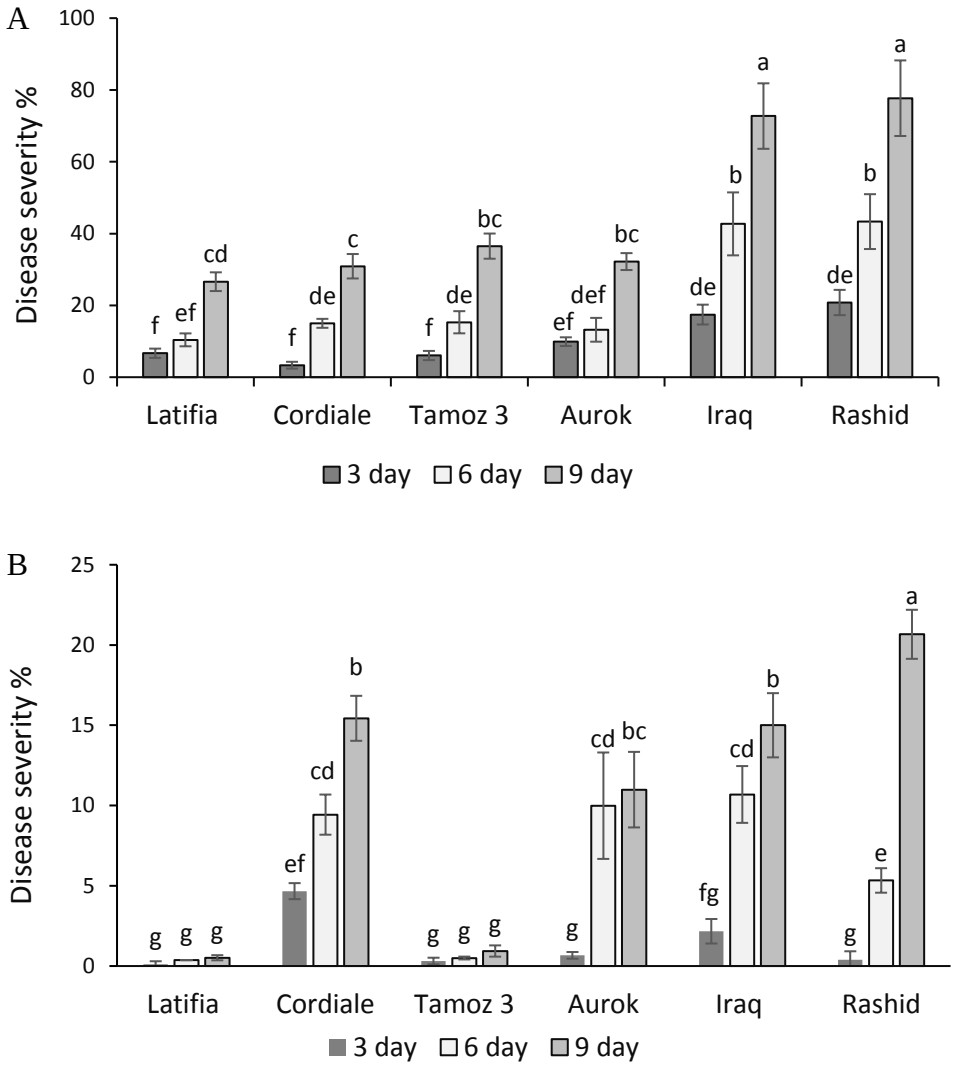


Figure 3.2. Increase in disease severity (%) over time on six wheat cultivars infected (A) at 36 days by the spray method and (B) at 46 days by the brush method.
Different letters among bars represent significant differences (ANOVA, two way, Tukey $P < 0.05$, n =6), error bars represent ± 1 standard deviation.

3.6.2 Genetic diversity of wheat cultivars used

To improve understanding of the wheat material included in this study, genetic diversity of 21 wheat cultivars was tested (6 Iraqi, 6 UK, 7 Syrian, one Chinese and one French wheat cultivar) (Table 2.2). The primers of 15 microsatellite markers (Xgwm 3, 18, 95, 155, 190, 261, 325, 357, 389, 408, 458, 513, 577, 631, 680) were tested amplifying to the respective microsatellite loci (Xgwm) that they were used by Roder *et al.* (2002) to construct a microsatellite database. The primers gave a single PCR product from most of the wheat chromosomes used. This number of markers was sufficient to distinguish between the majorities of the 21 varieties.

DNA extraction from wheat leaves was carried out as described in section 2.9, by adding three fluorescent dyes (FAM, HAM and HEX) in the PCR reaction to distinguish among results of

different samples during the same run in sequencing machine as shown in sections 2.12.2 and 2.19.

Genetic diversity of the 21 different wheat cultivars in this study was computed with GenALEx version 6.5 software (Peakall and Smouse, 2012) by Principal Coordinates Analysis (PCA). The results from Figure 3.3 show that Latifia and Tamoz3 (Iraqi spring wheat cultivars), which were more resistant to spot blotch, and more closely related to each other by PCoA analysis than Rashid and Aurok (Iraqi wheat cultivars) which showed high susceptibility to spot blotch.

Most of the UK (winter wheat cultivars) cultivars were located in a single group. Also, most Syrian wheat (spring wheat cultivars) were in a single group. Cordiale and Cadenza (UK winter wheats studied in this thesis) were genetically similar. These two cultivars showed slight differences in spot blotch severity in different experiments carried out in this study. In the PCoA analysis, the first two principal axis explain a total of 39.77% of unbiased genetic distance, with 14.35% and 25.42% for coordinates 1 and 2, respectively.

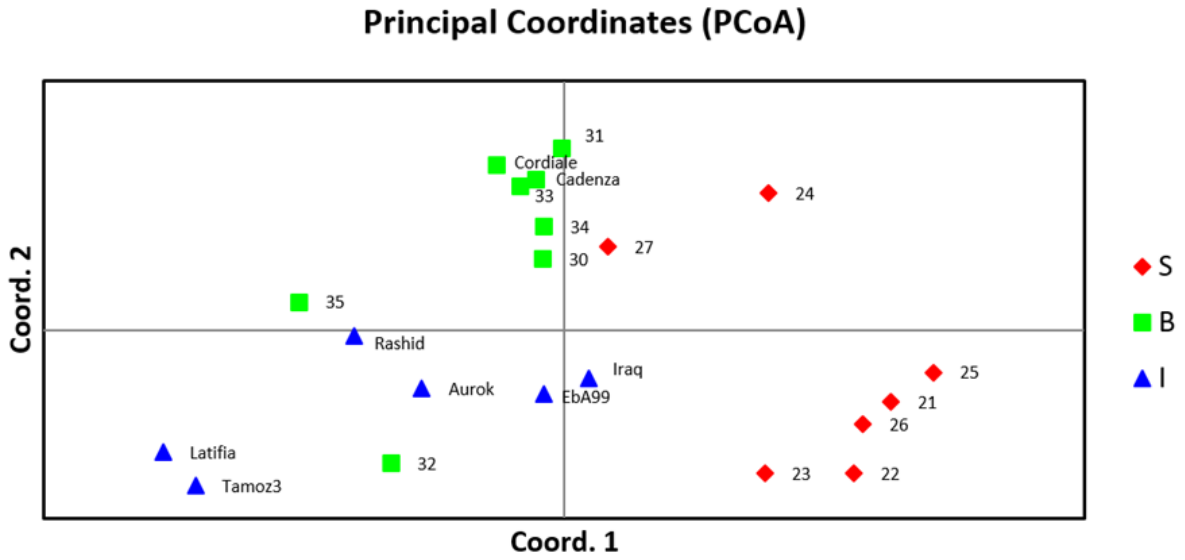


Figure 3.3. Principal Coordinates Analysis (PCoA) plot of the six Iraqi (I, blue), eight UK (B, green) and seven Syrian (S, red) bread wheat cultivars.
The plot is based on the first two principal coordinates (coord. 1 = 14.35% and coord. 2 = 25.42%). Sixteen microsatellite markers and Taglgap as reference gene used for each wheat. Iraqi and Syrian wheat are spring wheat cultivars, the UK wheat is winter wheat.

3.6.3 Reduced nitrogen shows reduced growth in infected and uninfected Iraqi wheat

The effect of reducing nitrogen input from standard level 7.5 mM to 3.75 mM and 0.75 mM nitrate was investigated. The growth at 18 days old of infected (the second leaf of each plant brushed at 4 dpi) and uninfected Rashid and Latifia wheat cultivars was measured. Shoot height (Figure 3.4 A) was significantly lower (ANOVA, Tukey, $P < 0.05$) in severely reduced nitrogen

compared to the standard level of nitrogen input in uninfected and infected Rashid and infected Latifia, with no difference in uninfected Latifia cultivar. There were no significant (ANOVA, Tukey, $P > 0.05$) differences in shoot height between the severely reduced and moderately reduced nitrogen levels in uninfected and infected wheat.

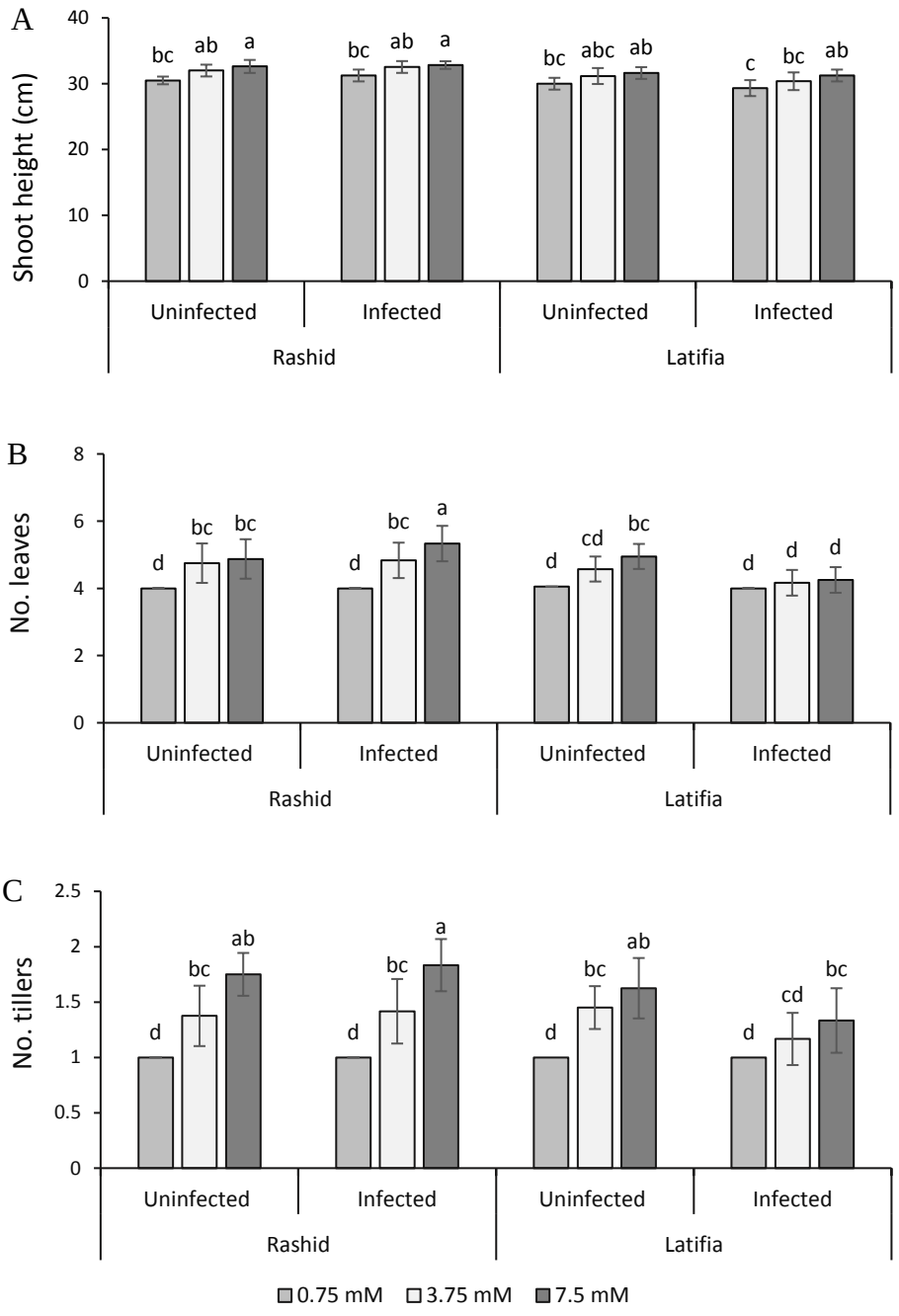


Figure 3.4. Effect of three levels of nitrogen supply (0.75, 3.75 and standard level 7.5 mM nitrate) on (A) shoot height (cm), (B) Number of leaves and (C) Number of tillers in uninfected and infected (four dpi by spot blotch) Rashid and Latifia wheat cultivars at 18 days old.
Different letters on bars represent significant differences (ANOVA, three way, Tukey, $P < 0.05$, n= 9), error bars represent ± 1 standard deviation.

However, shoot height showed no significant difference ($P > 0.05$) between uninfected and infected Rashid and Latifia cultivars when the comparison was between the same wheat at the same nitrogen level. The mean numbers of leaves were significantly lower (ANOVA, Tukey, $P < 0.05$) in severely stressed plants than at the standard level of nitrogen in uninfected and infected Rashid and uninfected Latifia, but not in infected Latifia (Figure 3.4 B). Generally, the number of tillers was greater increased with increasing nitrogen input. Uninfected Latifia had a significantly ($P < 0.05$) higher number of tillers when grown with optimum nitrogen (7.5 mM) than infected plants with moderately reduced nitrogen (3.75 mM) and severely reduced nitrogen (0.75 mM) (Figure 3.4 C). Plants grown with severe nitrogen stress had the lowest number of tillers (average of 1 tiller), while high nitrogen (7.5 mM) gave the highest number of tillers (1.63 and 1.33 tillers for uninfected and infected Latifia, respectively, and in Rashid cultivar 1.83 tiller for infected plants and 1.75 tiller in the uninfected plants).

Chlorophyll content index (Figure 3.5) with the control nitrogen level was significantly different in uninfected Rashid (ANOVA, Tukey, $P < 0.05$) compared to severely and moderately reduced nitrogen, while in uninfected Latifia it was only significantly different with severely reduced nitrogen. Rashid and Latifia wheat showed significant differences between uninfected and infected plants at the same nitrogen level. Uninfected Rashid had significantly higher chlorophyll content index than Latifia at 7.5 mM nitrogen.

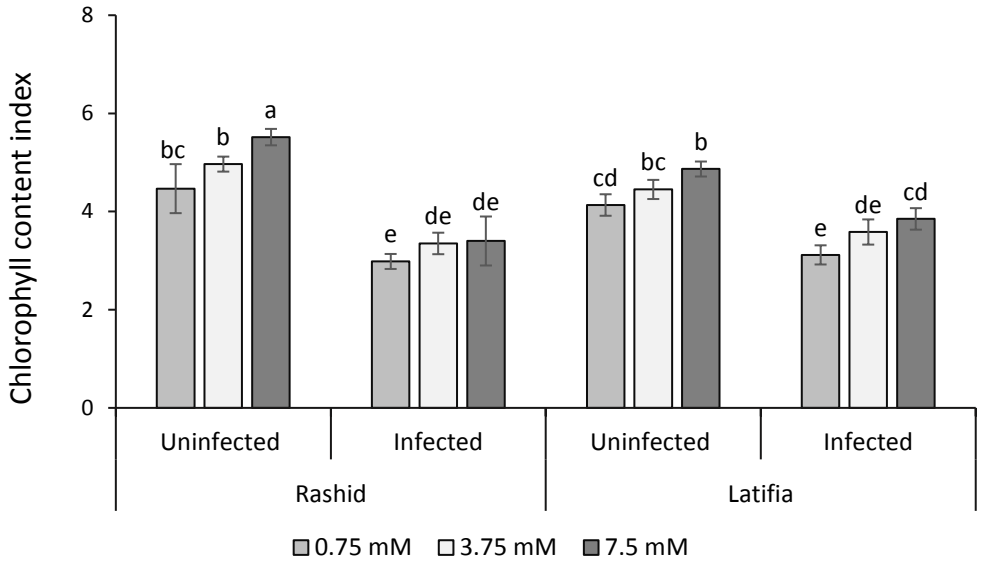


Figure 3.5. Effect of three levels of nitrogen supply (0.75, 3.75 and standard level 7.5 mM nitrate) on chlorophyll content index in uninfected and infected (four dpi by spot blotch) Rashid and Latifia wheat cultivars at 18 days old.
Different letters among bars represent significant differences (ANOVA, three way, Tukey $P < 0.05$, n= 9), error bars represent ± 1 standard deviation.

Average leaf area index was significantly (ANOVA, Tukey, $P < 0.05$) lower with severely reduced nitrogen level than with standard nitrogen input, but not significantly lower (ANOVA, Tukey, $P > 0.05$) with moderately reduced nitrogen for both Rashid and Latifia cultivars (Figure 3.6). There were no significant differences (ANOVA, Tukey, $P > 0.05$) for moderately reduced nitrogen compared to both optimum level and severely reduced nitrogen in both infected and non-infected Rashid and Latifia cultivars.

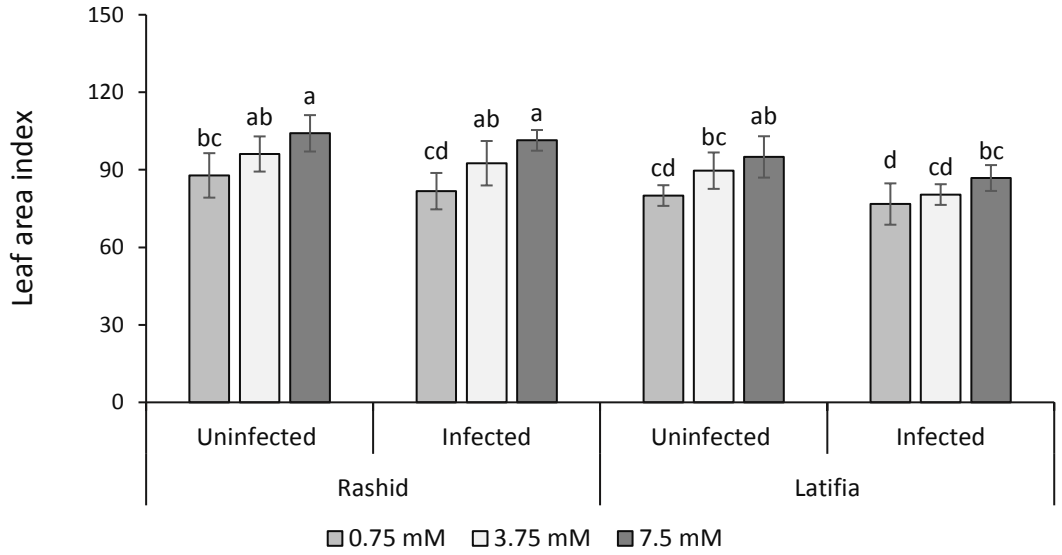


Figure 3.6. Effect of three levels of nitrogen supply (0.75, 3.75 and standard level 7.5 mM nitrate) on Leaf area index in uninfected and infected (four dpi by spot blotch) Rashid and Latifia wheat cultivars at 18 days old.
Different letters on bars represent significant differences (ANOVA, three way, Tukey $P < 0.05$, n= 9), error bars represent ± 1 standard deviation.

Wheat grown with 0.75 mM nitrate (representing severe nitrogen stress) had the lowest spot blotch disease symptoms. The lowest extent of necrotic and chlorotic lesions was observed on infected plants, with mean score 9.33 and 2.08%, followed by 3.75 mM nitrate (mean disease scores 14.0, 2.48% in Rashid and Latifia respectively) as shown in Figure 3.7.

The concentration 7.5 mM nitrate gave the highest disease severity of spot blotch: the highest extent of necrotic and chlorotic lesions was observed on infected plants, with mean scores of 23.0 and 3.83% in Rashid and Latifia respectively. However, scores for Rashid were significantly different (ANOVA, Tukey, $P < 0.05$) from scores for Latifia at the same level of nitrogen input. Different effects of differences in nitrogen availability on spot blotch disease severity were recorded in Rashid and Latifia wheat cultivars at 18 days old.

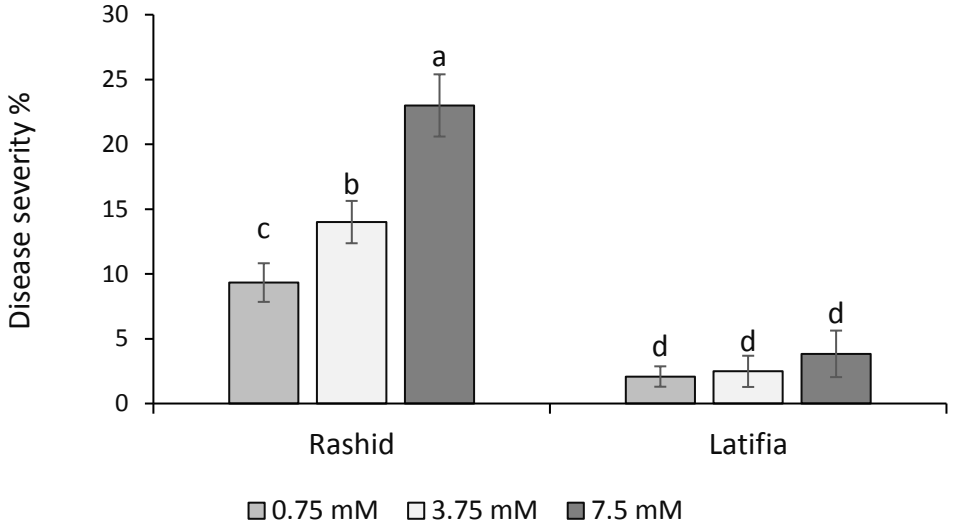


Figure 3.7. Effect of three levels of nitrogen supply (0.75, 3.75 and standard level 7.5 mM nitrate) on disease severity % at four days after infection by spot blotch in Rashid and Latifia wheat cultivars at18 days old.
Different letters on bars represent significant differences (ANOVA, two way, Tukey $P < 0.05$, n= 9), error bars represent ± 1 standard deviation.

Numbers of spores were measured on leaves of platns infected at 14 days old, kept for four days, then incubated for a further four days in wet conditions. The number was calculated from the haemocytometer count as the number of spores per ml after resuspension as described in section 2.6.2. Spore counts increased with increasing nitrogen input from 0.75 to 3.75 and 7.5 mM nitrogen (Figure 3.8). In infected Rashid, there was a significant difference (ANOVA, Tukey, $P < 0.05$) in mean spore counts between standard level (7.5 mM; mean 19200) and moderate (3.75 mM) and low (0.75 mM) levels (means 14300 and 12560, respectively). Rashid was significantly different from Latifia at all nitrogen levels. On Latifia there was no significant difference (ANOVA, Tukey, $P > 0.05$) due to reducing nitrogen input from standard nitrogen level (mean 11800) to moderately reduced (mean 9400), and from moderately reduced to of severely reduced nitrogen (mean 7600). However, the count with high nitrogen was significantly different from the count with low nitrogen in Latifia.

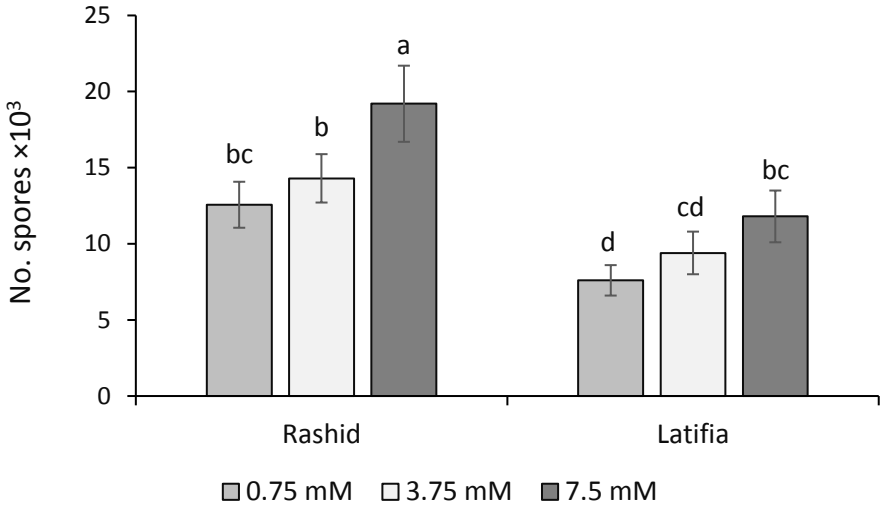


Figure 3.8. Effect of three levels of nitrogen supply (0.75, 3.75 and standard level 7.5 mM nitrate) on the number of spores on infected leaves with spot blotch transferred to high humidity at four dpi then incubated for four days for Rashid and Latifia wheat cultivars.
Different letters on bars represent significant differences (ANOVA, two way, Tukey, $P < 0.05$, n= 9), error bars represent ± 1 standard deviation.

3.6.4 Correlation between disease severity and other parameters

To understand the relationship between disease severity and other growth parameters, which is necessary to achieve our understanding of the role of these plant parameters in effect on plant responding to spot blotch infection, correlations between results were investigated. The results in (Table 3.1) showed positive correlation values of disease severity with most parameters studied, except chlorophyll content in leaves, which was negative. The positive values of correlation mean that disease severity symptoms increased with increasing size and biomass of plants at high nitrogen input. However, the correlation was only significant ($P < 0.05$; $r = 0.736$) for leaf area in Rashid wheat. Chlorophyll content showed a negative correlation value that was significant ($P < 0.05$) in susceptible Rashid that is mean chlorophyll content in leaves decreased with increasing disease severity symptoms in plants under increasing nitrogen availability. However, the number of spores showed a great correlation with disease severity; it was significant ($P < 0.05$ and $P < 0.01$) in Latifia and Rashid wheat cultivars, respectively. The coefficient of determination (R^2) for the relationship between leaf area and disease severity was higher at 54% in Rashid than in Latifia at 40%. R^2 values between disease severity and number of spores were 89% and 64% in Rashid and Latifia, respectively. The high values of coefficient of determination can be explained by a linear relationship between the number of spores and disease severity. By contrast, chlorophyll content, which had a high correlation with disease severity, has an R^2 value of 52% in Rashid, meaning that 52% of the total variation in values of chlorophyll content in leaves can be explained by the linear relationship between

chlorophyll content and disease severity in Rashid cultivar. This means that chlorophyll content in leaves decreased with increasing disease severity symptoms in plants under increasing nitrogen availability.

Table 3.1. Correlation values (r) and coefficient of detection (R^2) of disease severity display with other growth parameters in infected Rashid and Latifia wheat cultivars across nitrogen input (critical r value is 0.666 and 0.798 at P < 0.05*, 0.01**, df=n-2=7).

Wheat cultivar		Shoot height	No. leaves	No. tillers	Chlorophyll content	Leaf area index	No. spores
Rashid	**r**	0.617	0.504	0.496	-0.721*	0.736*	0.943**
	R^2	0.381	0.254	0.246	0.520	0.542	0.889
Latifia	**r**	0.364	0.484	0.318	-0.282	0.637	0.797*
	R^2	0.132	0.234	0.101	0.079	0.403	0.635

3.6.5 Physiological measurements in uninfected and infected Brachypodium

The physiological measurements in *Brachypodium distachyon* (Bd21) were carried out to develop our understanding of the effects of different levels of nitrogen input on plant growth and the presence and absence of spot blotch infection at 31 day old plants. Shoot height was the greatest at the standard level of nitrogen, with mean values 11.5 cm and 11.3 cm (Figure 3.9 A) in the uninfected and infected plants, respectively. These results were significantly different (ANOVA, Tukey, P < 0.05) in the infected plants between severely reduced stress and standard input, with no significant difference of both from moderate nitrogen input (ANOVA, Tukey, P > 0.05). However, uninfected plants showed no statistical difference among treatments. Numbers of leaves and numbers of tillers were high with moderate nitrogen, with mean scores 18.5 and 18 leaves Figure 3.9 B, 8.3 and 7.75 tillers Figure 3.9 C in the uninfected and infected plant, respectively. These results were significantly different compared with severely reduced nitrogen stress (0.75 mM), whereas they were not significantly different from the standard level of nitrogen (7.5 mM) in uninfected and infected plants.

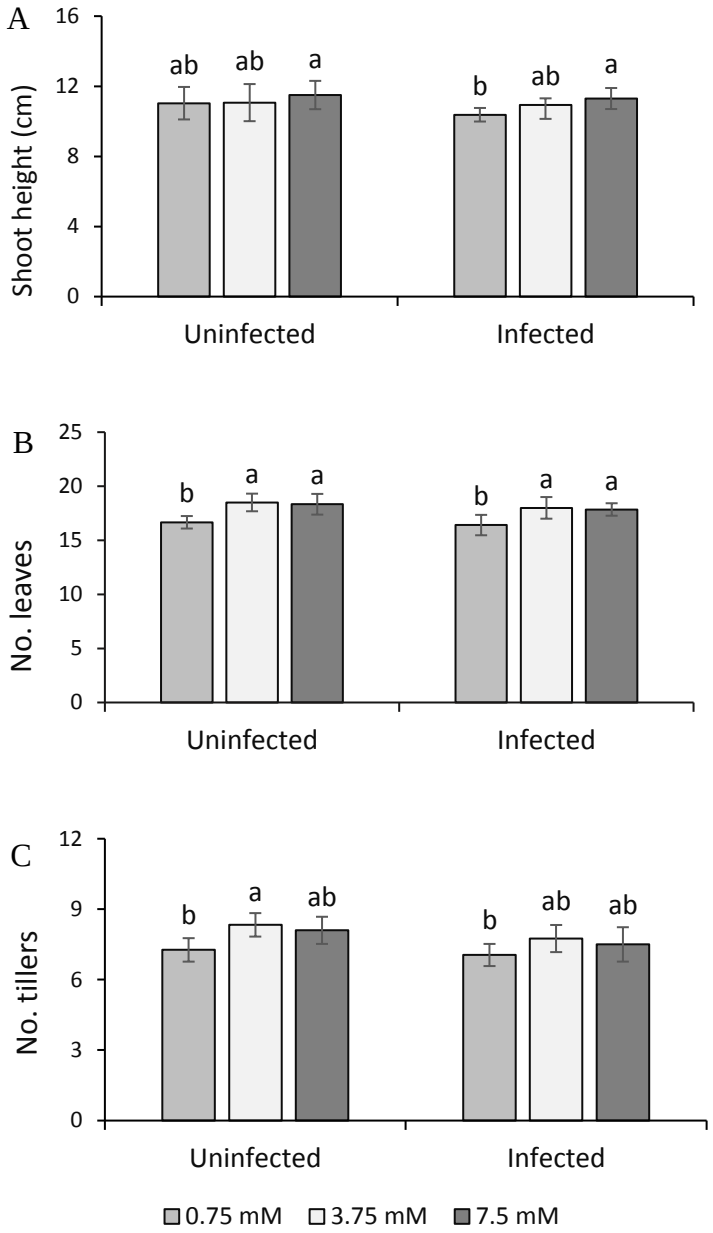


Figure 3.9. Shoot height (cm) (A), Number of leaves (B) and Number of tillers (C) in Brachypodium was assessed for their consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM in uninfected 31 old and infected plant (four dpi) by spot blotch.
Different letters on bars represent significant differences (ANOVA, two way, Tukey $P < 0.05$, n= 9), error bars represent ± 1 standard deviation.

Values of chlorophyll content index were high at the standard level of nitrogen, with mean scores 4.33 and 3.75 (Figure 3.10) in the uninfected and infected plants, respectively. These results were significantly different (ANOVA, Tukey, $P < 0.05$) from the infected plants with severely and moderately reduced nitrogen levels, and no significant difference in uninfected Brachypodium (ANOVA, Tukey, $P > 0.05$).

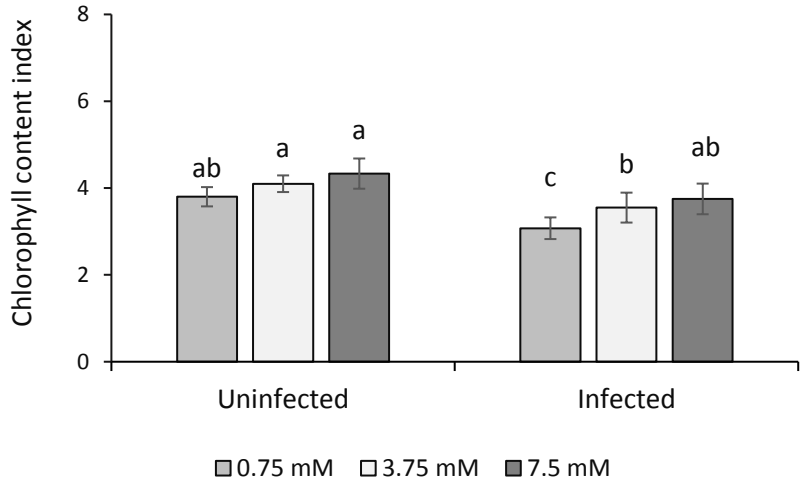


Figure 3.10. Chlorophyll content index (CCI) in Brachypodium was assessed for their consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM in the uninfected and infected plant (four dpi) at 31 days old of the plants.
Different letters on bars represent significant differences (ANOVA, two way, Tukey $P < 0.05$, n= 9), error bars represent ± 1 standard deviation.

Disease severity was measured in Brachypodium to *Bipolaris sorokiniana* under nitrogen stress (Figure 3.11). Plants grown with severely reduced nitrogen (0.75 mM) had significantly lower disease severity % (ANOVA, Tukey, $P < 0.05$) than plants growth with moderately reduced nitrogen and standard nitrogen level. There was no difference between the last two concentrations (3.75 mM and 7.5 mM nitrogen). From these results, it was determined that the reduction in disease severity was clear by reducing nitrogen input in both wheat (Rashid and Latifia) and Brachypodium plants. However, plants were grown under severely reduced nitrogen level (0.75 mM) had the lowest spot blotch attack.

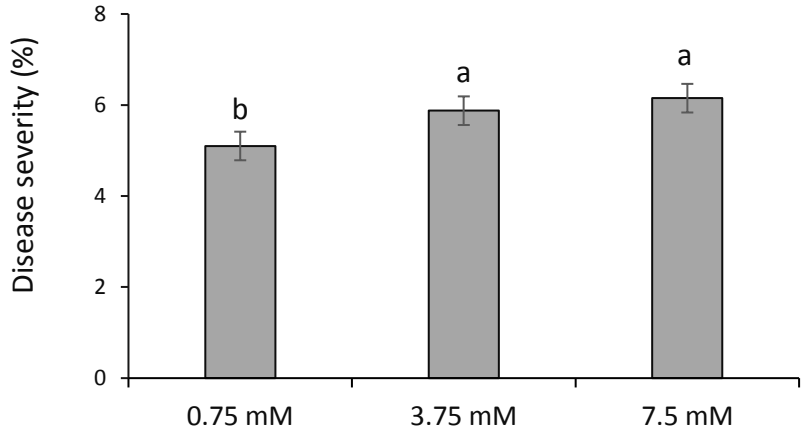


Figure 3.11. Disease severity (%) in Brachypodium was assessed for their consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM nitrate after four days infected plants at 31 days old.
Different letters on bars represent significant differences (ANOVA, one way, Tukey $P < 0.05$, n= 9), error bars represent ± 1 standard deviation.

3.6.6 Validation of qPCR as a method of pathogen quantification

Expression of the internal transcribed sequence (ITS) of rRNA was tested with the non-specific primer pair *ITS1-ITS4* and two primer pairs specific to *Bipolaris*, *Coch* and *BipITS*, which amplify sequences of 357 and 107 bp (NCBI accession No: FJ609737.1), respectively. Figure 3.12A shows expression of *Bipolaris*, *Coch ITS1-ITS4* and *BipITS* primers were normalised to wheat endogenous control (Eukaryotic Initiation Factor 4A, *eIF4A*) and (Figure 3.12 B) shows expression of *Bipolaris*, *Coch ITS1-ITS4* and *BipITS* primers were normalised to *B. sorokiniana* 28S rRNA in Rashid cultivar. Wheat grown with low nitrogen (0.75 mM), which had the lowest level of *B. sorokiniana* symptoms, was used as the reference condition in comparison with moderate (3.75 mM) and standard nitrogen availability (7.5 mM), that showed high disease severity compared with 0.75 mM nitrogen. Quantitative real-time PCR showed that expression of the ITS region as measured with all three primer pairs increased with increasing nitrogen availability, associated with increased disease severity and increased sporulation (Figure 3.7). Relative expression was significantly upregulated (confidence intervals, $P < 0.001$), 8.8-fold for *Coch*, 5.5-fold for *ITS1-ITS4* and 5.3-fold for *BipITS* region at optimum nitrogen level (7.5 mM) compared to low nitrogen input (0.75 mM) (Figure 3.12 A). For moderately reduced nitrogen compared to low nitrogen level, Coch showed significantly upregulated (CI, $P < 0.01$) with 3.4-fold, whereas, *ITS1-ITS4* showed significantly upregulated (CI, $P<0.05$) with 2.5-fold, but there were no significant changes in *BipITS* expression when grown under reduced nitrogen with 2.4-fold. The effect of nitrogen input was much less for expression relative to *B. sorokiniana 28s rRNA*. However, the difference in expression of three genes when the comparison between severely reduced nitrogen level and optimum nitrogen availability was only 2.2-fold for *Coch*, 1.4-fold for *ITS1-ITS4* and 1.3-fold for *BipITS*. Expression of all three genes (*Coch*, *ITS1-ITS4* and *BipITS*) were not significant in the severely reduced nitrogen and moderately reduced nitrogen level (Figure 3.12 B)

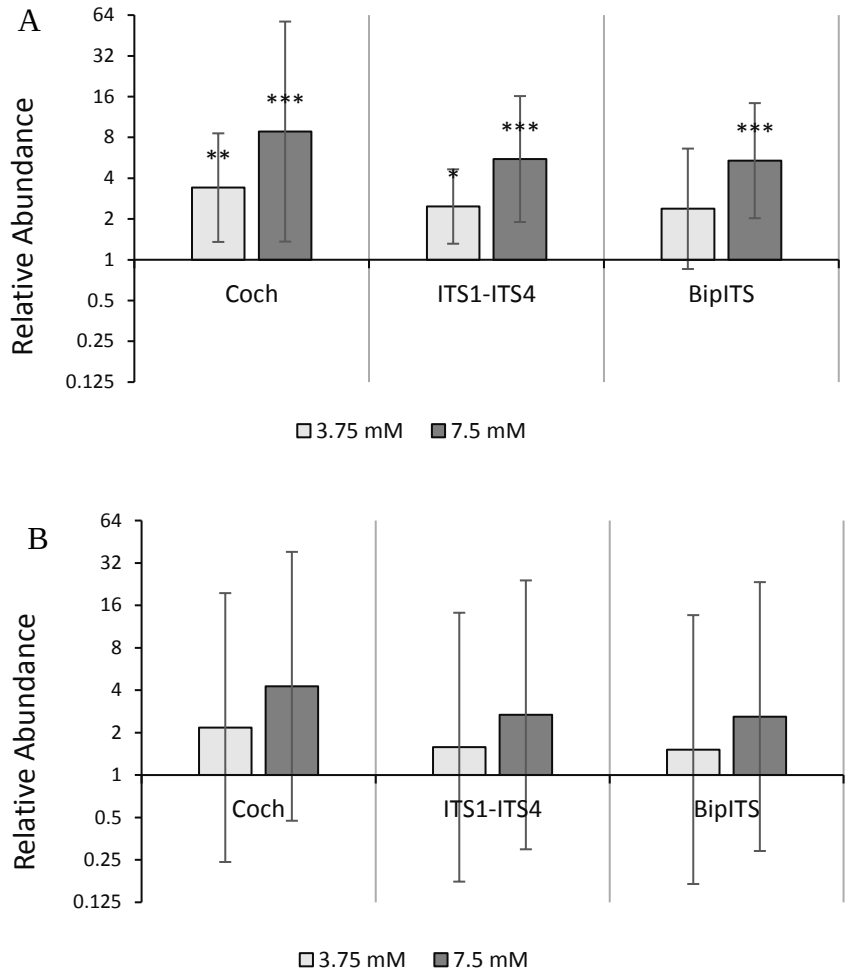


Figure 3.12. Relative abundance of *Bipolaris sorokiniana* RNA amplified with *Coch*, *ITS1-ITS4* and *BipITS* primers in infected Rashid were normalised to (A) wheat endogenous control (*eIF4A*), (B) *Bipolaris sorokiniana* endogenous control (*Bs28S rRNA*).
Expression was measured with different nitrogen availability (7.5 mM, 3.75 mM compared to 0.75 mM nitrate) in infected Rashid wheat with spot blotch four dpi. Error bars represent confidence intervals; bars that do not cross the x-axis indicate the difference is statistically significant at a t-test ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***, n= 9) within each gene.

For Latifia wheat cultivar, again, wheat grown with low nitrogen (0.75 mM nitrate), which had the lowest levels of *Bipolaris sorokiniana* symptoms, was used as the reference condition in comparison with moderate reduced nitrogen level (3.75 mM) and standard nitrogen availability (7.5 mM). Relative RNA abundance increased with increasing disease severity and increasing nitrogen availability (Figure 3.13 A). The difference in expression was significant (CI, $P < 0.01$) with *Coch* and *BipITS* primers. Fold differences were 5.2-fold with *BipITS*, 4.6-fold with *Coch* and 3.8-fold with *ITS1-ITS4* for standard nitrogen level compared with low nitrogen input. With moderate nitrogen, the fold changes were 3.4-fold with *Coch*, 3.3-fold with *BipITS*, both of which were significant ($p < 0.05$), but the difference with *ITS1-ITS4* primers was 2.6-fold and non-significant (CI, $P > 0.05$). Normalise to *B. sorokiniana* 28S rRNA, differences in relative RNA abundance between plants grown with low and standard nitrogen input were small (Figure

3.13 B), but *BipITS* and *Coch* primers showed significant up-regulation (CI, $P < 0.05$) with 2.4-fold change for *BipITS* , 2.1-fold for *Coch* and 1.7-fold for *ITS1-ITS4*. Differences between low and moderate nitrogen were not significant (confidence interval, $p > 0.05$), with 1.5-fold for *Coch*, 1.4-fold for *BipITS* and 1.1-fold for *ITS1-ITS4*.

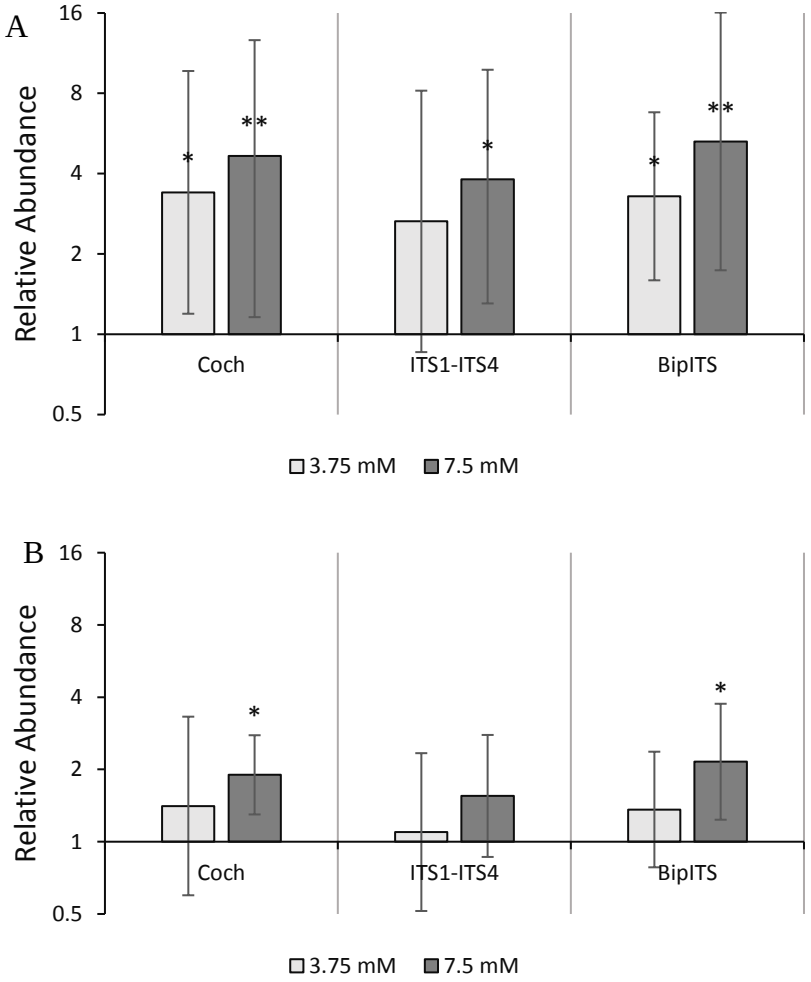


Figure 3.13. Relative abundance (fold difference) of *Bipolaris sorokiniana* RNA (*Coch, ITS1-ITS4* and *BipITS*) in infected Latifia is displayed normalised to (A) endogenous control in wheat (Eukaryotic Initiation Factor 4A (*eIF4A*), (B) *Bipolaris sorokiniana* endogenous control (28S ribosomal rRNA –*Bs28S rRNA*).
Expression was measured with different nitrogen availability (7.5 mM, 3.75 mM compared to 0.75 mM nitrate) in infected Rashid wheat cultivars with spot blotch four dpi. Error bars represent confidence intervals; bars that do not cross the x-axis indicate the difference is statistically significant at a t-test ($P < 0.05$*, $P < 0.01$**, n= 9) within each gene.

Abundance of *Bipolaris sorokiniana* RNA was examined in infected Brachypodium plants with *Coch, ITS1-ITS4* and *BipITS* primers were normalised to Brachypodium endogenous control, Elongation Factor 1α -*BdEF1α* (Figure 3.14 A) and *Bs28S rRNA* (Figure 3.14 B). Relative RNA abundance with the three primers pairs was tested under the combined effects of reduced nitrogen availability (7.5 mM, 3.75 mM and 0.75 mM) in infected Brachypodium. Brachypodium assessed as having low spot blotch symptoms was used as the reference

condition (0.75 mM nitrogen). Quantitative real-time PCR showed that the three candidate genes increased in expression with increasing *Bipolaris sorokiniana* prevalence by increasing nitrogen availability from severely reduced to moderate and standard level (0.75 mM, 3.75 mM and 7.5 mM) (Figure 3.14 A and B).

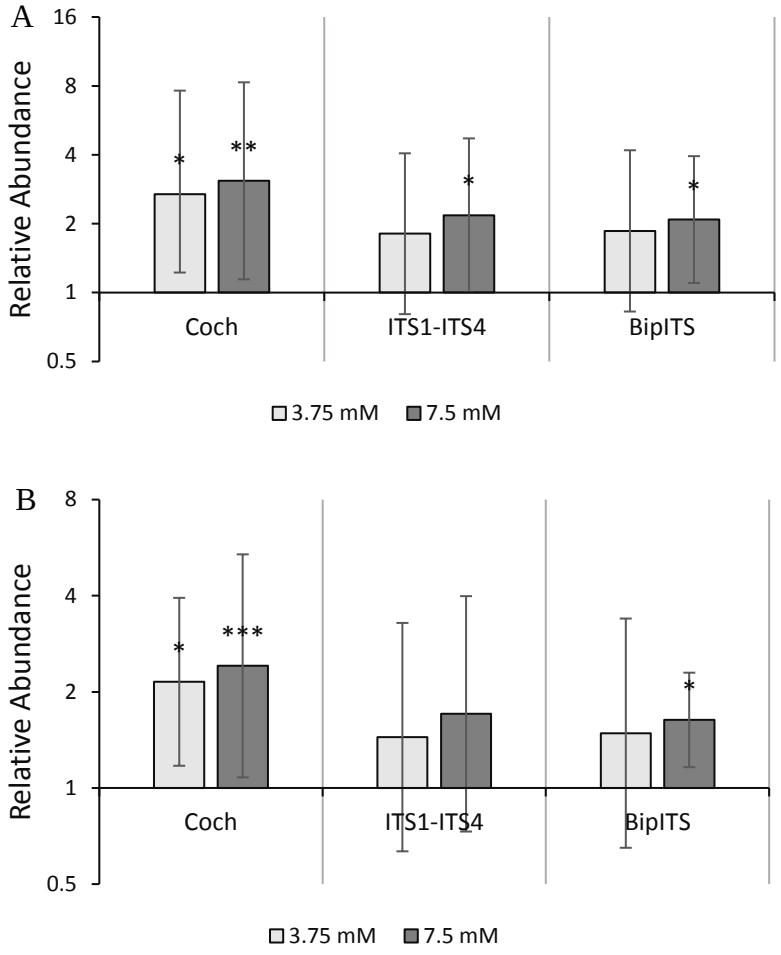


Figure 3.14. Relative abundance (fold difference) of *Bipolaris sorokiniana* RNA (*Coch*, *ITS1-ITS4* and *BipITS* primers) in infected Brachypodium is displayed normalised to endogenous control (A) Brachypodium Elongation Factor 1α (*BdEF1α*) and (B) *B. sorokiniana* 28S ribosomal rRNA (*Bs28S rRNA*).
Expression was measured with different nitrogen availability (7.5 mM, 3.75 mM compared to 0.75 mM nitrate) in infected Rashid wheat cultivars with spot blotch four dpi. Error bars represent confidence intervals; bars that do not cross the x-axis indicate the difference is statistically significant at a t-test ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***, n= 9) within each gene.

Fold difference in abundance of other *Bipolaris sorokiniana* endogenous RNAs (*BsGPD*, *Bs18S rRNA* and *Bs28S rRNA*) in infected wheat is normalised to wheat endogenous control (Eukaryotic Initiation Factor 4A, *eIF4A*) (Figure 3.15). Wheat assessed as having very low levels of spot blotch symptoms was used as the reference condition that was severely reduced nitrogen level (0.75 mM nitrate). Quantitative real-time PCR showed that relative abundance of the three RNAs increased in with increasing *Bipolaris sorokiniana* prevalence associated

with increasing nitrogen availability from severely reduced to the standard level (0.75 mM, 3.75 mM and 7.5 mM nitrate) in infected wheat with spot blotch.

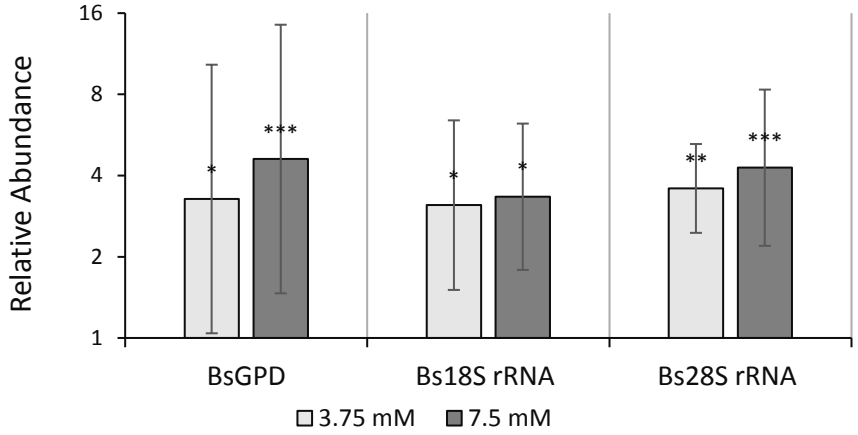


Figure 3.15. Fold difference of *Bipolaris sorokiniana* endogenous control genes (*BsGPD, Bs18S rRNA* and *28S rRNA*) is normalised to wheat endogenous control (Eukaryotic Initiation Factor 4A (*eIF4A*) in infected wheat.
Expression was measured with different nitrogen availability (7.5 mM, 3.75 mM compared to 0.75 mM) in infected Rashid wheat with spot blotch four dpi. Error bars represent confidence intervals; bars that do not cross the x-axis indicate the difference is statistically significant at a t-test ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***, n= 9) within each gene.

3.6.7 Validation of used genes in different conditions

Testing amplification of used genes is necessary to build our knowledge about these genes in the conditions in which they are to be used shows higher amplification of *rbcL* (coding for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) in healthy leaves (very prominent band) than in infected wheat leaves with spot blotch. Infected leaves in the nitrogen level 7.5 mM gave a slightly lighter band than with 0.75 and 3.75 mM nitrogen (Figure 3.16). Expression of *rbcL* expression (LT576864.1) is studied in the next chapters. However, amplification with the *ITS1-ITS4* (internal transcribed spacer) primers showed a distinct case when it was examined under different conditions. In infected leaves, there were two different amplification products of the *ITS1-ITS4* region, 586 bp and 687 bp, whereas in uninfected plants there was only one band at 687 bp as expected (FJ609737.1). In DNA extracted from spores of *B. sorokiniana*, *ITS1-ITS4* primers gave one band at 586 bp (KY985464.1). Therefore, *ITS1-ITS4* can amplify sequences from both organisms, wheat and *B. sorokiniana*. The integrity of the genes was analysed by separation on 1.5% (w/v) agarose gels, and bands were visualized by ethidium bromide staining under UV.

Figure 3.16. Agarose gel of amplified RNA products from uninfected and infected Rashid wheat cultivar.
Sizes of the *rbcL* RNA (653bp), *ITS1-ITS4* region RNA (687 bp in the uninfected plant, 586 bp in DNA of *B. sorokiniana* spores). Rashid was grown with 0.75, 3.75 and 7.5 mM nitrate (Hoagland's solution), NTC = water.

To analyse the expression of *ITS1-ITS4* in uninfected and infected Rashid and Latifia by spot blotch with decreasing nitrogen input from 7.5 mM to 3.75 mM to 0.75 mM nitrate. Relative levels of gene transcript were normalised against Eukaryotic Initiation Factor 4A (*eIF4A*). Figure 3.17 shows that in uninfected plants of Rashid and Latifia, there was no significant up-regulation of expression measured with *ITS1-ITS4* with either severely reduced or moderately reduced nitrogen compared to the standard level of nitrogen (7.5 mM). In infected Rashid, the RNA abundance measured with *ITS1-ITS4* was significantly lower with severely and moderately reduced nitrogen (CI, $P < 0.01$, $P < 0.05$, respectively) than with standard nitrogen. RNA abundance measured with *ITS1-ITS4* was also lower in Latifia with both severely and moderately reduced nitrogen, but the difference from standard nitrogen was only significant with the severe stress (CI, $P < 0.05$).

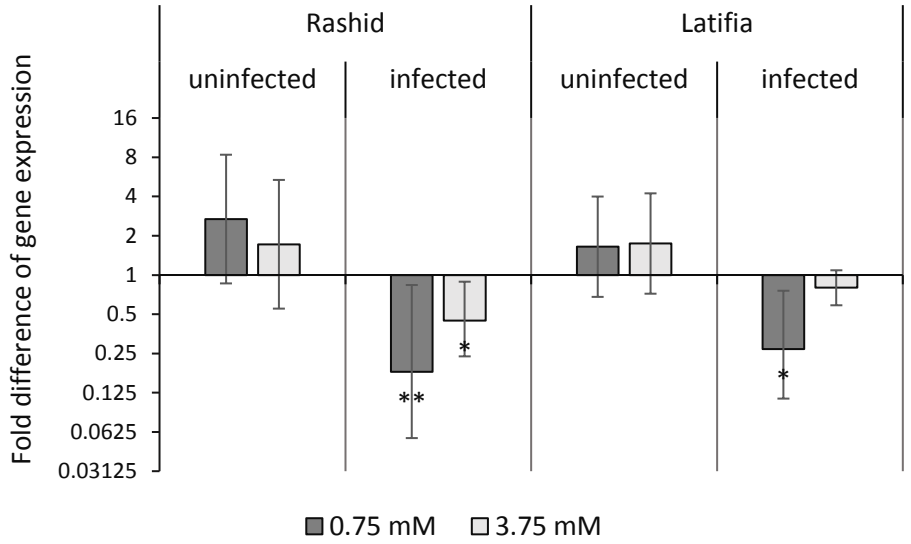


Figure 3.17. Expression of internal transcribed spacer amplified with the *ITS1-ITS4* primer pair across nitrogen treatments in uninfected and infected wheat.
Reduced nitrogen treatments (0.75 mM, 3.75 mM) were compared to 7.5 mM NO_3^-. Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised against wheat endogenous control gene (*eIF4A*). Error bars show confidence intervals ($P < 0.05$*, $P < 0.01$**, $n = 9$).

This finding is perhaps more pertinently explained by analysing changes in RNA abundance measured with *ITS1-ITS4* primers in response to *Bipolaris sorokiniana* infection for plants that were grown at each nitrogen condition separately (Figure 3.18). Fold differences in infected wheat are displayed relative to uninfected plants grown at the same nitrogen concentration. RNA abundance was greater in infected plants at all nitrate concentrations, confirming that *ITS1-ITS4* expression responds to spot blotch impact. Besides, fold changes in Rashid wheat, which showed more susceptibility to spot blotch, were higher than in Latifia wheat. The increases in relative RNA abundance were significant in Rashid wheat (CI, $P < 0.01$) with both severely reduced nitrogen with 3.1-fold (low disease severity) and moderately reduced nitrogen with 4.7-fold (moderate disease severity), and highly significant (Cl, $P < 0.001$) at 7.5 mM nitrogen (high disease severity), where the highest fold change was 10.3. In Latifia, RNA abundance measured with *ITS1-ITS4* showed significant up-regulation with 2.2-fold (Confidence Intervals, $P < 0.05$) in moderately reduced nitrogen, and significant with 4.3-fold (CI, $P < 0.01$) in standard nitrogen input. With severely reduced nitrogen (low disease severity), RNA abundance measured with *ITS1-ITS4* was not significantly higher (CI, $P > 0.05$), with the lowest fold difference in expression, only 1.8-fold. These differences in *ITS1-ITS4* expression confirm the effect of spot blotch infection. However, expression of *ITS1-ITS4* showed significantly approximately 50% higher transcribed (ANOVA, $P < 0.05$) in Rashid cultivar compared to expression in Latifia wheat grown at the same nitrogen concentration. This finding

ensures that increases in the expression of ITS regions result from the increase in spot blotch prevalence in leaves of susceptible Rashid that was more than in resistant Latifia wheat.

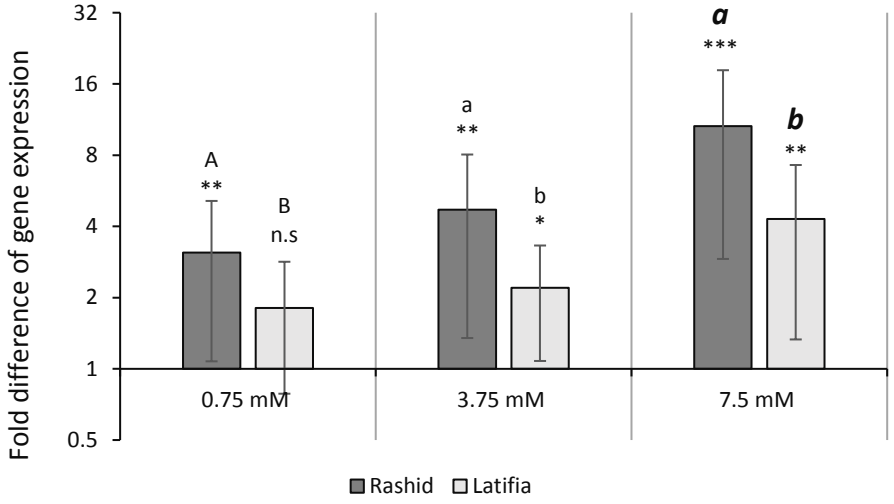


Figure 3.18. Fold difference in expression of internal transcribed spacer sequences amplified with the *ITS1-ITS4* primer pair in infected Rashid and Latifia wheat cultivars. Fold differences are displayed relative to uninfected plants grown at the same nitrogen concentration.
Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised against wheat endogenous control gene (*eIF4A*). Error bars (confidence intervals) that do not cross the x-axis indicate the difference is statistically significant at a t-test (P< 0.05*, P< 0.01**, P< 0.001***, n = 9). Different letters on bars represent significant differences displayed comparison between Rashid and Latifia cultivars grown at the same nitrogen concentration (ANOVA, one way, Tukey P < 0.05, n= 9).

3.7 Discussion

Nitrogen input affects the pattern of growth of plants, (Hinzman *et al.*, 1986; Byrnes, 1990) and is usually in the form of fertiliser application as an input, with crop yield as the output (Delogu *et al.*, 1998), as discussed in more depth in Chapter 1, section 1.11. The purpose of this study was not to establish that nitrogen input has an effect on the growth of wheat, but to investigate the effect of increased nitrogen on spot blotch severity. However, it was important to ensure that including nitrogen was appropriate for the current study. The preliminary findings in this study generally verified that reducing the nitrogen concentration from the standard level of 7.5 mM (optimum) to 3.75 mM reduced growth, and that reduction to 0.75 mM resulted in much less growth. However, only plants grown with 0.75 mM nitrogen were statistically significantly smaller than those grown with standard nitrogen, and they did not consistently differ from plants grown with 3.75 mM nitrogen input at 18 days old. Plants grown with low nitrogen input had significantly fewer leaves and tillers, lower chlorophyll content index and leaf area index and shorter shoots than those supplied with 3.75 mM or 7.5 mM nitrate. These results are consistent

with Hinzman *et al.* (1986) who found a lower chlorophyll content and reduced leaf area with reduced nitrogen input.

The total surface area of plants was greater at high nitrogen input than at reduced nitrogen levels in uninfected and infected Rashid and infected Latifia, which was accompanied by a higher chlorophyll content. This could potentially be an important factor in determining spot blotch resistance and possibly help to explain the increased spot blotch severity under higher nitrogen. Therefore, it was necessary to analyse the relationship between nitrogen supply and plant size, and the effect of plant size on spot blotch disease severity, as well as the effects of disease severity on the chlorophyll content of infected plants that had different defence responses to spot blotch. Plants grown with severely reduced nitrogen showed a significant reduction in leaf area index, with smaller surface size compared to the moderate and standard levels of nitrogen for both Rashid and Latifia at 18 days old. Chlorophyll content in infected plants that were grown with low nitrogen was significantly lower than with the standard nitrogen level but not significantly lower than with moderate nitrogen input in Rashid and Latifia. When the association between chlorophyll content and disease spread was examined, the results showed that the percentage reductions in chlorophyll content due to disease were 38%, 32% and 34% in Rashid and 21%, 20% and 25% in Latifia at nitrogen levels 7.5, 3.75 and 0.75 mM, respectively, showing that the relative loss of chlorophyll content was higher in infected Rashid than Latifia.

The correlation coefficient (r) and the coefficient of determination (R^2) showed a strong relationship between disease severity and other traits. The relationship between disease severity and leaf area index had the highest correlation coefficient (r = 0.736; the positive relationship means increased leaf surface area is increasing disease impact). The coefficient of determination (R^2) was 54%, meaning that variation in leaf area contributed 54% of variation in disease severity in Rashid wheat. However, in Latifia, the correlation coefficient was 0.64 and coefficient of determination 40%. By contrast, in Rashid disease severity was negatively correlated with chlorophyll content (r = -0.721; negative relationship means increased disease severity was associated with more loss of chlorophyll caused by the pathogen) and the coefficient of determination was 52%. This relationship was largely absent in Latifia, with r = -0.28 and R^2, 8%. Thus the reduction in chlorophyll content appears to be a good indicator of disease severity.

In the current study, there was a trend to higher disease severity and number of spores in infected wheat at the higher nitrogen input, particularly for the more susceptible Rashid at 7.5

mM nitrate. This finding is consistent with Loyce *et al.* (2008) who found that reducing nitrogen input decreased disease severity with several pathogens in wheat. However, other studies have found that higher levels of disease severity were associated with low soil fertility or low-N fertiliser treatment in field conditions (Sharma and Duveiller, 2004; Krupinsky *et al.*, 2007) making quantitative comparisons to the findings of this study difficult.

In addition to the many advantages of obtaining a quantitative measure by qPCR of disease severity, this method also enables detection of the pathogen in the early biotrophic phase or during the symptomless biotrophic phase (Wessling and Panstruga, 2012). The hyphae of spores need 12 hours to enable to penetrate the host's cuticle under appropriate conditions (Sahu *et al.*, 2016) and multiply rapidly, spreading into the intercellular space within the mesophyll tissue of the plant leaves (Acharya *et al.*, 2011), then 48 hours is enough to produce a new generation of conidia. However, quantification of fungal prevalence in the plant tissues can be measured by using qPCR based on the fungal load present in infected samples before conidia are produced. (Wessling and Panstruga, 2012).

It was possible to demonstrate that quantitative PCR can be used to assess load pathogen development in wheat. Samples of uninfected tissue gave no amplification products with *BipITS* and *Coch* primers, confirming the specificity of these primers for infected tissue. This result makes uninfected tissue unsuitable as a reference condition for relative gene expression analysis. To provide a suitable alternative reference for comparison between different conditions, low disease severity at low nitrogen input (0.75 mM nitrate) was chosen as a reference condition and assigned a value of 1. The abundance of RNA regions amplified with *BipITS* and *Coch* primers with moderate (moderate disease severity) and high nitrogen input (high disease severity) was compared to abundance of both regions with low spot blotch prevalence at low nitrogen input (0.75 mM). Eukaryotic Initiation Factor 4A (*eIF4A*) and *Bipolaris sorokiniana 28S rRNA* genes were used as endogenous controls, as *eIF4A* has been demonstrated to be a suitable endogenous control (Jarošová and Kundu 2010) to normalise gene expression against total wheat cDNA. Quantitative real-time PCR showed the relative RNA abundance with *BipITS*, *Coch* and *ITS1-ITS4* primers increased with high *Bipolaris sorokiniana* prevalence at increasing nitrogen availability from 3.75 mM to 7.5 mM in infected Rashid and Latifia plants. The *BipITS* and *Coch* regions showed significantly higher relative RNA abundance when normalised with Eukaryotic Initiation Factor 4A (*eIF4A*) than a spot blotch reference gene (*28S rRNA*). Generally, there was a good similarity of expression measured with *BipITS and Coch* primers between different infected plants, but Rashid had higher values of expression than Latifia. Expression in Brachypodium showed some similarity to the expression

in Latifia. Overall, the evidence suggests that the abundance of *BipITS* and *Coch* regions increased as disease severity progressed. It was concluded that measuring the abundance of *BipITS* and *Coch* regions represents a reliable, quantitative method of assessing disease severity during the biotrophic phase of infection.

Amplification of the internal transcribed spacer with *ITS1-ITS4* primers showed a distinct case when it was examined under different conditions. These primers can be involved in the amplification of sequences from both organisms, wheat and *B. sorokiniana*. White *et al.* (1990) used rRNA sequences including rice (*Oryza sativa*) to design the *ITS1-ITS4* primer pair, and the same primer sequences were used in this study. The *ITS1-ITS4* pair was tested by measuring the coefficient of determination (R^2 =99%); this uses a serial dilution of cDNA and DNA from infected samples to predict the linearity of the Cq data and assesses the reliability seen across the technical replicates (Taylor *et al.*, 2010). Figure 3.17 shows that relative RNA abundance measured with *ITS1-ITS4* did not differ significantly with either severely reduced or moderate nitrogen availability compared to the standard nitrogen level of 7.5 mM in both uninfected Rashid and Latifia wheat cultivars. However, there was significantly lower relative abundance with severely and moderately reduced nitrogen, respectively, in infected Rashid, whereas the difference was only significant with the severely reduced nitrogen level in Latifia.

Because the *ITS1-ITS4* primer pair amplifies sequences from uninfected plants, more understanding of the results can be obtained by analysing differences in RNA abundance measured with *ITS1-ITS4* in response to *Bipolaris sorokiniana* infection of plants that were grown at all three nitrogen levels compared to uninfected plants (Figure 3.18). Relative expression was up-regulated at all nitrogen concentrations, confirming that differences in *ITS1-ITS4* expression are due to infection. Besides, the fold difference values with *ITS1-ITS4* were higher in Rashid than in Latifia. To summarise, *ITS1-ITS4, BipITS* and *Coch* primers provide a reproducible scale by which qPCR data could be directly mapped to absolute fungal load. Such a scale could offer rapid, reliable pathogen quantification in future studies.

In addition to the many advantages of obtaining a quantitative measurement of disease severity, this method also enables detection of infection during the biotrophic phase. Methods which rely on the observation of symptom (Simon *et al.*, 2003; Loyce *et al.*, 2008; Domiciano *et al.*, 2014) cannot be used during the symptomless biotrophic phase, and a similar problem is encountered when spore washes are used to assess the fungal load in an infected sample before conidia are produced. Therefore, quantitative PCR is a novel method for assessing the effect of nitrogen availability on spot blotch severity at an earlier stage than was previously possible.

Microsatellites provide readily detectable markers for agronomically important genes and quantitatively inherited traits and facilitate their handling in segregating breeding populations. The results of microsatellite genetic diversity analysis by Principal Coordinate Analysis (PCoA) showed some grouping of cultivars (Figure 3.3). The molecular marker results based on the PCR technique showed that the most resistant wheat (Latifia) was most closely linked to another resistant wheat (Tamoz3) in one group. Rashid and Aurok showed high susceptibility to spot blotch in a second group. Thus, there is a high possibility that these cultivars have similar gene pools and may contain genes for resistance to spot blotch, and cultivars derived from the same parents should be more similar genetically (Roder *et al.*, 2002). A similar observation was described by Plaschke *et al.* (1995), who found that two sister lines could not be distinguished using 23 wheat microsatellites. Thus, these products can be applied relatively easily in wheat breeding programmes against spot blotch.

3.8 Conclusions

To investigate the interaction between nitrate supply, spot blotch disease severity and regulation of defence gene expression in Iraqi commercial wheat. The susceptibility of five commercial Iraqi wheat varieties and one UK wheat variety (Cordiale) to spot blotch was tested. Rashid was the most susceptible and Latifia was the least. Rashid and Latifia cultivars were selected for further investigation.

Testing of the genetic diversity of wheat cultivars showed genetically similarity between Rashid and some other wheat cultivars with similar susceptibility, whilst the resistant Latifia cultivar was genetically similar to Tamoz3 cultivar, which showed high resistance to spot blotch. Rashid, Latifia and Brachypodium (model plant) supplied with 0.75 mM nitrate had significantly fewer leaves and tillers, lower chlorophyll content index, lower leaf area index and shorter shoots than those supplied with 3.75 mM or 7.5 mM nitrate. There was a trend to higher disease severity and number of spores in infected wheat at the higher nitrate levels, which was more marked for the more susceptible cultivar Rashid at 7.5 mM nitrate than for Latifia. The correlation coefficient was high between disease severity and other traits; it was positively correlated with number of spores and leaf area index, and highly negatively correlated with chlorophyll content in infected leaves. Quantitative real-time PCR analysis of expression of the *Bipolaris sorokiniana ITS* region in infected Rashid and Latifia wheat cultivars and Brachypodium showed that relative abundance of *ITS* region RNA increased with increasing spot blotch prevalence associated with increasing nitrogen availability from low to the standard nitrogen level (0.75 mM, 3.75 mM and 7.5 mM nitrate).

It is important to note that this work was carried out with wheat grown in growth room conditions, and the findings need to be verified with plants grown in the field in an agriculturally meaningful setting. For field studies, quantitative PCR may be considered as a novel method for assessing the effect of nitrogen availability on spot blotch development at an earlier stages than was previously possible. Furthermore, *Coch*, *ITS1-ITS4* and *BipITS* regions showed a quantitative increase in RNA abundance as disease severity increased; then qPCR can be used as a quantitative method of assessing pathogen load during the biotrophic phase of infection.

4 The effect of interaction between nitrogen supply and severity of *Bipolaris sorokiniana* on WRKY TFs expression in wheat cultivars

4.1 Abstract

The investigation of gene expression in response to stress is an excellent approach to crop improvement. The interaction between nitrogen supply, spot blotch disease severity and the regulation of WRKY TF defence gene expression in Iraqi commercial wheat and Brachypodium has investigated. Recent progress in research into the functions of these wheat WRKY genes and their homologues in Brachypodium is reviewed with a focus on their involvement in responses to abiotic and biotic stresses. Multiple tools have been tested in this project to optimise available candidate genes and the stability of endogenous control genes. Results for internal control gene-expression stability (*M*), the Bestkeeper Excel sheet tool and RefFinder suggest that *eIF4A* and *28S rRNA* are better normalisation candidates than *EF1a* and *18S rRNA*, and this conclusion is consistent across the three nitrogen treatments. Growth with reduced nitrogen resulted in the up-regulation of all seven *TaWRKY* genes and *PR1,* but only the *rbcL* gene showed down-regulation in both the Rashid and Latifia wheat cultivars. Expression of the WRKY genes in uninfected Brachypodium was highly similar to the expression of wheat WRKY genes.

There is a complex pattern of changes in the expression of genes coding for WRKY transcription factors in response to nitrate stress and infection by *B. sorokiniana*. There remains a great deal of potential in the use of WRKY genes to better understand how wheat responds to nitrogen stress and *B. sorokiniana* attack, and how the two might be linked. Continued investigation into WRKY gene function will provide a basis via which we can understand, interpret and potentially manipulate this link in a way which ensures wheat production is improved and ongoing global food security increased.

Keywords: WRKY transcription factor genes, Brachypodium, wheat, reducing nitrogen, *B. sorokiniana*, gene expression analysis, *rbcL*, *PR1,* housekeeping gene expression stability, quantitative RT-PCR.

4.2 Introduction

4.2.1 WRKY TF gene roles in response to stress

Gene expression changes in response to stress, and this response is essential for any living organism. Being sessile, plants have evolved the ability to respond rapidly and efficiently since they cannot move away from stressors to more suitable area with more optimal conditions. Furthermore, plants must adapt to survive when they encounter stress. Therefore, research to discover which genes are expressed in response to changing environmental conditions is needed in the field of molecular phytology.

The investigation of gene expression in response to stress is an excellent approach to crop improvement. Elucidating the genes that are differentially expressed in response to specific types of stress, can give insight into which genes are involved in the survival of plants under stress. Understanding genes that enable plants to withstand cold, drought or nitrogen stress (Mickelbart *et al.*, 2015), and bacteria, fungi, viruses and nematodes (Onaga and Wydra, 2016), for example, may allow us to use these genes as molecular markers in breeding programmes, or in engineering the overexpression of homologous genes in crop species to enhance tolerance.

Plant responses to protect themselves from attack by fungal pathogens are essential to maintaining the production of food crops (Ayliffe *et al.*, 2008). Equally, understanding the mechanisms by which some cultivars of the species are resistant to pest or pathogen, or how some conditions confer tolerance or resistance whilst others increase susceptibility, can allow us to transfer tolerance or resistance to other, commercially viable cultivars (Dangl *et al.*, 2013). *Bipolaris sorokiniana* is a serious pathogen of wheat grown in warm climates. It causes seedling blight, node cankers and spot blotch on wheat leaves as well as common root rot, depending on environmental conditions (Zillinsky, 1983). In the field, disease severity is increased by abiotic stresses such as low soil fertility (Sharma and Duveiller, 2004), Resistance in commercial wheat varieties is poor, thus improving resistance to spot blotch fungus is of major interest economically because it has the potential to improve food security for the future. It has been shown in this thesis that spot blotch severity and growth of *B. sorokiniana* are lower in plants grown under reduced nitrogen conditions than in those grown under conditions of high nitrogen availability, which appear to be more susceptible to the disease. Nitrogen availability is a critical factor in the cultivation of high-yield crops. Therefore it is not practical to grow wheat under reduced nitrogen availability in order to counter *B. sorokiniana*. However, if we can understand the mechanism which leads to increased tolerance under such conditions, we may be able to develop a novel approach to increase resistance to *B. sorokiniana* in the field. Understanding

this phenomenon starts with understanding the transcriptional reprogramming which occurs when the plant is grown under these stresses, and to determine what links them. In order to assess gene expression, several different techniques are commonly used. Furthermore, the pathogen is able to produce colonies within the host tissue 24 hours after infection and the hyphae produce conidiophores that emerge out of the host, giving rise to a succession of conidia by four days (Sahu *et al.*, 2016). Thus, four days is considered to be the end of the range of post-infection in this study.

This study focuses specifically on the expression of one family of proteins which have been identified as showing differential expression in response to spot blotch. Expression of WRKY transcription factor genes has been shown to be differentially regulated and these factors can act as activators or repressors in response to drought, cold, and salinity (Qin *et al.*, 2013; Wang *et al.*, 2013; Okay *et al.*, 2014), as well as to infection with Agrobacterium, powdery mildew, bacterial wilt, and rice blast (Deslandes *et al.*, 2003; Ding *et al.*, 2014; Liu *et al.*, 2016). A study by Bahrini *et al*. (2011b) indicated that *TaWRKY45* is involved in the defence systems for biotic stressors in wheat and that it may potentially be utilised to improve disease resistance, and it was observed that *TaWRKY45* was upregulated in response to benzothiadiazole (BTH), which induces a characteristic set of systemic acquired resistance (SAR) genes. In response to abiotic stress, including drought, cold and salinity, 41 of the 103 WRKY genes in rice have been shown to be differentially expressed (Ramamoorthy *et al.*, 2008). The knock-out of *WRKY3* and *WRKY4* expression in Arabidopsis has been shown to result in susceptibility to pathogens such as *Botrytis cinerea* (Lai *et al.*, 2008). It is apparent that this family of transcription factors, which is one of the largest in the plant kingdom (Bakshi and Oelmuller, 2014), and it is central to the response of plants to both abiotic and biotic stress.

4.2.2 Gene expression analysis

Gene expression analysis studies are increasingly important in various fields of biological research. The accurate normalisation of gene expression levels is essential for reliable results, especially when subtle differences in gene expression are biologically significant. Real-time PCR is the most commonly used tool to quantify the mRNA levels of different target genes (Vandesompele *et al.*, 2002). However, RT–qPCR experiments require normalisation to control for error between samples when measuring RNA expression and to control for confounding sources of variability in the experimental protocol, which involves the input sample, RNA extraction, reverse transcription and finishes with RT–qPCR data analysis (Tenea *et al.*, 2011). There are many methods used to control for this error. One approach is to normalise to total

RNA. The method most widely used is to normalise RNA levels by qPCR to an internal reference or housekeeping gene (De Spiegelaere *et al.*, 2015; Melgar–Rojas *et al.*, 2015; Gong *et al.*, 2016).

The level of expression of reference genes should be stable in the tissues of interest, and its expression level should not change over time or under different experimental conditions. Using unstable RNAs can strongly compromise data reliability (Vandesompele *et al.*, 2002; Melgar–Rojas *et al.*, 2015). However, commonly used reference genes can be differentially regulated (Tenea *et al.*, 2011). Therefore, it is critical to identify suitable reference genes for RT–qPCR data normalisation for each new experimental design in order to obtain precise results.

The stability of expression of the candidate reference genes can be examined by using three different algorithms developed for Microsoft Excel, such as geNorm (Vandesompele *et al.*, 2002), NormFinder (Andersen *et al.*, 2004) and BestKeeper (Pfaffl *et al.*, 2004). In addition, RefFinder (http://leonxie.esy.es/RefFinder/) is web-based to compare and rank candidate reference genes. Thus, using rankings from each program, an appropriate weight is assigned to an individual gene, and the geometric mean of their weights is calculated to give the overall final ranking. This study focuses on geNorm and BestKeeper and the Cq values from RT–qPCR Cq were transformed into relative quantity values (Q) using the ΔCq method. The geNorm value shows the most stable expression level of the reference genes across the samples, and the lowest value across different reference genes is the most stable and important gene. BestKeeper is based on the principle that appropriate endogenous genes should display similar expression patterns and should be highly correlated (Pfaffl *et al.*, 2004; Melgar–Rojas *et al.*, 2015).

The analysis in the geNorm method is based on the precept that the experimental conditions should not affect the ratio of expression values of two or more suitable reference genes and expression be stable under various treatments considered for comparison (Pfaffl *et al.*, 2004). However, the pairwise variation of each candidate reference gene is calculated against all other genes examined to determine the stability value (*M)* for each reference gene as the mean of pairwise variation. The lowest *M* value would be the gene which is most stably expressed. Beside this, geNorm calculates the minimal number of reference genes for accurate normalisation from the pairwise variation (*V*) between two sequential normalisation factors containing an increasing number of genes tested. Vandesompele *et al.* (2002) decided to use a cut–off value of 0.15 so that a (Vn/n+1) pairwise variation value below 0.15 indicates that (n) genes are sufficient for normalisation and the gene (n+1) should not be included (Pfaffl *et al.*, 2004; Melgar–Rojas *et al.*, 2015).

As mentioned above, BestKeeper is based on the principle that the appropriate reference genes should display similar expression patterns and must be highly correlated. The Excel sheet calculates statistical parameters for each candidate reference gene from raw Cq values, and the most important are (1) coefficient of correlation (r), (2) coefficient of determination (R^2), (3) coefficient of variation (CV%) and (4) standard deviation (SD) of the Cq values. However, BestKeeper does not indicate which of these values is most relevant (Melgar–Rojas *et al.*, 2015). Pfaffl *et al*. (2004) found that CV and SD values give an important first estimation of reference gene stability. The most stably expressed genes should have the lowest variation (lowest CV and SD), while highly correlated genes with high r and R^2 values are putatively stably expressed (Melgar–Rojas *et al.*, 2015).

In the present study, quantitative the real-time polymerase chain reaction (qPCR) was used to assess the gene expression of WRKY transcription factors in wheat under various stress conditions. This technique is fast and cheap method for the analysis of the expression of specific genes and requires only part of the gene's coding sequence to be known. This allowed a determination of the relative expression of members of the WRKY transcription factor family, which had previously been identified as showing differential expression in response to nitrogen stress and spot blotch which is a disease caused by the fungus *Bipolaris sorokinana*.

4.3 Hypothesis:

1. WRKY transcription factors regulate differently by reducing nitrogen input.
2. WRKY TFs regulate different foliar disease resistance mechanisms against spot blotch to different nitrogen availability in resistant and susceptible wheat.

4.4 Aims:

1. To determine whether housekeeping genes have stable responses against exposure to various treatments.
2. To determine the best reference genes for the validation of gene expression in the WRKY TFs family.
3. To determine if wheat plants grown with 0.75 mM nitrogen and 3.75 mM nitrogen exhibit differential expression of WRKY genes compared to in wheat and Brachpodium grown with 7.5 mM nitrogen.
4. To investigate the effects of the interaction of different concentrations of nitrogen and pathogen infection on the expression of defence genes in wheat and Brachpodium using qRT-PCR.

4.5 Results

4.5.1 Testing the *T. aestivum* homologues for specificity

Before the detailed testing of gene expression optimisation condition and stability was conducted for each of the normalisation candidates, a simple statistical analysis is very important to ensure that no candidates showed extreme variation when tested in all of the different treatments required for this experiment. For data obtained from qPCR experiments to be accurate, they must be normalised against internal control genes for differences in cDNA quality and quantity between samples. The qPCR assay is a well-established technique that has been performed on a large number of different species and tissue types and, as a result, the experiments conducted in other species are able to inform the choice of reference genes used in these experiments. Vandesompele *et al.* (2002) have previously suggested that the use of only one reference gene can lead to high levels of error, and so it would be preferable to find a number of reference genes that could be used for normalisation. Table 4.1 lists candidate genes from which a potential *Triticum aestivum* homologue could be found, and to what level they are similar to *Brachypodium distachyon*, *Hordeum vulgare*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays* homologue.

Table 4.1. Refined list of candidates for gene normalisation. The table indicates the *T. aestivum* gene acronym, the chosen acronym for the cereals gene homologue, and the percentage similarly of the cDNA sequences.

T. aestivum		***B. distachyon***	***H. vulgare***	***O. sativa***	***Z. mays***
eIF4A	GenBank	XM_003563460	AK365761	XM_026026114	NM_001111404
	%similarity	90%	95%	87%	88%
28S rRNA	GenBank	XR_002961475	HQ825319.1	XR_003238828	XR_002750196
	%similarity	98%	99%	97%	96%
			25S rRNA		
EF1α	GenBank	XM_010230696	Z50789	D63581	EU960821
	%similarity	94%	97%	92%	90%
18S rRNA	GenBank	XR_002961464	AK251731.1	XR_003238822	XR_002748746
	%similarity	99%	99%	99%	99%

For each gene, primer pairs were designed that ensure only cDNA targets were amplified, where a negative control using water instead of DNA and positive control of untreated leaves and spores for each gene were used in PCR and qPCR runs. The nucleotide sequences of primers are presented in Appendix B1, B2, B3 and B4. The sequence of each primer was checked against the *Triticum aestivum* genome sequence to ensure that they would not amplify similar genes; however, due to gaps in the wheat genome sequence it was deemed necessary to test all

primer pairs under Taq PCR conditions (see section 2.12) to determine if all produced single bands. Figure 4.1 shows the bands and sizes of amplification of some genes used in this study. Wheat and fungal genes were examined with RNA from Rashid wheat cultivar, whereas Brachypodium genes with RNA product from Brachypodium leaves. The integrity of the genes was analysed by separation on 2% (w/v) agarose gels, and bands were visualized by ethidium bromide staining under UV.

Primer pairs were tested by deriving the coefficient of determination (R^2), which uses a serial dilution of cDNA and DNA to predict the linearity of the Cq data and allows reliability to be assessed across the technical replicates (Taylor *et al.*, 2010). The R^2 value of over 0.980 suggests that a primer pair has suitably high amplification efficiency (Taylor *et al.*, 2010) and the results are shown in (Figure 4.2) and (Table 4.2). A melt peak analysis in which only one distinct peak is present, and hence a product of only one size is being amplified, was also essential for a primer pair to be considered acceptable (Figure 4.3). If the reaction is working at 100% efficiency, then a plot of Cq value against $\log_2$ (relative concentration) would produce a straight line with a gradient of -1 where amplification efficiency = $[10^{(-1/slope)}] - 1$. This would confirm that halving the concentration of the sample results in an increase of 1 in the Cq value. As well as covering a range of abundance levels, the efficiency of the PCR was confirmed at a range of dilutions, from undiluted to 1 in 64. The Cq values for *Ta28S rRNA* were between 5.76 and 10.56, demonstrating a very high abundance, the *Bs28S rRNA* expression levels ranged from 8.65 to 14.71, and the Cq value of *BdUbi18* was between 19.09 and 24.91. Meanwhile, abundance of *TaWRKY3* transcript was much lower, with Cq values from 25.44 to 31.46, for *WRKY19* Cq was 17.27 to 23.07, *WRKY45* Cq was 23.52 to 29.71, *WRKY46* Cq was 18.17 to 24.36 and the values for *WRKY68a* were between 24.01 and 29.37. The coefficient of linear regression of Cq against $\log_2$ (relative concentration) for *Ta28S rRNA*, *Bs28S rRNA*, *BdUbi18* and *TaWRKY3, 19, 45, 46 and 68a* primers were -1.002, -0.980, -0.968, -1.003, -0.967, -0.996 and -1.001, respectively (Figure 4.2).

For example: *Ta28S* $E = (10^{(-1/-3.316)}) - 1 = -1.002$

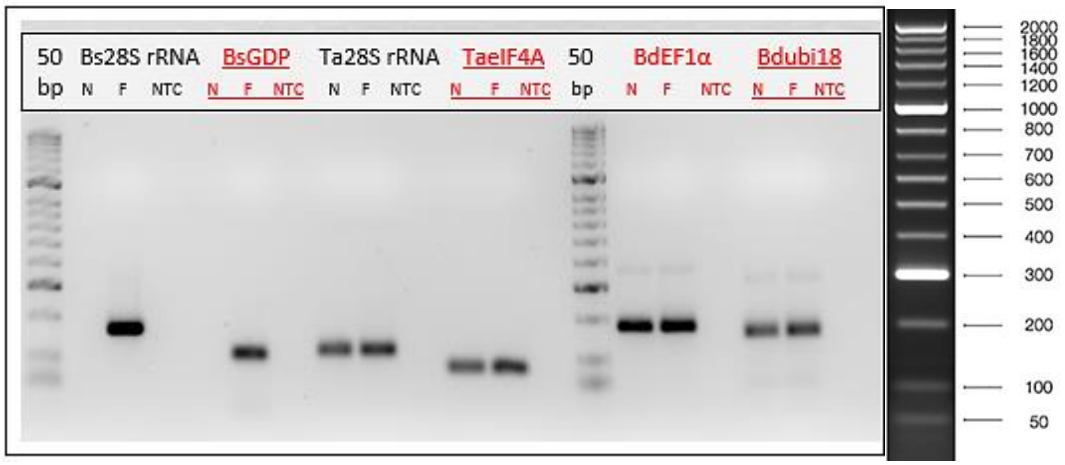


Figure 4.1. Agarose gel of amplified RNA products of housekeeping genes in Rashid wheat and *Brachpodium* leaves grown in 7.5 mM nitrate (Hoagland's solution).
Bipolaris sorokinana (*Bs28S rRNA*: 165bp, *BsGDP**: 98bp), *Triticum astivum* (*Ta28S rRNA*:107 bp, *TaeIF4A*: 73bp) and *Brachypodium distachyon* (*BdEF1α*: 181bp, *Bdubi18*:159bp). N= non-infected, F= spot blotch infected plants, Control=NTC. Relevant sizes of bands from the 50 bp ladder are indicated as size markers.
* Source of BsGDP from (Pogorelko *et al.*, 2013).

A melt peak analysis in which only one distinct peak is present, and hence a product of only one size is being amplified, was also essential for the primer pair to be considered acceptable. The results obtained with the pairs of primers *Ta28S rRNA* and *Bs28S rRNA* in infected wheat and spores of spot blotch are shown in (Figure 4.3). Primer pairs that did amplify efficiently for the remaining primer dilution curves are listed in Table 4.2. Primers that gave no amplification product or gave more one band have been excluded, such as *WRKY1*, *WRKY2*, *WRKY8*, *WRKY13*, *WRKY53* and *WRKY60*.

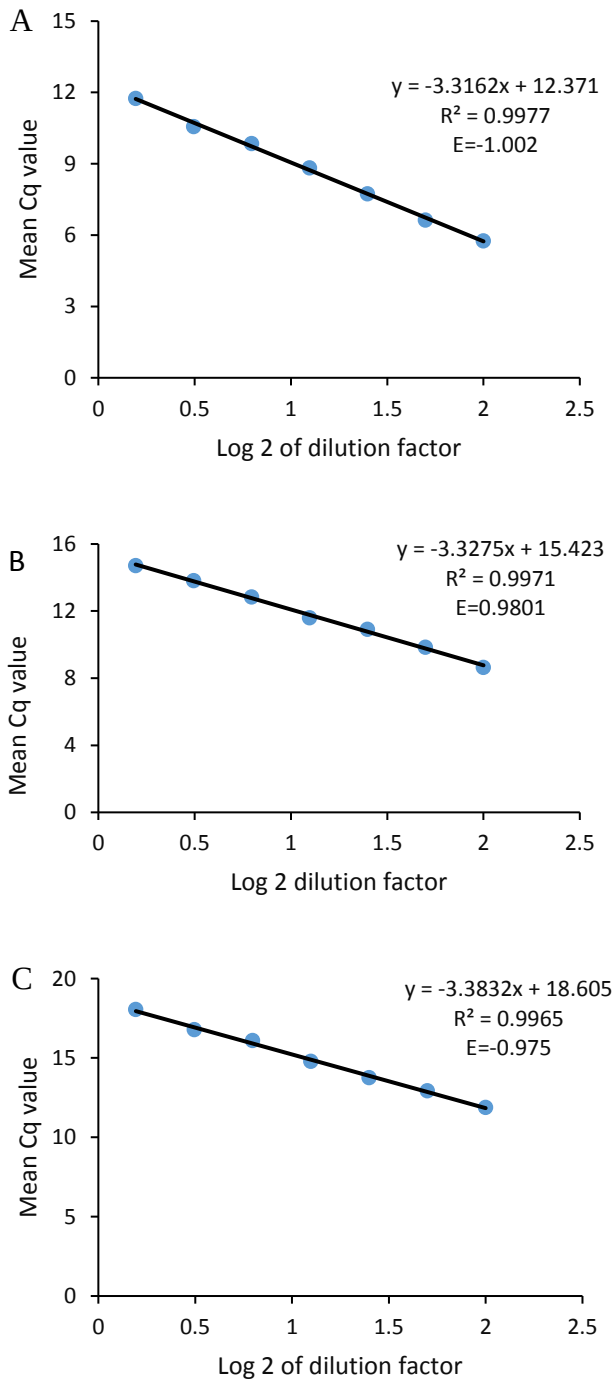


Figure 4.2. The efficiency of *Ta28S rRNA* primers in wheat cDNA (A), *Bs28S rRNA* in both infected wheat spot blotch (B) and DNA of spot blotch spore (C).
Dilution factor plotted against the average Cq value, performed on three technical replicates for each cDNA or DNA dilution.

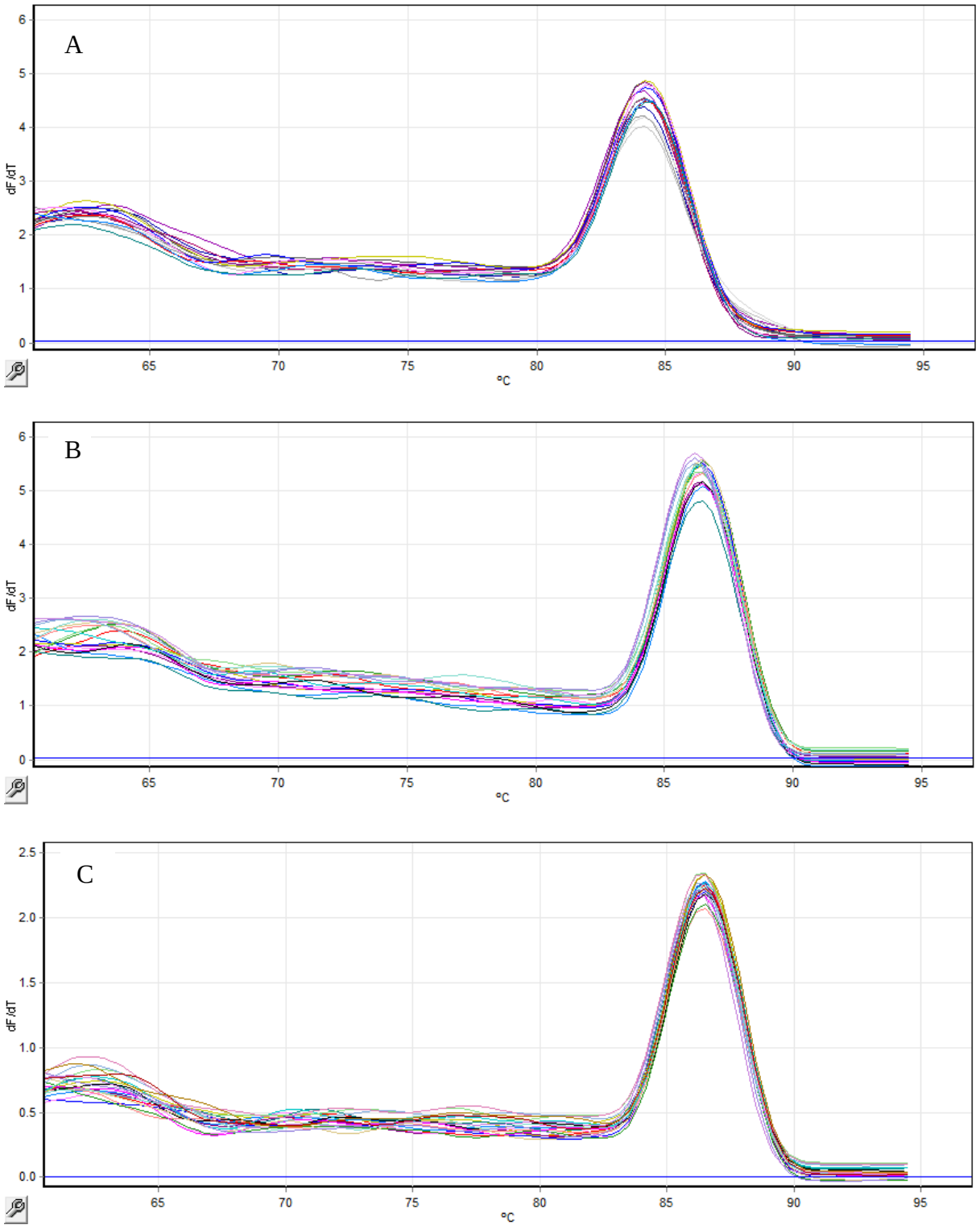


Figure 4.3. Melt peak analysis of (A) *Ta28S rRNA* primers in wheat cDNA, *Bs28S rRNA* in both infected wheat spot blotch (B) and DNA of spot blotch spores (C).
Melt peak analysis results are of dilution factors on three technical replicates for each cDNA or DNA.

Table 4.2. Regression analysis of Cq values of different genes against log 2 (relative concentration).

Ta28S rRNA versus Log Concentration		*Bs28S rRNA* versus Log Concentration		*BdUbi18* versus Log Concentration		*TaWRKY3* versus Log Concentration	
R^2	Slope Coef	R^2	Slope Coef	R^2	Slope Coef	R^2	Slope Coef
99.7 %	-1.002	99.6 %	-0.980	99.0%	-0.978	99.6%	-1.003

TaWRKY19 versus Log Concentration		*TaWRKY45* versus Log Concentration		*TaWRKY46* versus Log Concentration		*TaWRKY68a* versus Log Concentration	
R^2	Slope Coef	R^2	Slope Coef	R^2	Slope Coef	R^2	Slope Coef
99.3 %	-0.967	99.6 %	-1.021	99.2 %	-0.996	99.1 %	-1.001

4.6 Potential for normalisation

For gene to be suitable candidate for normalisation, it must be expressed to a highly similar level in all tissue samples being investigated. A number of software tools have been developed to statistically verify the stability of potential normalisation genes by way of pairwise testing for varying from the most simples, such as the ΔCq method (Silver *et al.*, 2006) to complex pair-wise analyses that are able to select more than one optimal reference gene, such as geNorm NormFinder (Vandesompele *et al.*, 2002; Anderson and Cairney, 2004) and BestKeeper analysis (Pfaffl *et al.*, 2004). All of the available tests perform slightly different analyses to verify the stability of gene expression, and a strategy of using several of these methods to obtain the best combination of reference genes has been widely used (De Spiegelaere *et al.*, 2015; Shivhare and Lata, 2016). This study identified several potential normalisation genes, and these were narrowed down to four excellent candidates for which there are primer pairs that have been shown to efficiently amplify only one product of wheat *eIF4A, 28S rRNA, EF1α,* and *18S rRNA* genes. The levels to which these genes are expressed in the uninfected and infected wheat leaves were tested, and the stability of expression for each gene is compared against other potential candidates.

All housekeeping genes were validated for their consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM nitrate for uninfected and infected wheat with spot blotch. Four endogenous control genes in wheat (*eIF4A*, *28S rRNA*, *EF1α* and *18S rRNA*) (Figure 4.4) and and two in Brachypodium Elongation Factor 1α (*BdEF1α*) and Ubiquitin 18 (*BdUbi18*) (Figure 4.5) showed non-significant differences (ANOVA, Tukey, P > 0.05) in Cq values between six treatment conditions for each endogenous control when they were assessed for their consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM. *Bipolaris sorokiniana* endogenous controls (glyceraldehyde-3-phosphate dehydrogenase -*BsGDP*, ribosomal RNA – *Bs18S rRNA* and *Bs28S rRNA*), were validated for their consistency across three biological replicates at standard nitrogen availability in infected wheat (Figure 4.6). Differences of Cq values between the six treatment conditions were non-significant for each endogenous control (ANOVA, Tukey, P > 0.05). Therefore, their proven stability in different conditions of nitrogen stress and the presence or absence of spot blotch means that these represent ideal candidates to act as controls for gene expression normalisation by qPCR.

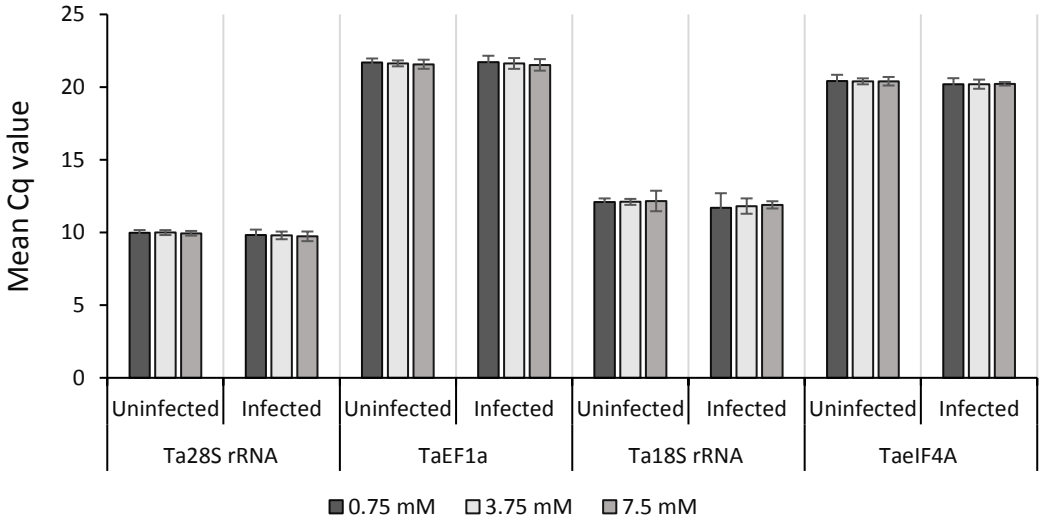


Figure 4.4. Four candidate wheat endogenous control genes were assessed for their consistency across nitrogen levels in uninfected and infected wheat.
Triticum aestivum 28S rRNA, Elongation Factor 1α (*EF1α*), *18S rRNA* and Eukaryotic Initiation Factor 4A (*eIF4A*) were assessed for their consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM in uninfected and infected plants (ANOVA, Tukey: P > 0.0.5, n= 9) within each gene, error bars represent ± 1 standard deviation.

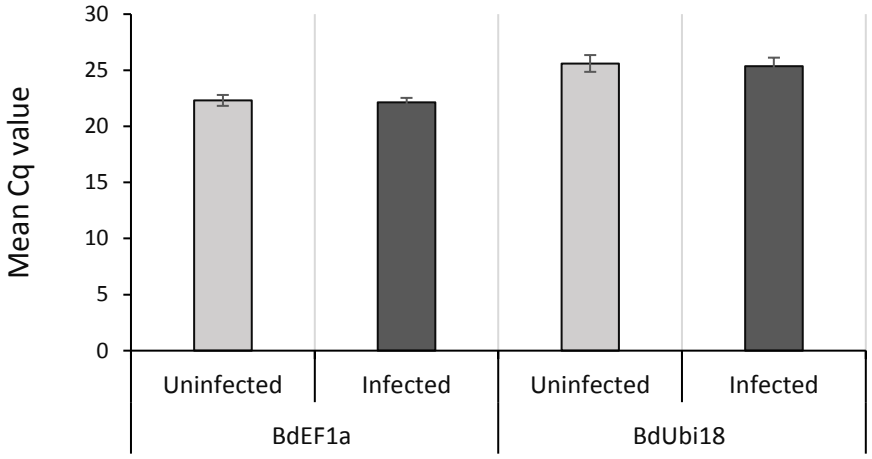


Figure 4.5. Expression of two candidate endogenous control genes was assessed in uninfected and infected Brachypodium.
Brachypodim distachyon Elongation Factor 1α (*BdEF1α*) and Ubiquitin 18 (*BdUb18*) were assessed for their consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM in uninfected and infected plants (ANOVA, Tukey: P > 0.0.5, n= 9) within each gene, error bars represent ± 1 standard deviation.

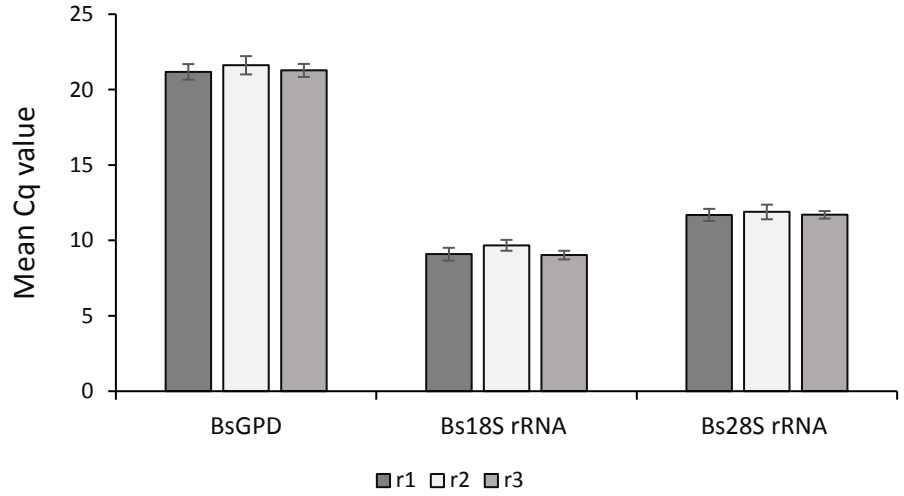


Figure 4.6. Expression of three candidate *Bipolaris sorokiniana* endogenous control genes across different biological replicates.
Bipolaris sorokiniana GPD, Bs18S rRNA and *Bs28S rRNA* were assessed for their consistency across three biological replicates in standard nitrogen availability in infected wheat (ANOVA, Tukey: P > 0.0.5, n= 3) within each gene, error bars represent ± 1 standard deviation.

A *Bipolaris sorokiniana* endogenous control was tested for consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM in infected wheat, showing significant differences (ANOVA, Tukey, P < 0.05) in Cq values between the three treatment conditions for each endogenous control (Figure 4.7). There were high Cq values for severely reduced nitrogen level samples and these were significantly different (ANOVA, Tukey, P < 0.05) from moderate and standard nitrogen levels for all three endogenous controls, where only *Bs18S rRNA* showed no significant difference (ANOVA, Tukey, P > 0.05) between moderate stress and standard

nitrogen input. These results correspond well to the differences in growth of *B. sorokiniana* described in Chapters 3 and 5.

Based on the above results for all tests conducted the chosen genes provided excellent tools to exclude any undesirable effects in reducing the accuracy of gene expression of these genes. It is possible to use them in investigations of expression under different stresses via qPCR, so that the results will be more accurate and acceptable. The following sections use other methods to gain more information about genes to be used in this study, and especially in considering a housekeeping gene to be a suitable candidate for normalisation which must be expressed to a highly similar level in all tissue samples investigated.

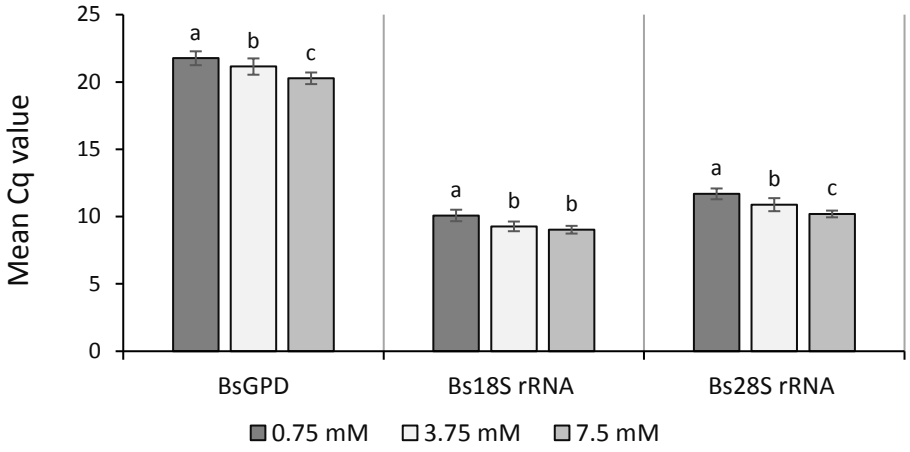


Figure 4.7. Expression of three candidate *Bipolaris sorokiniana* endogenous control genes across nitrogen concentrations in infected plants.
Bipolaris sorokiniana GPD, Bs18S rRNA and *Bs28S rRNA* were assessed for their consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM. Different letters represent statistically significantly different means (ANOVA, one way, Tukey: P > 0.0.5, n= 9) within each gene, error bars represent ± 1 standard deviation.

4.7 Reference gene stability analysis

4.7.1 Analysis of raw Cq values

A suitable candidate housekeeping gene must be expressed to a highly similar level in all of the tissue samples under different treatments. A number of statistical parameters can be used to verify the stability of potential normalisation genes by way of pairwise testing (Silver *et al.*, 2006; Gong *et al.*, 2016). The standard deviations of raw Cq values (threshold value from qPCR assay), as suggested by De Spiegelaere *et al.* (2015) are highly acceptable for normalisation with values down 1.5.

The standard deviation (SD) of all raw Cq values within each biological replicate was calculated, and the results are shown in (Table 4.3). The *28S rRNA* and *eIF4A* genes display the least variation, with the mean standard deviation of all three biological replicates being only 0.186 Cq units for *28S rRNA* and 0.218 for *eIF4A*. The *EF1a* shows the next highest level of variation in Cq value with a mean standard deviation of 0.250, and *18S rRNA* shows the highest SD of 0.442. All values were below the exclusion threshold of 1.5 and as a result, all potential normalisation genes were subjected to pair-wise testing to more accurately determine the stability of each gene across the different nitrogen stress levels.

Table 4.3. Raw Cq analysis of normalisation genes showing standard deviation (SD) of the raw Cq values (housekeeping genes- wheat *eIF4A, 28S rRNA, EF1α,* and *18S rRNA*) as measure of gene expression variability across biological replicate samples.

Gene name	Standard deviation of the Cq values			
	Biological replicate 1	Biological replicate 2	Biological replicate 3	Mean
eIF4A	0.255	0.168	0.232	0.218
28S rRNA	0.185	0.163	0.212	0.186
EF1α	0.286	0.221	0.243	0.250
18S rRNA	0.465	0.589	0.273	0.442

4.7.2 BestKeeper analysis

Further validation of housekeeping genes used in this study, additional statistic parameters needed to be used that will be very useful to select best housekeeping gene. Pfaffl *et al.* (2004) described BestKeeper method which is based on the principle that appropriate endogenous genes should display high similarity in expression patterns and should be highly correlated (Melgar–Rojas *et al.*, 2015). The Excel-based tool was used to obtain the best reference gene.

Previously, the standard deviations of Cq values across biological replicates showed acceptable values for all genes used, where *eIF4A* had the lowest value of standard deviation which means that it is the most suitable gene. At this stage, the standard deviation was used to ensure the validation of genes across the three nitrogen treatments used in this study. The coefficient of correlation (r) and coefficient of determination (R^2) showed the highest values of endogenous genes when two genes were used to validation them. The analysis showed strong correlation ($0.773 < r < 0.887$) for all four candidates. Vandesompele *et al*. (2009) suggested that highly correlated genes with high r value would be putatively stably expressed. The coefficients of

determination (R^2) were 0.884 and 0.814 for *eIF4A* and *28S rRNA* respectively, and both showed high R^2 values when all four housekeeping genes and three housekeeping genes were compared. These genes also gave lower R^2 values (0.787 and 0.734) when four genes were compared. Furthermore, *eIF4A* and *28S rRNA* genes also had highly significant P-values ($P < 0.001$) indicating very little difference across treatments.

The results for standard deviation (SD) and standard error (SE) also showed lower values when comparing two genes than when four genes were tested. Analysis across nitrogen treatments showed that the lowest calculated coefficients of variation (CV%) were 0.879 and 0.369 for *eIF4A* and *28S rRNA* respectively (Table 4.4). However, *EF1a* showed r and R^2 values similar to those of *eIF4A* and *28S rRNA*, but the other values of standard deviation, standard error and coefficient of variation were higher (0.31, 0.103 and 2.579 respectively). By contrast, *18S rRNA* presented the lowest correlation coefficient (r) and coefficient of determination (R^2) and the highest standard deviation (SD), standard error (SE) and coefficient of variation (CV), indicating that it was the least stable of the four housekeeping gene candidates (Julian *et al.*, 2016). Besides this, there were a large decreases in values of the standard of deviation and coefficient of variation in the selected two bestkeepers that have the lowest values for both statistical parameters (SD = 0.21, CV = 1.51) compared with three bestkeepers genes (SD = 0.27, CV = 1.98) and four bestkeeper genes (SD =0 .29, CV = 2.32) for the candidate reference genes.

Table 4.4 Statistical parameters of four candidate housekeeping genes (HKG) based on their crossing point (CP) values.
Nitrogen levels used as crossing points and the comparison was assessed between all four genes (wheat *eIF4A, 28S rRNA, EF1α,* and *18S rRNA*): 4, 3 and 2 housekeeping genes by Bestkeeper Excel sheet.

Parameter	No. HKG	Intervals control gene name- Cq				Bestkeeper (n=4)	Bestkeeper (n=3)	Bestkeeper (n=2)
		eIF4A	28S	EF1α	18S			
Coefficient of correlation r	4	0.887	0.857	0.871	0.773			
	3	0.925	0.892	0.919				
	2	0.940	0.902					
Coefficient of determination R^2	4	0.787	0.734	0.759	0.598			
	3	0.855	0.796	0.844				
	2	0.884	0.814					
P- value	4	0.002	0.001	0.006	0.126			
	3	0.001	0.001	0.001				
	2	0.001	0.001					
Standard deviation CP	4	0.14	0.11	0.34	0.45	0.29		
	3	0.08	0.08	0.31			0.27	
	2	0.1	0.06					0.21
Standard error	4	±0.073	±0.053	±0.113	±0.162			
	3	±0.093	±0.006	±0.103				
	2	±0.077	±0.005					
Coefficient of variation%	4	1.033	0.693	2.188	3.497	2.32		
	3	1.013	0.591	2.079			1.98	
	2	0.879	0.369					1.51
Min CP		21.73	9.28	20.47	11.37	14.83	13.05	13.81
Max CP		22.39	9.74	22.09	12.51	15.57	13.85	14.57
Min x-fold		-1.12	-1.35	-1.56	-1.79			
Max x-fold		1.23	1.17	1.41	1.71			
SD dev x-fold		1.08	1.10	1.34	1.36			
Geometric mean		21.82	9.45	21.53	12.01	15.22	13.42	14.19
Arithmetic mean		21.84	9.45	21.57	12.03	15.22	13.43	14.19

4.7.3 Internal control gene (geNorm) analysis

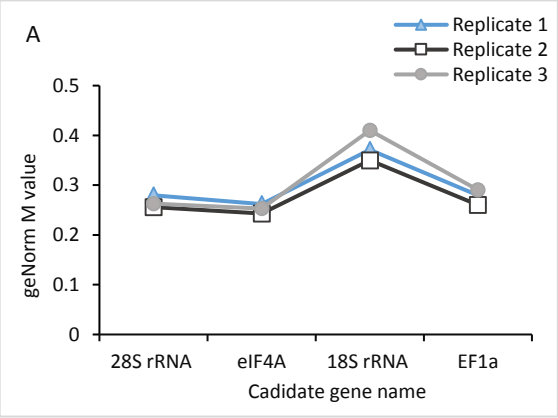
The internal control gene-stability measurement *M* can give each normalisation candidate a 'stability value' (*M*) across the different samples being tested (Vandesompele *et al.*, 2002), *M* represents the geNorm gene stability measure for a particular reference gene (Hellemans *et*

al., 2007). Genes with the lowest *M* values have the most stable expression across the samples (Anderson and Cairney, 2004; Julian *et al.*, 2016).

The analysis of qPCR data combines the frequently used statistical analysis of geNorm (Vandesompele *et al.*, 2002). The equation given in section 2.16.1 uses an improved version of the original internal control gene-stability algorithm to provide a stability value *M* for each of the candidate normalisation genes being tested, and suggested optimal number and the combination of normalisation genes. Genes that have an *M* value of < 0.15 are considered to be 'highly stable' (Vandesompele *et al.*, 2002).

All candidate normalisation genes were tested on each of the biological replicates, and the internal control gene-stability value *M* (Vandesompele *et al.*, 2002) was compared for the four normalisation genes. The results are shown in (Figure 4.8 A and B). *M* values for *TaeIF4A*, *Ta28s rRNA* and *TaEF1α* across three biological replicates were consistent, and their mean values were close (0.253, 0.266 and 0.277) for *eIF4A*, *28s rRNA* and *EF1α* genes, respectively. However, *Ta18S rRNA* showed the highest mean *M* value (0.381), with the highest variation between replicates and so is the least stable of the candidate genes and can be excluded.

Comparison for each gene across biological replicates showed the high stability values of *eIF4A* are the most consistent, followed by those for *28S rRNA* and *EF1α*. The values give more confidence to use these genes as endogenous control genes for studying the response of WRKY TF genes in wheat to reduced nitrogen availability and *Bipolaris sorokiniana* stress by using qPCR assay in the next step, whereas *18S rRNA* shows the highest variation of the three genes and can be excluded.



B

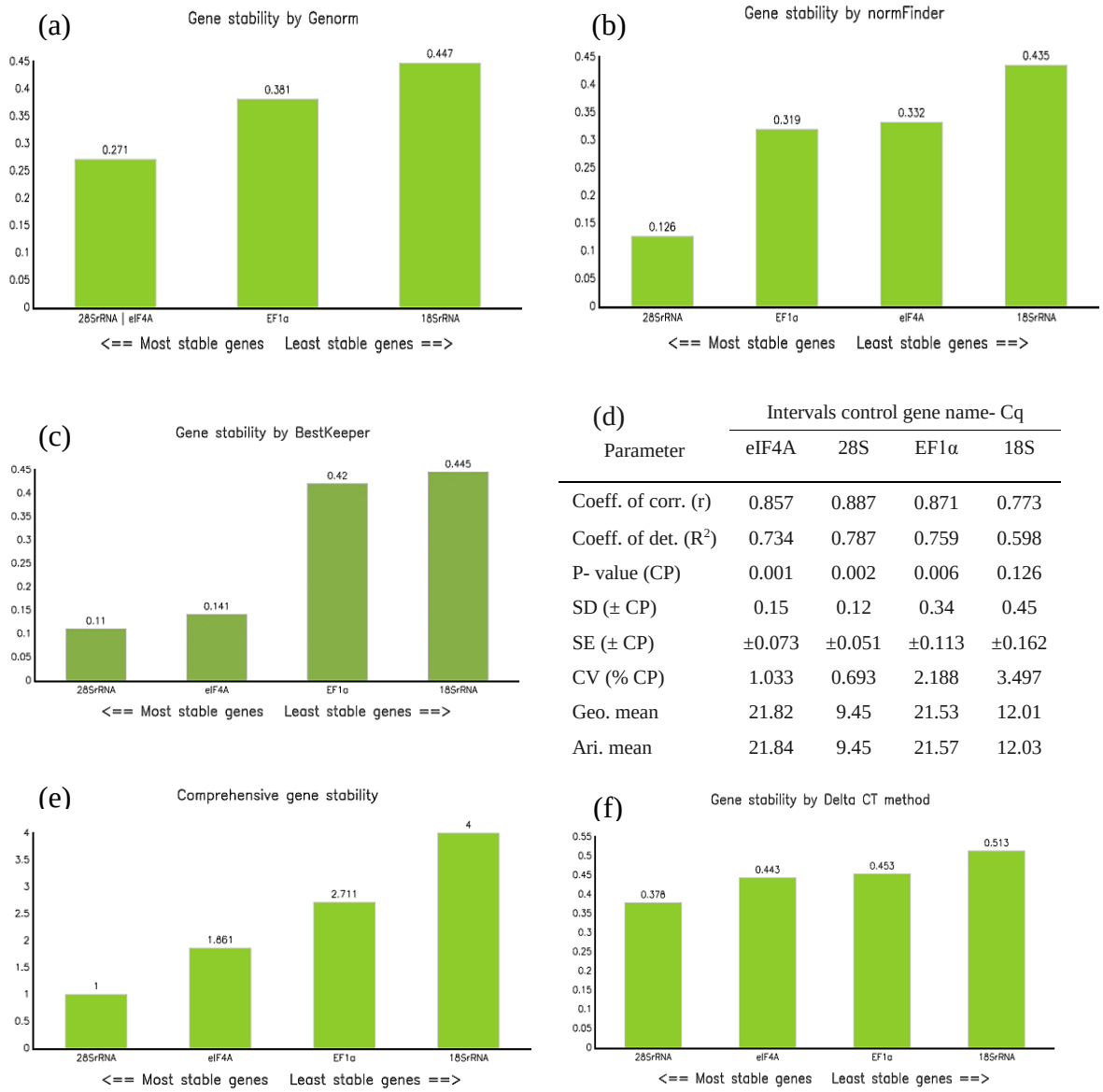
Gene name	Internal control gene-stability measure (*M*) values			
	Biological replicate 1	Biological replicate 2	Biological replicate 3	Mean
eIF4A	0.262	0.245	0.253	0.253
28S rRNA	0.279	0.256	0.262	0.266
18S rRNA	0.371	0.352	0.412	0.381
EF1α	0.279	0.260	0.291	0.277

Figure 4.8. Comparison of internal control gene-stability measure (*M*) values based on their crossing point (CP) values in the three biological replicates. (A), and geNorm M values of the candidate normalisation genes (wheat *eIF4A*, *28S rRNA*, *EF1α*, and *18S rRNA*) (B).

4.8 RefFinder: Evaluating reference gene expression

RefFinder is a user-friendly package developed to facilitate the evaluation and screening of reference genes from extensive experimental qPCR data (http://leonxie.esy.es/RefFinder/). It integrates the frequently used statistical analysis of geNorm (Vandesompele *et al.*, 2002), and the RefFinder software uses an original geNorm algorithm to provide the stability value (*M*) for each of the candidate normalisation genes being tested, and suggested optimal number and the combination of normalisation genes. By this measure, genes that have an *M* value of < 0.15 are considered 'highly stable. Thus, the geNorm analysis indicates that *28S rRNA*/*eIF4A* is the most suitable genes for the normalisation of RT-qPCR data with the lowest *M* values (Figure 4.9 a). Also, RefFinder package provides a stability value (*S*) from the NormFinder algorithm (Andersen *et al.*, 2004) by analysing intra-and inter-group variances. NormFinder also suggests the best combination of reference genes and calculates the stability value for this combination (Melgar–Rojas *et al.*, 2015). Thus, NormFinder analysis showed (see Figure 4.9 b) that the best reference gene was *28S rRNA* with the lowest value of (*S*, 0.126), followed by the *eIF4A* gene (*S*, 0.319).

Besides, RefFinder software provides BestKeeper analysis which calculates several key statistics for each reference gene from raw Cq values. The values of coefficient of variation (CV%) and standard deviation (SD) give the first estimation of reference gene stability (Pfaffl *et al.*, 2004). Therefore, the candidate reference gene which is the most stable expressed would exhibit the lowest variation in the CV% and SD (Melgar–Rojas *et al.*, 2015). The *28S rRNA* showed the highest r-value of 0.887 with the lowest CV and SD values of 0.693 and 0.12 respectively (Figure 4.9 d). Furthermore, the RefFinder package provides the Cq method, which uses the relative expression of pairs of candidate normalisation genes to determine the stability of each reference gene based on the raw Cq values from all samples. Each gene is normalised against all other genes undergoing testing, and the stability values for candidate genes are ranked from the most to the least stable based on how much variation is seen between the samples (Silver et al., 2006). Thus, *28S rRNA* showed the lowest SD value of 0.11, followed by *eIF4A* with an SD value of 0.141 (Figure 4.9 f).



(d)

Parameter	Intervals control gene name- Cq			
	eIF4A	28S	EF1α	18S
Coeff. of corr. (r)	0.857	0.887	0.871	0.773
Coeff. of det. (R^2)	0.734	0.787	0.759	0.598
P- value (CP)	0.001	0.002	0.006	0.126
SD (± CP)	0.15	0.12	0.34	0.45
SE (± CP)	±0.073	±0.051	±0.113	±0.162
CV (% CP)	1.033	0.693	2.188	3.497
Geo. mean	21.82	9.45	21.53	12.01
Ari. mean	21.84	9.45	21.57	12.03

Figure 4.9. Stability values of four wheat candidate reference genes (*eIF4A*, *28S rRNA*, *EF1α*, and *18S rRNA*) expression estimated by RefFinder software based on their crossing point (CP) values: (a) geNorm; (b) normFinder; (c-d) BestKeeper; (e) Cq; and (f) comprehensive gene stability. These are used to determine the stability of each candidate normalisation gene based on the raw Cq values from all samples.

4.9 Summary of methods used:

The different methods of assessing the normalising capability of the four candidate genes, *eIF4A, 28S rRNA, EF1α* and *18S rRNA*, produced results which differ slightly. However, the main conclusions that can be drawn are the same for all methods. The first is that *18S rRNA* shows consistently less stable expression across the samples when compared to the other three genes, and as a result should be discarded immediately as a candidate for normalisation between wheat grown in different nitrogen treatments. The second is that all three of the remaining genes, *eIF4A, 28S rRNA* and *EF1α*, are suitable for use as normalisation genes. The

combination in which they should be used, however, is not immediately clear. The methods used were Cq, correlation (r), coefficient of detection (R^2), standard error (SE), standard deviation (SD), coefficient of variation (CV%) and internal control gene-stability (*M*), and all suggested that *eIF4A* and *28S rRNA* were better normalisation candidates than *EF1α*, and this was consistent across the three nitrogen treatments.

Thus far, the investigation into the stability of the candidate normalisation genes has shown that *eIF4A*, *28S rRNA* and *EF1α* all show high levels of stability. However, this study aims to identify the best combination of genes. Due to the disagreement found between the methods used, the further test of normalisation was carried out by using the expression data in an Excel-based spreadsheet software application named BestKeeper across the three different nitrogen input levels. Samples were first normalised using the geometric mean of *eIF4A*, *28S rRNA*, and *EF1α*, based on the previous results with higher stability components. The three genes *eIF4A*, *28S rRNA* and *EF1α* were selected, which have higher stability components than four genes. Finally, the best two genes (*eIF4A* and *28S rRNA*) were selected which had the highest stability after *EF1α* was excluded due to its low component stability. This finding gave increased confidence in using *eIF4A* and *28S rRNA* in the main study focusing on the detection of the expression of genes of interest relative to internal control genes in this study.

Furthermore, RefFinder software provides tools to select the most stable reference gene under different conditions from a number of candidate normalisation genes. Stability values provided, include the geNorm stability value (*M*) for each of the candidate normalisation genes being tested, and suggested optimal number and combination of normalisation genes. The stability value (*S*) from the NormFinder algorithm is derived by analysing intra-and inter-group variances. In addition BestKeeper analysis calculates several key statistics for each reference gene from raw Cq values, where the coefficient of variation (CV) and standard deviation (SD) values give a first estimation of reference gene stability. The findings of all tools in the RefFinder package highly supported the previous conclusions from the primary tools used. Jarosova and Kundu (2010) also found that the 28S ribosomal RNA (*28S rRNA*) and Eukaryotic Initiation Factor 4A (*eIF4A)* were suitable endogenous controls to normalise gene expression against total wheat cDNA.

4.10 Testing the candidate normalisation genes

The expression dynamics of the normalisation gene combinations discussed above were examined across the nitrogen input levels in wheat using the BestKeeper algorithm. Firstly, the normalising ability of the four combinations (*TaeIF4A, Ta28S rRNA, TaEF1α* and *Ta18S*

rRNA) genes were analysed, and from this, the changes in expression of WRKY genes found by BestKeeper against changes in nitrogen input were assessed. Meanwhile, it was necessary to ensure that no product was seen in the wheat cDNA sample when amplification with the WRKY TF primer sequences were tested in standard PCR conditions. Once this had been ascertained, the primers were subjected to the tests described above. Briefly, the expression levels of target genes were first normalised to the average level of the candidate reference genes, and the geometric mean was used as recommended by Vandesompele *et al.* (2002).

Variations according to nitrogen treatments across the biological replicates were assessed in infected Rashid wheat cultivar with spot blotch. The seven WRKY genes, *rbcL* and *PR1* were normalised against the geometric mean of four reference genes (*eIF4A*, *28S rRNA*, *EF1α* and *18S rRNA*) (Figure 4.10 A), three reference genes (*eIF4A*, *28S rRNA* and *EF1α*) (Figure 4.10 B), two *eIF4A* and *28S rRNA* (Figure 4.10 C) and for a single endogenous control gene *28S rRNA* (Figure 4.11 A) and *eIF4A* (Figure 4.11 B). The results show that there is more similarity between groups (geometric mean) when the two endogenous control genes (*eIF4A* and *28S*) are used as shown in (Figure 4.10 C) with the three reference genes group (*eIF4A*, *28S* and *EF1α*) (Figure 4.10 B) and a single endogenous control gene (*eIF4A*-Figure 4.11 and *28S* -Figure 4.11 B, separately). These results display the same pattern especially to *eIF4A* and *28S rRNA*, suggesting that either combination of normalisation genes will result in the same final trend being found. Thus, it is possible to use the geometric mean of both or only one to normalise the WRKY TF genes' response to *Bipolaris sorokiniana* in wheat under reduced nitrogen availability.

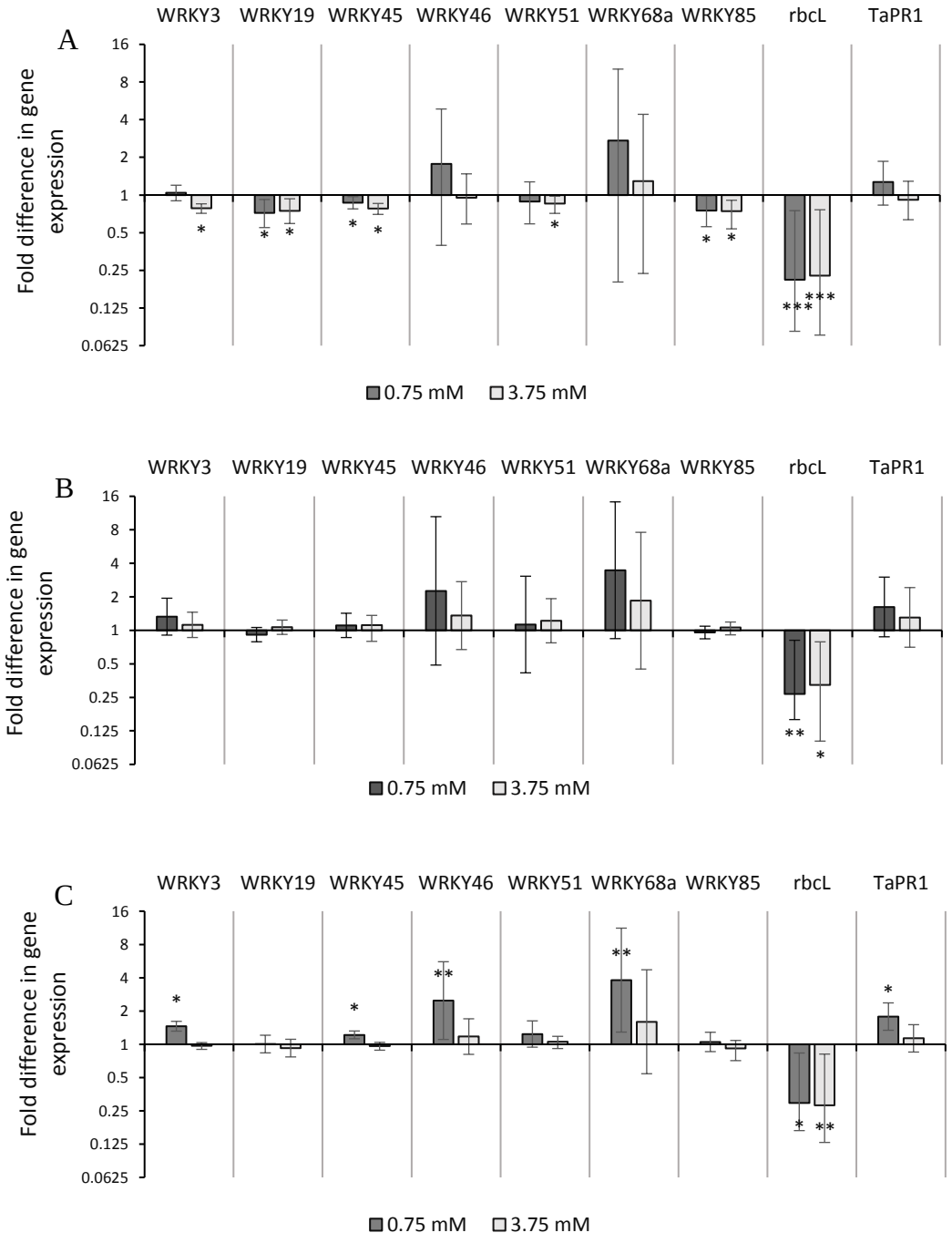


Figure 4.10. Expression of WRKY TF, *rbcL* and *PR1* genes across the nitrogen treatments in wheat. Reduced nitrogen levels (0.75mM, 3.75 mM) are displayed relative to 7.5 mM nitrate.
Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised against the geometric mean of validated normalisation wheat genes (A) *eIF4A*, *28S rRNA*, *EF1α* and *18S rRNA*, (B) *eIF4A*, *28S* and *EF1α* and (C) *eIF4A* and *28S*. Error bars (confidence intervals) that do not cross the x-axis indicate that the corresponding comparison of means is statistically significant (t-test, P < 0.05*, 0.01**, 0.001***, n= 9) within each gene.

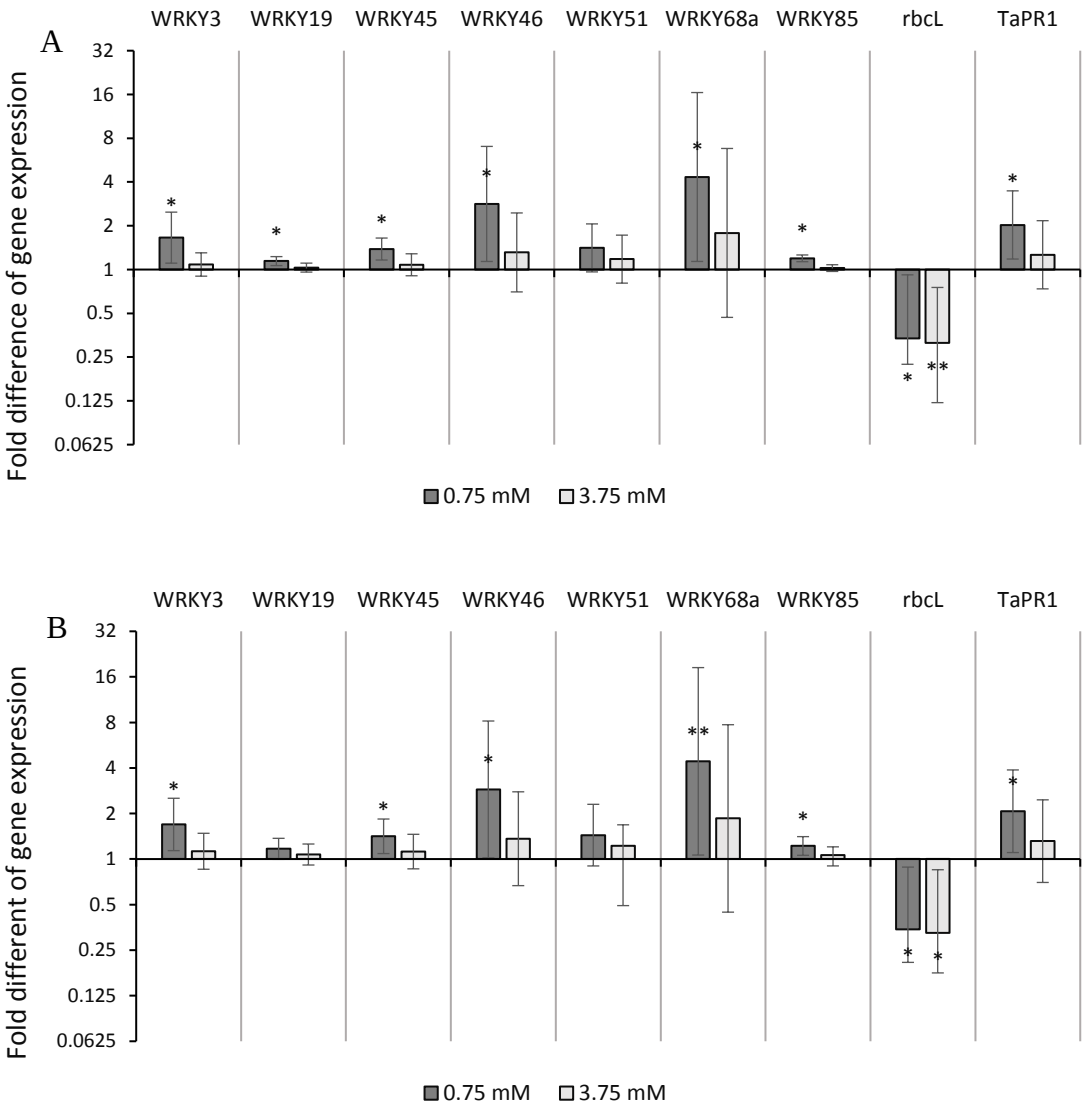


Figure 4.11 Expression of WRKY TF, *rbcL* and *PR1* genes across the nitrogen treatments in wheat. Reduced nitrogen levels (0.75 mM, 3.75 mM) are displayed relative to 7.5 mM nitrate. Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised against (A) *28S rRNA* and (B) *eIF4A*. Error bars (confidence intervals) that do not cross the x-axis indicate that the corresponding comparison of means is statistically significant (t-test, P < 0.05*, 0.01**, n= 9) within each gene.

4.11 Gene expression response to reduce nitrogen in Rashid, Latifia and Brachypodium

Seven WRKY TF genes (*WRKY3, 19, 45, 46, 51, 68a* and *85*), in addition to ribulose-1,5-bisphosphate carboxylase/oxygenase-RuBisCO large subunit (*rbcL*) and pathogenesis-related protein-1 (*PR1*) were investigated for their differential expression in response to reduced nitrogen input (0.75 and 3.75 mM), relative to the standard nitrogen input of 7.5 mM nitrate. These genes had previously been shown to produce desirably high amplification efficiency, as shown in section 4.5.1. The expression of all seven WRKY TF genes was up-regulated in Rashid and Latifia cultivars grown with reduced nitrogen for 18 days old (Figure 4.12 A and

B, respectively). The severely reduced nitrogen level in Rashid cultivar gave significant up-regulation of expression (CI, $P < 0.01$) for *WRKY68a* and (CI, $P < 0.05$) for *WRKY3*, *WRKY 45* and *WRKY46*, whilst at reduced nitrogen to moderate level the up-regulation was not statistically significant (CI, $P > 0.05$). Expression of the *PR1* gene was significantly up-regulated expression only in severely reduced nitrogen. The *rbcL* gene was the only one gene that was significantly down-regulated (CI, $P < 0.05$) at moderately and severely reduced nitrogen levels.

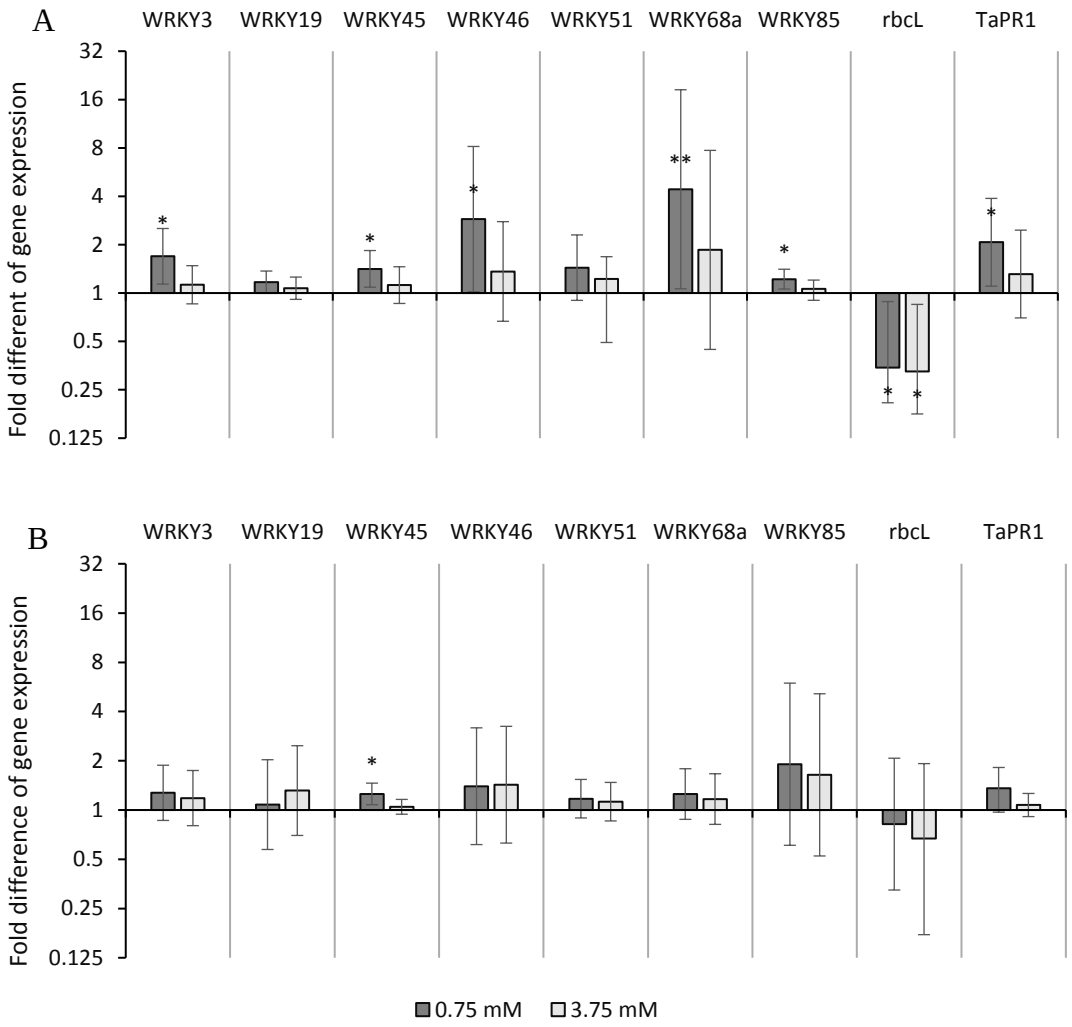


Figure 4.12. Fold differences in seven WRKY TF, *rbcL* and *PR1* genes expression in uninfected leaves in (A) Rashid and (B) Latifia wheat cultivar grown with reduced nitrogen availability (0.75 and 3.75 mM) compared with standard level (7.5 mM nitrate).
Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised against the geometric mean of the validated normalisation wheat genes (*TaeIF4A* and *Ta28S rRNA*). Error bars (confidence intervals) that do not cross the x-axis indicate that the corresponding comparison of means is statistically significant (t-test, $P < 0.05$*, 0.01**, n= 9) within each gene.

The WRKY TF genes, *rbcL* and *PR1* in Latifia wheat were investigated for their response to reduced nitrogen input at 0.75 and 3.75 mM (Figure 4.12 B above). Fold differences in the expression of the WRKY TFs and *PR1* showed up-regulation of expression with both moderately and severely reduced nitrogen, relative to standard nitrogen input of 7.5 mM. The only gene to show a statistically significant (CI, $P < 0.05$) difference was *WRKY45* with severely reduced nitrogen. Meanwhile, there was significant down-regulation of *rbcL* expression (CI, $P < 0.05$) with moderate nitrogen, but non-significant up-regulation with more severe nitrogen stress. There was non-significant up-regulation of *PR1* expression with both severely and moderately reduced nitrogen.

The WRKY TF genes in Brachypodium were obtained from the homology of *Triticum aestivum WRKY* sequences with *Brachypodium distachyon* WRKY sequences. In addition, the *rbcL* and *PR1* genes from Brachypodium were investigated as shown in (Figure 4.13).

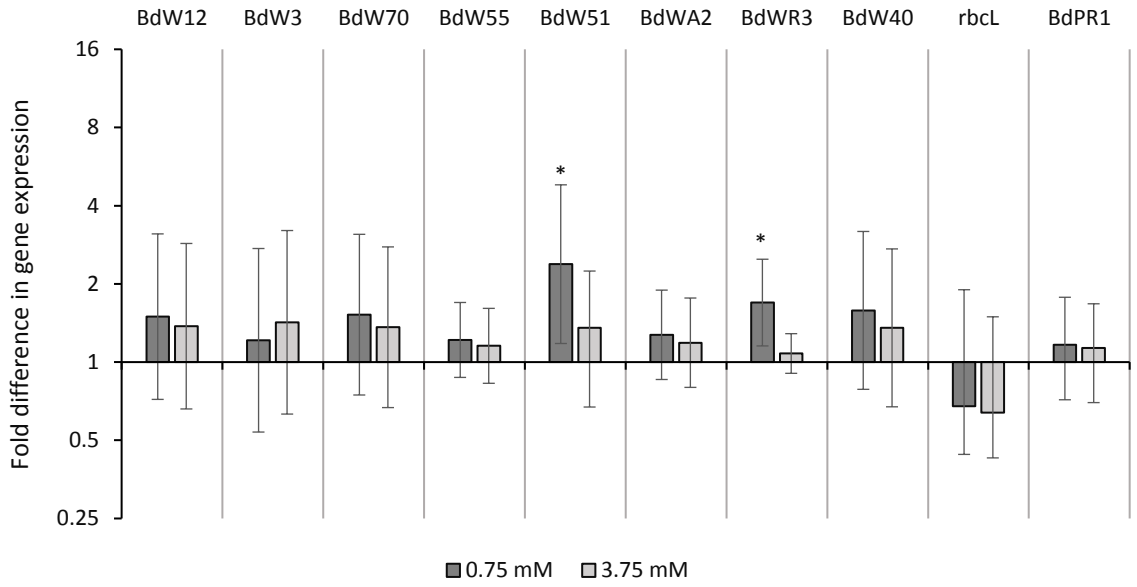


Figure 4.13. Fold differences in seven WRKY TF, *rbcL* and *BdPR1* gene expression in uninfected Brachypodium leaves grown with reduced nitrogen availability (0.75 and 3.75 mM) compared with standard level (7.5 mM nitrate).
Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised against the geometric mean of validated normalisation genes (*BdEF1α* and *BdUbi18*). Error bars (confidence intervals) that do not cross the x-axis indicate that the corresponding comparison of means is statistically significant (t-test, $P < 0.05$*, n= 9) within each gene.

Expression of the Brachypodium WRKY TF, *rbcL* and *PR1* genes was examined in plants grown with reduced nitrogen availability (0.75 and 3.75 mM nitrate), were displayed relative to standard nitrogen input of 7.5 mM nitrate. All seven Brachypodium WRKY genes used in this study showed no statistical difference in expression between moderately reduced and standard nitrogen levels. However, the expression of *BdWRKY51* and *BdWR3* was significantly

up-regulated (CI, $P < 0.05$) with severely reduced nitrogen. Relative expression of *rbcL* was lower with moderately and severely reduced nitrogen compared with standard nitrogen, but the difference was not significant. Also, *PR1* showed not significant upregulation of expression (CI, $P > 0.05$) in both severely and moderately reduced nitrogen levels.

4.12 Gene expression response to *Bipolaris sorokiniana* in Rashid and Latifia grew under reduced nitrogen availability

Gene expression was analysed four days post-infection at18 day old plants when the infection still in the biotrophic phase. The observed differences in gene expression can perhaps be interpreted by analysing the changes in gene expression in response to *Bipolaris sorokiniana* infection for plants that were grown with each nitrogen condition separately in Rashid and Latifia wheat cultivars. The expression of seven WRKY TFs, *rbcL* and *PR1* was assessed for *B. sorokiniana* infected to uninfected Rashid and Latifia grown in same nitrogen conditions (0.75, 3.75 mM and 7.5 mM nitrate). Samples scored differently for *B. sorokiniana* severity as either low in severe nitrogen stress, medium in the moderate and high in the standard nitrogen input according to the amount of leaf surface covered by symptoms of *B. sorokiniana* (see Figure 3.11).

Expression of four (*WRKY19, 45, 46* and *85*) of the seven WRKY TF genes appeared to be down-regulated at all nitrogen concentrations, with significant down-regulation mainly at lower nitrogen concentrations, confirming that changes in WRKY TFs expression in response to spot blotch are dependent on nitrogen concentration (Figure 4.14 A). The *WRKY3* expression was down-regulated with severely reduced nitrogen and up-regulated at both moderate and standard nitrogen levels. For *WRKY51* relative expression levels were similar across all concentrations but up-regulation was only significant (CI, $P < 0.05$) with moderate and standard nitrogen levels, confirming changes in *WRKY51* expression in response to spot blotch impact. In contrast, *WRKY46* expression was only significantly down-regulated in plants grown with severely and moderately reduced nitrogen (CI, $P < 0.01$ and CI, $P < 0.05$, respectively). Also, there was significant down-regulation of *WRKY85* expression (CI, $P < 0.05$) only with moderately reduced nitrogen and non-significant with both severely reduced stress and standard nitrogen input. Changes in *rbcL* expression were negative across all concentrations but there was only significant down-regulation with standard nitrogen (CI, $P < 0.05$), confirming that changes in *rbcL* expression in response to spot blotch are dependent on nitrogen concentration. However, there was similar but not significant up-regulation of *PR1* expression across all nitrogen levels under test.

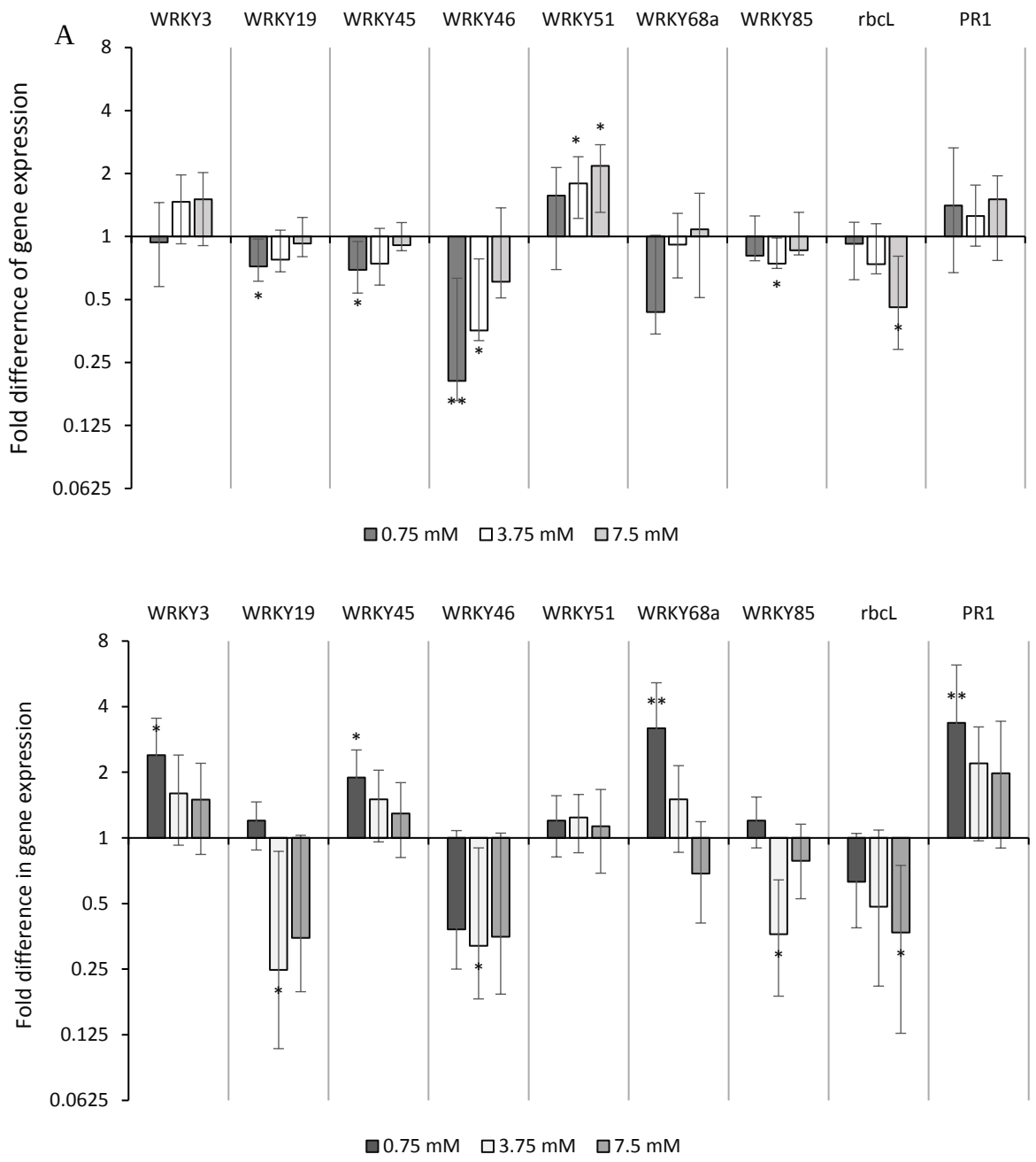


Figure 4.14. Fold differences in expression of seven WRKY TF, *rbcL* and *PR1* genes in (A) Rashid and (B) Latifia wheat cultivars. Fold differences are displayed relative to uninfected plants grown in the same nitrogen concentration.
Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised against the geometric mean of the validated normalisation genes (*TaeIF4A* and *Ta28S rRNA*). Error bars (confidence intervals) that do not cross the x-axis indicate the corresponding comparison of means is statistically significant (t-test, P <0.05*, 0.01**, n= 9) within each gene.

In infected Latifia wheat cultivar, WRKY gene expression changed in response to *Bipolaris sorokiniana* infected Latifia are displayed relative to uninfected plants grown in the same nitrogen concentration (Figure 4.14 B above). The genes used in this study showed different

expression levels of spot blotch response depending on nitrogen treatments. *WRKY3, WRKY45, WRKY51* and *PR1* expression appeared to be up-regulated across all nitrogen concentrations, with significant up-regulation of expression of all nitrogen concentrations. For *WRKY19, WRKY68a* and *WRKY85*, expression was not consistent between the three nitrogen concentrations. Expression of the *WRKY19* and *WRKY85* genes appeared to be up-regulated only with severe nitrogen stress, whereas expression of *WRKY68a* was significantly up-regulated (CI, $P < 0.05$) with severely reduced nitrogen and not significantly (CI, $P > 0.05$) with moderately reduced nitrogen. Expression of *WRKY46* was significantly down-regulated (CI, $P < 0.05$) only with moderately reduced nitrogen and non-significantly down-regulated with both severely reduced and standard nitrogen input. Expression of *rbcL* showed little change with nitrogen input, but only the standard nitrogen level gave significant down-regulation (CI, $P < 0.05$), confirming that changes in *rbcL* expression in response to *B. sorokiniana* are dependent on the nitrogen concentration.

To summarise the expression of seven WRKY TF, *rbcL* and *PR1* genes in Rashid and Latifia wheat cultivars. Fold differences of genes in infected Rashid and Latifia were displayed relative to uninfected plants grown in the same nitrogen concentration (Figure 4.15). Expression of *WRKY3, 45, 68* and *PR1* was significantly upregulated for four days after infection in severely reduced nitrogen level in Latifia cultivar and no change in Rashid. *TaWRKY3* was upregulated with high significance in infected Latifia under severe nitrogen stress but not under medium and high levels of fungal infection and nitrogen supply. However, when *WRKY3* expression was compared in infected Rashid, it was not differently up-regulated in high and moderate nitrogen levels, whilst under severe nitrogen stress expression was non-significantly down-regulated.

There was no change in expression of *WRKY19* in susceptible Rashid wheat cultivar in moderate reduced level and standard nitrogen input, but significant in severely reduced nitrogen. Expression of *WRKY19* in Latifia was significantly down regulated for in plants subjected to moderate nitrogen deficiency, but there was no change at the severe level and standard nitrogen.

Expression of *TaWRKY45* was upregulated in the resistant Latifia cultivar and significantly only in severely reduced nitrogen. These differences in the response of *WRKY45* expression confirm the effect of spot blotch infection, whereas in Rashid cultivar, expression of *WRKY45* showed down-regulation and significantly only at low nitrogen.

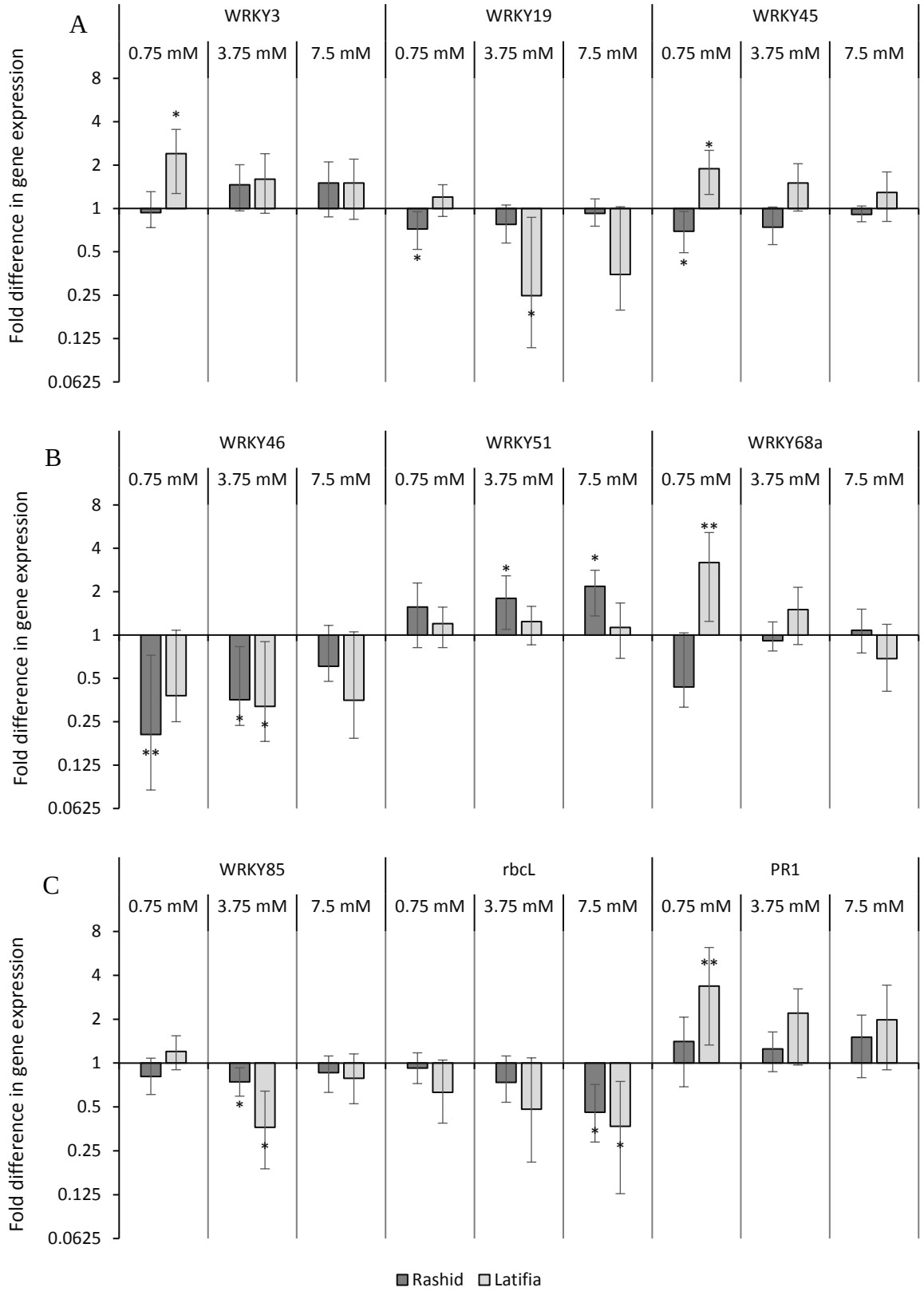


Figure 4.15 Fold differences in expression of (A) *WRKY3, WRKY19* and *WRKY45* (B) *WRKY46, WRKY51* and *WRKY68a* and (C) *WRKY85, rbcL* and *PR1* genes in Rashid and Latifia wheat cultivars. Fold differences are displayed relative to uninfected plants grown in the same nitrogen concentration. Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Confidence intervals used to comparison between values of fold changes within each gene and wheat cultivar (t-test, P <0.05*, 0.01**, n= 9) is statistically significant.

Whereas, *WRKY46* showed significantly down-regulation in expression in susceptible Rashid in severely and moderately reduced nitrogen, but not in standard nitrogen input. In resistant Latifia, *WRKY46* exhibited significant down regulation only in moderate level nitrogen. which means that the expression in response to infection is dependent on nitrogen concentration in both cultivars. Expression of *WRKY51* in Rashid was significantly upregulated in moderately and standard nitrogen availability. While, expression of *WRKY51* in Latifia showed weak upregulated.

The expression of *WRKY68a* was significantly up-regulated in the presence of a low level of spot blotch infection and severe nitrogen stress in the resistant Latifia wheat. However, for plants infected with spot blotch, the main change in gene expression with reduced nitrogen was a down-regulation of expression, although again neither effort reached statistical significance compared to expression at 7.5 mM nitrogen in the susceptible Rashid. Fold difference in *WRKY85* expression was significantly higher in moderately reduced nitrogen in Latifia than Rashid cultivar. Whereas, the expression of *WRKY85* was upregulated only in severe nitrogen stress.

However, *rbcL* showed the greatest significant down-regulation of expression only at standard nitrogen level in response to spot blotch infection in both Rashid and Latifia. Whereas, Expression of *PR1* showed high significant up-regulation in the resistant Latifia and no change in susceptible Rashid under severe nitrogen stress. Whereas, there were no change in *PR1* expression at the moderate reduced nitrogen and standard nitrogen input.

4.13 Discussion

The use of gene expression profiles in sustainable agriculture comes mainly from the large interest of researchers in identifying potential diagnostic genes that can improve the defence efficiency of wheat against pathogens when nitrogen availability is reduced. RT– qPCR remains one of the most sensitive techniques for quantifying gene expression and is widely used, due to its ability to calculate the precise fold change in a comparative expression analysis(Joseph *et al.*, 2018). The investigation of relative gene expression via the ΔΔCq method based on the Cq value from RT– qPCR in response to stress provides an excellent approach to understanding changes in gene expression that can be applied to crop improvement. The determination of which genes allow plants to withstand cold, drought or nitrogen stress(Mickelbart *et al.*, 2015), as well as bacteria, fungi, viruses or nematodes (Onaga and Wydra, 2016), for example, may allow us to use these genes as molecular markers in breeding programmes, or to engineer the overexpression of homologous genes in crop species so as to improve tolerance.

It is important when using the ΔΔCq method that the fundamental assumptions it employs are shown to be true. This is an absolute prerequisite for reliable results, especially when the biological significance of subtle differences in gene expression is being studied. Therefore, it is vital to show that the PCR is running at 100% efficiency. The ΔΔCq method relies on the assumption that, during every cycle, the number of copies of the transcript for the gene of interest doubles so that every two-fold dilution of cDNA results in an increase in Cq value of 1 for all genes tested using the formula Cq = $-\log_2$ (concentration) + c. The linear regression of Cq values against $\log_2$ (cDNA concentration), as shown in (Table 4.2), gave values of the coefficient of regression of -1.002, 0.980, -1.003 and -1.02 for *Ta28S*, *Bs28S*, *TaWRKY3* and *TaWRKY45* respectively. This confirms that halving the concentration of the sample results in an increase in the Cq value of 1. Genes showed different abundance levels. The Cq values for *Ta28S rRNA* were between 5.76 and 10.56, demonstrating a very high abundance. Meanwhile, abundance of *TaWRKY3* RNA was much lower abundance, and its Cq values ranged from 25.44 to 31.46.

Furthermore, the accurate normalisation of gene expression levels is also crucial for reliable results, especially when the biological significance of subtle differences in gene expression is studied (Vandesompele *et al.*, 2002). For the gene to be a suitable candidate for use in normalisation, it must be expressed at a highly similar level in all of the tissue samples being investigated and should show the least variation among treatments in a given experiment (Joseph *et al.*, 2018). The expression of the target gene is evaluated according to the expression level of the reference gene. Therefore, use of a reference gene whose expression is unstable could lead to inaccurate evaluation of target gene expression (Wan *et al.*, 2017). All housekeeping genes used in this study were validated for consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM nitrate for uninfected and infected wheat. The expression levels of four wheat reference genes (*28S, EF1α, eIF4A* and *18S*) were shown to be consistent across six treatment conditions in terms of Cq values in RT-qPCR, making them appropriate reference genes under these conditions (Figure 4.4). Conversely, when *Bipolaris sorokiniana* endogenous controls were validated for consistency across nitrogen concentrations of 7.5 mM, 3.75 mM and 0.75 mM in infected wheat, Cq values were significantly different between the three treatment conditions for each endogenous control. Quantitative real-time PCR showed that the expression of *BsGPD, Bs28S* and *Bs18S* increased in expression with increasing spot blotch prevalence at high nitrogen input in comparison to reduced nitrogen reduced stresses that have been shown to lead to low spot blotch severity. It is apparent that increasing *BsGPD, Bs28S* and *Bs18S* expression indicates increasing spot

blotch severity and indeed provides a quantitative measure of disease severity. Thus, *BsGPD, Bs28S* and *Bs18S* are not suitable to use as endogenous control genes in infected plants

Researchers have studied the validation of housekeeping genes in considerable detail. A number of statistical parameters have been suggested to verify the stability of potential normalisation genes by way of pairwise testing (Silver *et al.,* 2006; Gong *et al.,* 2016). Data presented by De Spiegelaere *et al*. (2015) determined that a standard deviation value (SD) of reference genes in different conditions of more than 1.5 is unacceptable for normalisation. Standard deviations calculated in this study from all raw Cq values representing different nitrogen conditions within each biological replicate were less than 1.5, which means that all candidate genes may be acceptable for normalisation (Table 4.3). However, *28S rRNA* and *eIF4A* displayed the lowest variation, with the means of all three biological replicates being only 0.186 Cq units for *28S* and 0.218 for *eIF4A*. The *EF1α* showed the next highest level of variation in Cq, with a mean standard deviation of 0.250, and *18S* showed the highest SD at 0.442. All values lie under the exclusion threshold of 1.5 and, as a result, all of these potential normalisation genes were subjected to pair-wise testing, in order to more accurately determine the stability of expression of each gene across the different nitrogen stress levels.

For further validation of the housekeeping genes for normalisation, the BestKeeper tool (Pfaffl *et al.*, 2004) that has been widely used which includes many statistical parameters (Melgar–Rojas *et al.*, 2015) indicated that endogenous genes should display similar expression patterns that should be highly correlated. Also, they suggested that the lowest value of standard deviation and coefficient of variation are ideal measurements to determine how stable expression of a reference gene is under various conditions and its validation for normalisation. Vandesompele e*t al*. (2009) also pointed out highly correlated genes with high r values would be putatively stably expressed. High R^2 values of 0.884 and 0.814 were found here for *eIF4A* and *28S* respectively, which were the two best genes, but both showed a decrease in R^2 values when a comparison was made between all four housekeeping genes. The lower R^2 values were 0.787 and 0.734 for *eIF4A* and *28S* respectively. Also, *eIF4A* and *28S* showed the lowest standard deviation (0.879 and 0.369) and coefficient of variation (0.31, 0.103). When the least stable reference gene from the four candidates was tested, *18S* gave an r value of 0.773, SD of 0.45 and CV of 3.497.

Another method available to validate reference genes across different samples or conditions is the internal control gene-stability measurement (*M*), which can give each normalisation candidate a ‘stability value’ (*M*) across different samples being tested, and it is then possible to

rank each gene by normalisation ability (Vandesompele *et al*., 2002). When each gene was compared across biological replicates (Figure 4.9), the stability values of *eIF4A* were the most consistent, followed by those of *28S* and *EF1α*, whereas *18S* showed the highest variation among the genes tested. The RefFinder package also includes many tools to normalise reference genes from the RT-qPCR assay. The results from RefFinder (geNorm, NormFinder, BestKeeper and ΔΔCq logarithm) supported the preliminary results obtained using different methods, as demonstrated above. This result is consistent with the finding by Mascia *et al.* (2010) that *18S rRNA* and *EF1α* demonstrated highly variable expression levels that should discourage their use for normalisation. The *eIF4A* was selected for "hormone stimuli" under abiotic stress among nine candidate reference genes, but the less stable reference genes *EF1α* showed expression levels which varied for this reference gene as demonstrated by (Tian *et al.*, 2015). Finally, a review by Joseph *et al.* (2018) gave a comprehensive update for a good number of reference genes that have been tested by researchers in different conditions to select most suitable expression to normalisation to estimate expression in interested target genes.

The normalisation gene combinations discussed above by using different methods were analysed in terms of expression dynamics using the Excel-based spreadsheet software application the BestKeeper algorithm across the nitrogen input in wheat. The results showed in (Figure 4.10 A, B and C) that the group of two genes (geometric mean in the two endogenous control genes used (Figure 4.10 C) were more similar in gene expression with single endogenous control genes (Figure 4.11 A and B) in Rashid cultivar than the geometric mean of four reference genes. These results display the same pattern especially for *eIF4A* and *28S rRNA*, suggesting that using either of these genes or a combination of the two will result in the same final trend being displayed.

Overall, from the different methods used to assess the normalising capability of the four candidate genes, *eIF4A, 28S rRNA, EF1α* and *18S rRNA* produced results which differ slightly. However, the main conclusions that can be drawn are the same for all methods. The first is that *18S* showed consistently less stable expression across the samples compared to the other three genes examined, and as a result should be excluded immediately as a candidate for normalisation between wheat grown in different nitrogen treatments. The second is that all three of the remaining genes, *eIF4A*, *28S* and *EF1a* are suitable for use as normalisation genes in this study. The combination in which they should be used, however, is not immediately clear. The methods used were Cq, correlation coefficient, coefficient of determination, standard error, standard deviation, coefficient of variation and internal control gene-stability (*M*): all these methods suggested that *eIF4A* and *28S* are better endogenous control genes for normalisation

than *EF1a*, based on a high consistency of *eIF4A* and *28S* expression across the three nitrogen treatments.

The major objective of this research is to determine whether or not reducing nitrogen input leads to differential expression of WRKY transcription factors compared to wheat grown with optimum nitrogen input (7.5 mM) in the presence or absence of *Bipolaris sorokiniana* infection. It is known that transcription factors (TFs) mediate environmental influences on gene expression. WRKY TFs, containing a highly conserved WRKY domain, are one of the important plant-specific TF groups and are involved in regulation of various physiological programs including defences against biotic and abiotic stress.

To expand the available resources for the prediction of the wheat WRKY genes function, we identified homologies between wheat WRKY genes in the NCBI databases and WRKY genes in monocot species (Brachypodium, rice, maize, barley and sorghum) and used the homologous genes to construct a phylogenetic tree based on the Neighbour-Joining method by MEGA7 software (see Figure 2.3). The results clearly showing protein relationships based on sequence similarity among WRKY gene in monocot crops. Because homologous *Triticum aestivium TaWRKY* genes share high similarity with *Brachypodium distachyon BdWRKY* sequences, the Brachypodium proteins can also be expected to function in similar ways. Thus, data on the expression of homologous genes could provide evidence for prediction of WRKY gene functions (Ding *et al.*, 2014).

Differential changes in the expression of a range of WRKY TF genes were seen with reduced nitrogen conditions in Rashid and Latifia cultivars and Brachypodium as shown in (Figure 4.12 A and B) and (Figure 4.13), respectively. All seven WRKY genes and the *PR1* gene showed indications of up-regulation at a nitrogen level of 0.75 mM compared to 7.5 mM, whilst no differential expression was seen with moderately reduced nitrogen (3.75 mM), suggesting that these genes play a role in plants' responses to more severely reduced nitrogen supply. This result is consistent with the findings of Wu *et al.* (2008), who demonstrated that wheat WRKY genes are involved in leaf senescence and abiotic stresses.

In the Rashid cultivar, *WRKY3* was significantly up-regulated with severely reduced nitrogen, but in Latifia and Brachypodium up-regulation was not significant. Therefore, it is possible that *WRKY3* has a role in the response of plants to the quantitative effects of reduced nitrogen, possibly by controlling key growth parameters, and this was the case in Rashid wheat more than Latifia.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is a key enzyme in photosynthesis and the most abundant leaf protein, accounting for 15–30% of total leaf N content in C3 species (Suzuki and Makino, 2013). It has been reported that Rubisco gene expression is disturbed by leaf senescence, thus, Chlorophyll degradation is a key step in the senescence process (Suzuki and Makino, 2013). Additionally, Rubisco is likely a source of carbohydrates for respiration under conditions of environmental stress that reduce photosynthesis (Araujo *et al.*, 2010; Ono *et al.*, 2013). The analysis of *rbcL* gene expression showed significant down-regulation of expression for both severely and moderately reduced nitrogen levels (CI, $P < 0.05$). This result further validates the suggestion of Dann and Pell (1989) who indicated that Rubisco is degraded during leaf senescence. At reduced nitrogen availability, growth was reduced as was explained in Chapter 3, and there was an apparent increase in leaf senescence with high chlorosis symptoms in leaves grown with severely reduced nitrogen. Bielenberg *et al.* (2001) have previously suggested that reduced nitrogen results in increased leaf senescence along with reduced growth. Also, it has been reported that Rubisco gene expression is disturbed by leaf senescence (Suzuki and Makino, 2013). Besides, the function of defence faces an essential demand for resources under severe stress, which reduces growth according to the "Growth vs Defence" hypothesis or "Growth-Differentiation Balance", as discussed in Chapter 1 section 1.11. Therefore, negative effects on growth could result from a reduction of photosynthesis, decreasing energy reserves, and also from diversion of resources away from growth and towards defence. If defence capabilities are deficient, a pathogen could destroy the plant population, and so plant must achieve a balance growth and defence to optimise fitness, although a full discussion of the functions of these genes lies beyond the scope of this study.

Expression of *WRKY19* was down-regulated at both reduced nitrogen levels in the Rashid cultivar. In Latifia wheat, *WRKY19* had a slightly higher level of expression with moderately reduced nitrogen than with the severely reduced level. This finding suggests that the magnitude of differential expression of *WRKY19* is small compared to some other genes, and so whether there is biological significance to these changes should be questioned, especially in the case of reduced nitrogen stresses. Niu *et al.* (2012) found that the overexpression of *TaWRKY19* conferred tolerance to salt, drought and freezing stresses in transgenic Arabidopsis plants. Meanwhile, Okay *et al.* (2014) found that *WRKY19* expression in the leaves of wheat under drought stress was up-regulated in some wheat cultivars and down-regulated in others.

In contrast, *WRKY45* expression in the present study exhibited a dose-dependent increase in response to reduced nitrogen in both wheat cultivars, with significant up-regulation in plants under severe nitrogen stress. Therefore, it is possible that *WRKY45* mediates the response of wheat to the quantitative effects of reduced nitrogen, and that it has a similar and stable response in different wheats. This finding is different from Wu *et al.* (2008) who reported that expression of *WRKY45* was not responsive to any of the abiotic stresses they tested (low and high temperatures, osmotic stress and high salinity).

Expression of the *WRKY46* gene was up-regulated in both Rashid and Latifia under nitrogen stress. The response in the susceptible Rashid was greater than in the resistant Latifia. The expression of *WRKY68a* exhibited a dose-dependent increase in response to reduced nitrogen. Under severe stress, the expression of *WRKY68a* increased 4.43-fold, whereas under moderate nitrogen stress, expression increased only 1.8-fold in Rashid wheat. However, in Latifia, no difference in expression of *WRKY68a* was found between the two reduced nitrogen levels. The response was similar to that of *WRKY3* but with 60% higher expression. Expression of these genes may respond to various types of stress, as Wu *et al*. (2008) reported that the expression of *WRKY46* and *WRKY68a* was up-regulated in wheat leaves under osmotic stress from 20% polyethylene glycol (PEG) treatment for 4 hours.

Although much evidence is available about the response of *PR1* gene expression to different abiotic stresses, in this study *PR1* exhibited different patterns of expression in the two wheats. In Rashid, *PR1* expression increased more than 2-fold with severely reduced nitrogen, with a more highly significant difference than in moderately reduced nitrogen (1.3-fold difference). Furthermore, *PR1* expression was no different in Latifia and Brachypodium grown under different nitrogen stresses. In studies carried out with maize (Xie *et al.*, 2010), and rice (Jain and Kumar, 2015) a high response of expression of genes coding for pathogenesis-related proteins (*PR*) to different types of abiotic stress was found.

Changes in gene expression in the plants as a response to the pathogen are not surprising. Numerous wheat genes have been reported responding to spot blotch disease give different patterns of expression when analysed using RNA-Seq. In a recent study by Kumar *et al.* (2016) who indicated to 4925 wheats in India that were screened for spot blotch fungus, and 300 genes were detected in *Bipolaris sorokiniana* after isolation by genome sequencing. Thus, it is not surprising to see different levels of expression of WRKY transcription factor genes in the cross talk between biotic (spot blotch) and abiotic (nitrogen concentration) stress responses, and

responses to individual stresses may not be additive when the plants must deal with combinations of stresses.

Results from the combined stress assay showed that expression of *TaWRKY3* was upregulated with high significance in infected Latifia under severe nitrogen stress but not under medium and high levels of fungal infection and nitrogen supply. However, when *WRKY3* expression was compared in infected Rashid, it was not differently up-regulated in high and moderate nitrogen levels, whilst under severe nitrogen stress expression was non-significantly down-regulated. Result of *WRKY3* expression suggest this gene may play an important role in defence of wheat against *B. sorokiniana*.

The analysis of the abundance of *rbcL* mRNA showed the greatest significant down-regulation of expression at standard nitrogen level in response to spot blotch infection. This indicates that *rbcL* RNA may have been degraded during leaf senescence, with less down-regulation in infected plants grown with severely reduced nitrogen due to lower fungal damage in those plants than in plants grown with moderate and high nitrogen levels. Similarly, Jantasuriyarat *et al.* (2005) found that in the interaction between rice and the rice blast fungus, the transcription of photosynthetic genes, such as ribulose 1,5-bisphosphate carboxylase and chlorophyll a/b-binding genes, was suppressed in both the susceptible and the resistant interaction. Thus, any reduction in plant fitness due to priming of defence pathways is outweighed by increased disease resistance in disease affected areas, because the defence is not activated before pathogen attack (van Hulten *et al.*, 2006).

Expression of *PR1* showed high significant up-regulation in the resistant Latifia and no change in susceptible Rashid under severe nitrogen stress. Whereas, there were no change in *PR1* expression at the moderate reduced nitrogen and standard nitrogen input. Yadav *et al*. (2016) have reported that PR protein accumulation is one of the best-characterised plant defence responses, and also PR gene expression has been observed in wheat seedlings infected with *Blumeria graminis*. However, PR proteins are associated with the hypersensitive response (HR) and with the systemic acquired resistance (SAR) defence programme. Dempsey *et al.* (1999) indicated that activation of PR expression, therefore, had been used as a convenient marker for the induction of SAR. Upregulation of PR proteins during senescence implies that there is an overlap between the two pathways, senescence and pathogen response (Lim and Nam, 2005). Although there are many exceptions now, SA is often considered to be effective signal for production of protective pathogenesis-related proteins (PR).

There was no change in expression of *WRKY19* in susceptible Rashid wheat in moderate reduced level and standard nitrogen input, but significant in severely reduced nitrogen. Expression of *WRKY19* in Latifia was significantly down-regulated for in plants subjected to moderate nitrogen deficiency, but there were no change at the severe level and standard nitrogen. Low nitrogen supply could suppress expression in susceptible Rashid or activate it in resistant Latifia wheat. Thus, active suppression or alteration of upstream signalling by the pathogen likely results in suppression of immune responses and proliferation of the pathogen, such as the suppression of expression of *WRKY19* in infected susceptible Rashid. From the results of *WRKY19* expression the response to spot blotch infection was dependent on nitrogen concentration, except for expression at severely reduced nitrogen levels in Latifia where there was no difference in up-regulation. *WRKY19* in wheat also shows a high similarity to the *AtWRKY3* sequence in Arabidopsis (Appendix A1). Functional analysis based on transgenic over-expression lines has indicated that *AtWRKY3* may play a positive role in plant resistance to necrotrophic pathogens and have negative effect on plant resistance to biotrophic pathogens (Lai *et al.*, 2008). However, homology between different genes may not mean conservation in function (Ding *et al.*, 2014). In study of We *et al*. (2014) found that although Brachypodium *BdWRKY38* is homologous to the rice *OsWRKY48* gene, they did not have similar expression patterns after three h jasmonic acid (JA) and salicylic acid (SA) treatments.

Expression of *TaWRKY45* was up-regulated for four days after infection in the resistant Latifia and significantly only in severely reduced nitrogen. These differences in the response of *WRKY45* expression confirm the effect of spot blotch infection, whereas in Rashid cultivar, expression of *WRKY45* showed down-regulation and significantly only at low nitrogen, which means that the expression in response to infection is dependent on nitrogen concentration. Findings by Shimono *et al.* (2007) and Bahrini *et al.* (2011a) suggested that constitutive over-expression of the *TaWRKY45* transgene confers enhanced resistance to *F. graminearum* in transgenic rice and wheat plants associated with benzothiazole (BTH)-induced and SA-mediated defence signalling respectively.

WRKY46 showed significantly down-regulation in expression in susceptible Rashid in severely and moderately reduced nitrogen, but not in standard nitrogen input. Whereas, in resistant Latifia, *WRKY46* exhibited significant down regulation only in moderate level nitrogen. which means that the expression in response to infection is dependent on nitrogen concentration in both cultivars. Expression of *WRKY51* in Rashid was significantly upregulated in moderately and standard nitrogen availability. But in Latifia showed weak upregulated. These differences in the response of *WRKY51* expression confirm the effect of spot blotch infection.

The expression of *WRKY68a* was significantly up-regulated in the presence of a low level of spot blotch infection and severe nitrogen stress in the resistant Latifia wheat. However, for plants infected with spot blotch, the main change in gene expression with reduced nitrogen was a down-regulation of expression, although again neither effort reached statistical significance compared to expression at 7.5 mM nitrogen in the susceptible Rashid. These changes in the response of *WRKY68a* expression confirm the effect of spot blotch infection. Meanwhile, expression was down-regulated in Rashid, which means that expression response to spot blotch dependent on nitrogen concentration.

Fold difference in *WRKY85* expression was significantly higher in moderately reduced nitrogen in Latifia than Rashid cultivar. Whereas, the expression of *WRKY85* was upregulated only in severe nitrogen stress. These differences in the response of *WRKY85* expression dependent on nitrogen concentration.

Overall, to confirm how infection changed the expression of WRKY TF genes, expression was compared between infected and uninfected plants grown under the same nitrogen concentration (Figure 4.14 and Figure 4.15). Expression of *WRKY3* and *WRKY51* was up-regulated when plants were infected with spot blotch under all three nitrogen treatments in both wheat cultivars. Expression of *WRKY3, 45, 68* and *PR1* was significantly upregulated in severely reduced nitrogen level in Latifia and no change in Rashid. However, expression of *WRKY45* and *WRKY68a* was up-regulated only at low and medium nitrogen levels in the resistant Latifia. These differences in the response of *WRKY68a* expression confirm the effect of spot blotch infection, with down-regulation in the susceptible Rashid, indicating that expression in response is dependent on nitrogen concentration.

4.14 Conclusion

The main focus of this study is the investigation of the interaction between nitrogen supply, disease severity of *Bipolaris sorokiniana* and the regulation of WRKY TF gene expression in Iraqi commercial wheat. Recent progress in research into the functions of these wheat WRKY genes and their homologues in Brachypodium is reviewed with a focus on their involvement in responses to abiotic and biotic stresses. Multiple tools have been tested in this project to optimise the choice of endogenous control genes. Results for internal control gene-stability (*M*), the Bestkeeper Excel sheet tool and RefFinder suggest that *eIF4A* and *28S rRNA* are better normalisation candidates than *EF1α*, and this conclusion is consistent across the three nitrogen treatments.

Growth with reduced nitrogen resulted in the up-regulation of all seven WRKY genes tested and *PR1*, but only the *rbcL* gene was down-regulated in both the Rashid and Latifia wheat cultivars. Expression of *WRKY3* and *WRKY51* in the responses of infected and uninfected plants grown under the same nitrogen concentrations showed upregulation when infected with spot blotch under all three nitrogen treatments in both Rashid and Latifia cultivars. However, *WRKY45* and *WRKY68a* showed upregulation in low and middle nitrogen levels in the resistant Latifia, and these changes in the response of *WRKY68a* expression confirm the effect of spot blotch infection, with down-regulation in the susceptible Rashid indicating that expression in response to spot blotch is dependent on nitrogen concentration. In Brachypodium plants, the expression of *BdWRKY* genes was more similar to WRKY gene expression in the resistant Latifia than the susceptible Rashid cultivar.

There is complex patterns of changes in the expression of genes coding for WRKY transcription factors in response to nitrogen stress and infection by *B. sorokiniana*. In addition, these results provide an opportunity to advance the understanding of the roles of WRKY proteins in wheat subjected to stress from nitrogen limitation and spot blotch pathogenesis and encourage their use in further study and continued investigation into WRKY TF function.

The following chapters in this study will address: firstly, the development of WRKY gene expression responses over time after infection by spot blotch; secondly, investigating the effects of TILLING mutations in the WRKYGQK domain; thirdly, exploration of the expression of WRKY genes in the presence and absence of biocontrol bacteria (*Micromonospora* sp.) in spot blotch pathogen-infected and uninfected wheat.

5 Association between development of infection with *Bipolaris sorokiniana* and physiology and time course of defence gene expression in two Iraqi wheat cultivars

5.1 Abstract

WRKY transcription factor genes that are involved in the early interaction between pathogen and host could be target genes for improvement of crops by manipulation of their functions using transgenic methods or genome editing. Conclusions obtained in this field are still limited, especially in hexaploid wheat. Effects of pathogen (*Bipolaris sorokiniana*) infection on WRKY gene expression at 0, 24, 48, 72 and 96 hpi were examined in susceptible (Rashid) and resistant (Latifia) wheat cultivars. Relative expression of five out of six WRKY TF genes tested was up-regulated earlier in Latifia than in Rashid compared to time zero. In Latifia, relative expression of *WRKY51* was up-regulated the most at 48 hpi, 8.1-fold. Development of the pathogen was assessed by measuring relative expression of the internal transcribed sequence (ITS) of ribosomal RNA and chitin content. Relative *ITS* abundance was approximately twice as high in Rashid as in Latifia at late times after infection. Assays of chitin content using wheat germ agglutinin WGA conjugate method showed an increase in fungal biomass (chitin content) in infected wheat as disease severity increased over time that was significantly higher in Rashid than in Latifia. *ITS* and chitin assays provide ways of measuring how fungal load is related to development of disease severity and spot blotch resistance. Also, analysis by fluorescence microscopy (WGA) showed ability to detect the pathogen during the biotrophic phase soon after infection. Furthermore, quantitative PCR assessing the expression of WRKY genes and *B. sorokiniana ITS* regions, in addition to WGA assay should be considered excellent candidates capable of detecting spot blotch presence even in the early biotrophic phase, when the fungal load is low, which would undoubtedly facilitate studies of fungal pathogenesis studies and resistant crop breeding.

Keywords: WRKY TF genes, wheat, *Bipolaris sorokiniana*, disease severity assessment, ITS, quantification RT-PCR, molecular interactions, earlier infection, WGA assay

5.2 Introduction

Research into molecular responses in the early stages of host-pathogen interactions is critical for studies of pathogenesis bedding of resistant crops. Defining the molecular events occurring early in the host-pathogen interaction stage is essential for understanding the initial mechanisms of interactions between pathogen and host (Shen *et al.*, 2017). The WRKY transcription factor genes that are involved in the early interaction between pathogen and host could be target genes for improvement of crops by manipulation of the functions of these genes using transgenic methods or genome editing. The conclusions obtained in this field are still limited, especially in wheat.

WRKY proteins have been identified as a class of DNA-binding proteins that recognise W-box elements (TTGAC(C/T)), which are found in the promoters of many plant defence-related genes (Rushton *et al.*, 2010). WRKY genes have not been reported in archea, eubacteria, eukaryotic fungi, or animals (Baranwal *et al.*, 2016). Furthermore, it has been found that recognition of W-box sequences by WRKY proteins is necessary for the genes containing W-boxes to be induced (Rushton *et al.*, 1996; Rushton *et al.*, 2010; Wang *et al.*, 2013; Wen *et al.*, 2014; Hwang *et al.*, 2016). Some studies have provided evidence that WRKY proteins have regulatory functions in plant defence responses to pathogen attack. Firstly, treatment of plants with pathogen elicitors or salicylic acid (SA) can induce rapid expression of WRKY genes (Rushton *et al.*, 1996; Chen and Chen, 2000; Eulgem and Somssich, 2007). Secondly, W-box elements are present in promoter regions of a number of defence-related genes, including the well-studied PR (pathogenesis-related protein) genes (Rushton *et al.*, 1996; Chen and Chen, 2000; Eulgem and Somssich, 2007). Pathogenesis-related (PR) proteins are absent in healthy plants but are up-regulated in pathogen-infected plants. Immunoblot analyses showed that two PR proteins accumulated in infected leaves, supporting a role in increasing the plant's resistance to fungal attack (Monteiro *et al.*, 2003). Maleck *et al.* (2000) analysed changes in gene expression in Arabidopsis under 14 different conditions that repressed or induced systemic acquired resistance with 10000 ESTs. The results provided evidence that WRKY proteins play critical roles in regulating expression of genes associated with systemic acquired resistance. A set of 26 genes including *PR1* was found to be coordinately induced by various pathogens and defence-inducing conditions, and all of these genes contained W-box elements (Maleck *et al.*, 2000). Currently, however, the functions of the WRKY genes in bread wheat (*Triticum aestivum*) have yet to be systematically explored.

Infection by *Bipolaris sorokiniana* is initiated by adhesion of the spores to the leaf surface, followed by germination of the spores and formation of germ tubes (Acharya *et al.*, 2011). After eight hours, the germ tubes swell sufficiently to produce an appressorium, from which infecting hyphae develop and penetrate tissues of the plant in the next stage (Jansson and Akesson, 2003). The hyphae start to penetrate the host's cuticle within 12 h, multiply rapidly and spread into the intercellular space within the mesophyll tissue of the leaf (Acharya *et al.*, 2011; Sahu *et al.*, 2016). The early-stage signalling events take place within the first 24 h after infection. The pathogen colonises the host tissue by 24 h. After as little as 48 h, hyphae produce conidiophores, giving rise to a new generation of conidia over four days (Sahu *et al.*, 2016).

Plant disease detection is required for many purposes, including prediction of yield losses, forecasting and monitoring of epidemics and evaluating host resistance, as well as for studying the fundamental biology of host-pathogen interactions. Generally, symptoms do not provide unambiguous evidence for comparisons between resistance and susceptibility. There are many methods used to estimate the impact of pathogens, some of them based on the estimation of the progression of plant disease symptoms, others based on quantitative differences in fungal growth between resistant and susceptible lines (Ayliffe *et al.*, 2013; Domiciano *et al.*, 2014). One method for measuring fungal growth is to measure DNA or RNA content using a quantitative polymerase chain reaction (PCR). This approach requires nucleic acid extraction, enzyme amplification, and primer optimisation. The can be problems with cross-contamination, and the approach is relatively labour intensive, and technically demanding for accurate quantitation to be obtained. Nevertheless, measurement of concentrations of internal transcribed spacer (ITS) sequences, for example, should provide a reproducible scale where qPCR data can be directly mapped to absolute fungal load.

Image scanning has been used to quantify disease symptoms, but this requires sophisticated instruments, is relatively low throughput, is only feasible once symptoms are visible (Wijekoon *et al.*, 2008; Goodwin and Hsiang, 2010). Transgenic pathogens expressing reporter genes have also been used to quantify pathogen biomass (Chen *et al.*, 2003; Papadopoulou *et al.*, 2005). However, this approach is not applicable in the field and is limited to transformable fungal pathogens. Enzyme-linked immunosorbent assay (Harrison *et al.*, 1990) or the conversion of a chromogenic substrate by the pathogen (Diener, 2012) have also been used to quantify fungal pathogens. Image analysis based on digital images has advantages over simple visual assessment, but artefacts such as shadows and or reflections can lead to errors. Image analysis is not yet sophisticated enough to distinguish between multiple diseases easily (Bock and Nutter Jr, 2012). Although each of these approaches has advantages, they suffer from being laborious,

technically demanding, pathogen-specific, not suitable for field studies, or only relatively low throughput.

Recently, a method for microscopic resolution of fungal infection structures in infected plant leaves by detection of chitin has been developed (Ayliffe *et al.*, 2011). The method uses the lectin wheat germ agglutinin (WGA) conjugated to a fluorophore such as Alexa Fluor 488 or fluorescein isothiocyanate (FITC). Because binding of WGA to chitin is highly specific, WGA conjugates have been used extensively for resolution of fungal infection structures (Meyberg, 1988; O'Connell and Ride, 1990; Ayliffe *et al.*, 2008; Ayliffe *et al.*, 2013). It has been reported that WGA specifically binds to trimers of N-acetylglucosamine but not to the closely related cell wall component chitosan (Meyberg, 1988; O'Connell and Ride, 1990; Ayliffe *et al.*, 2011). Ayliffe *et al.* (2013) further developed the WGA assay to estimate fungal biomass quantitatively. Their method is simple, rapid and they found that it is also sensitive and reliable. Accurate assessment of disease resistance is essential for the development of disease resistant plants. Scoring of resistance is particularly challenging when plants are only partially resistant. Plant disease assessment is frequently based upon qualitative estimates of pathogen growth. As known, chitin is a basic fungal cell wall component, thus chitin assay could be applicable to many pathogens. However, the WGA method is not a substitute for direct observation of diseased plants, which can describe disease status based upon the appearance of diseased plants.

5.3 Hypotheses:

1- WRKY transcription factor genes are differentially expressed over time after infection with *B. sorokiniana* in resistant and susceptible plants.

2- qPCR-based quantification of *B. sorokiniana* RNA can detect early infection stages.

3- The wheat germ agglutinin WGA conjugate method gives results comparable with those obtained by qPCR.

4- Development of *B. sorokiniana* infection over time is variable in the plants that are more or less resistant or susceptible as assessed by symptoms.

5.4 Aim:

To define the time course of changes in gene expression following pathogen attack in wheat and to determine which wheat genes are particularly induced at specific times after infection,

and to compare these changes in expression with the development of the pathogen in wheat leaves as assessed by qPCR and the WGA assay.

1- To investigate the effects of pathogen (*Bipolaris sorokiniana*) infection on host WRKY TF gene expression over time after infection (0, 24, 48, 72 and 96 h) in susceptible and resistant wheat cultivars using qRT-PCR.

2- To measure *B. sorokiniana* RNA development in infected leaves by qPCR over time after infection.

3- To measure fungal biomass in infected leaves by chitin measurements using wheat germ agglutinin WGA conjugate.

4- To observe pathogen development by fluorescence microscopic analysis of samples treated with wheat germ agglutinin WGA conjugate.

5- To relate wheat responses to infection and pathogen development in susceptible and resistant wheat to physiological differences resulting from infection.

5.5 Results

5.5.1 Time course of WRKY TF gene expression after infection with *B. sorokiniana*

Changes in WRKY TF genes expression were measured over time after infection with *Bipolaris sorokinana* in cultivars Rashid and Latifia by using RT-qPCR to ensure that subsequent studies were applied on the appropriate stages. Plants were infected at 14 days old and the expression of genes was measured at 24 hpi (penetration and early invasive growth), 48 hpi (in planta growth with symptoms), 72 hpi (increased growth with symptoms) and 96 hpi (increased disease severity), relative to 0 hpi (uninfected wheat).

In the susceptible cultivar Rashid, expression of all six WRKY TF genes did not change significantly at 24 hpi (Figure 5.1 A). At 72 hpi, there were significant increases in expression of *WRKY3, 19, 45* and *68a* (CI, $P < 0.05$), by 5.5, 3.3, 4.2 and 2.9-fold respectively, but no significant change for *WRKY46* and *WRKY51*. However, expression of these genes decreased 96 h after *Bipolaris sorokiniana* inoculation, so that *WRKY19* and *WRKY46* showed down-regulation of expression. Only *WRKY51* showed significant up-regulation of expression (CI, $P < 0.05$) at 96 hpi, by 2.7-fold. The *PR1* gene showed the highest increase in expression, up to 10-fold (CI, $P < 0.01$) at 72 hpi, but its expression was up-regulated (CI, $P > 0.05$) around 5-fold at 48 hpi and 96 hpi. Expression of the *rbcL* gene coding for the large subunit of ribulose-

1,5-bisphosphate carboxylase/oxygenase decreased significantly (CI, P < 0.05) at 72h and 96h (CI, P < 0.001) post infection.

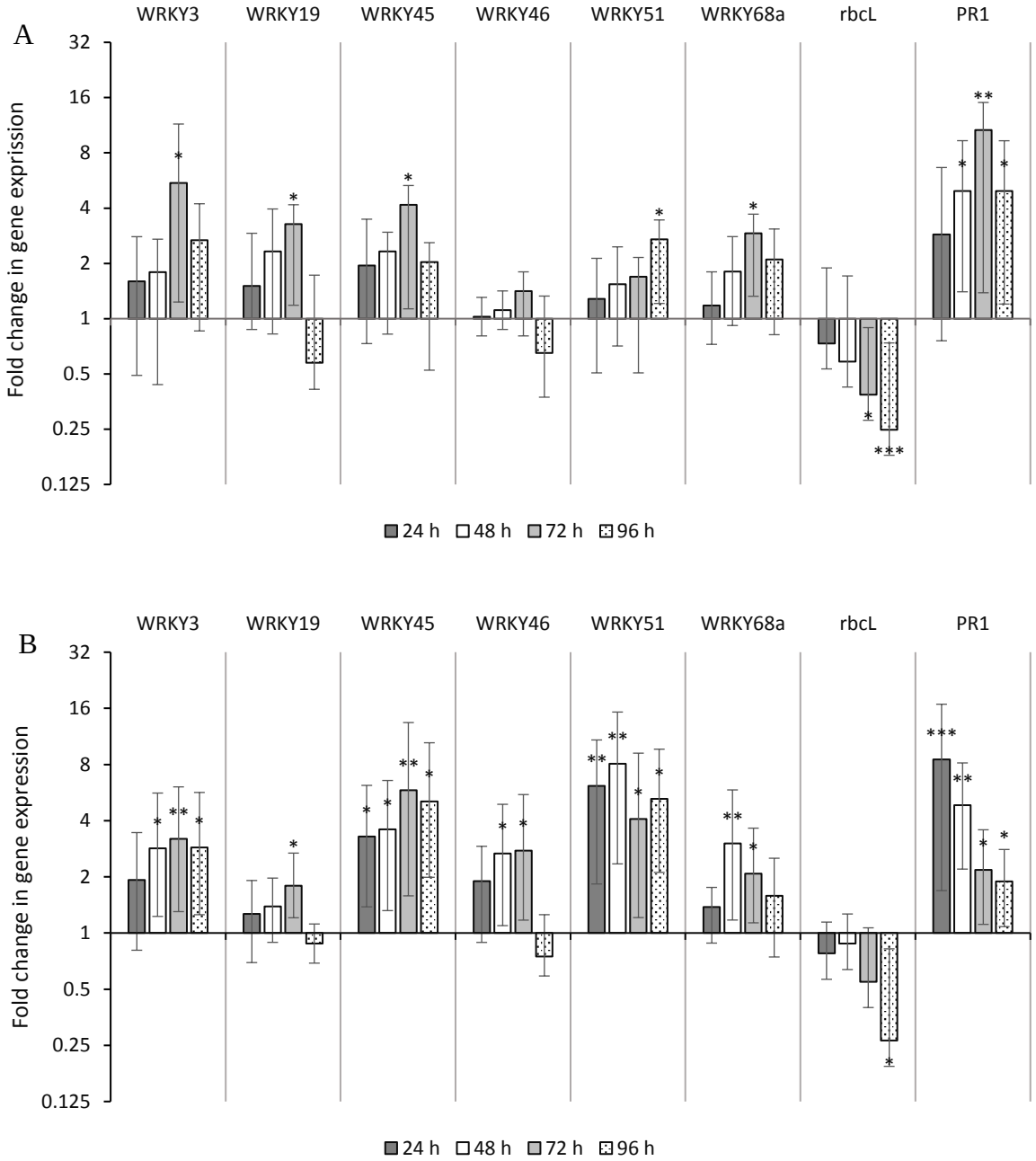


Figure 5.1 Fold changes in expression of six WRKY TFs, *rbcL* and *PR1* in infected leaves of (A) Rashid (susceptible) and (B) Latifia (resistance) wheat cultivars at 24, 48, 72 and 96 h after infection compared with 0 h as control, determined by qPCR.
Fold changes in gene expression were calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). *Ta28S rRNA* was used as endogenous control. Data are presented as means of three biological replicates with three technical replicates for each one. Error bars (confidence intervals) that do not cross the x-axis indicate that the difference from the control is statistically significant at a t-test (CI, P < 0.05*, 0.01**, n = 9) within each gene.

In the resistant cultivar Latifia, there was an early response of WRKY TF genes to infection with *Bipolaris sorokiniana* (Figure 5.1 B above). Up-regulation of expression of *WRKY51* and *WRKY68a* was highest at 48 h, 8.1-fold and 3-fold, respectively. Relative expression of *WRKY3, WRKY19, WRKY45* and *WRKY46* was highest at 72 h after infection (up-regulation 3.2, 1.8, 5.8 and 2.8-fold, respectively), but these values were only slightly higher than expression 48 h after infection. Also, the relative expression of *WRKY51* was high (6.2-fold up-regulation) early (24 hpi). The *PR1* gene showed the highest up-regulation of expression (CI, $P < 0.001$) early, at 24 hours after infection, but relative expression decreased over time, so that it was lowest at 96 h after infection (1.9-fold up-regulation) but still significantly above time zero (CI, $P < 0.05$). Relative expression of the *rbcL* gene decreased over time; relative expression at 96 hpi was significantly less (CI, $P < 0.05$) than at time zero.

To summarise of expression of *TaWRKY, rbcL* and *PR1* genes in susceptible Rashid compared to resistance Latifia (Figure 5.2). Expression of all WRKY TFs showed early higher significant response to spot blotch in Latifia than Rashid cultivar, expect *WRKY19* had no change till 72 hpi was significantly (CI, $P < 0.05$). Whereas, WRKY68a showed significant regulation (CI, $P < 0.01$) in Latifia, but not in Rashid at 48 hpi. The analysis of the abundance of *rbcL* mRNA showed the greatest significant down-regulation of expression at late response to spot blotch infection in Rashid than Latifia. However, Expression of *PR1* showed early high significant up-regulation in the resistant Latifia and no change in susceptible Rashid. Whereas, there were higher significant upregulation in *PR1* expression Rashid cultivar than Latifia cultivar.

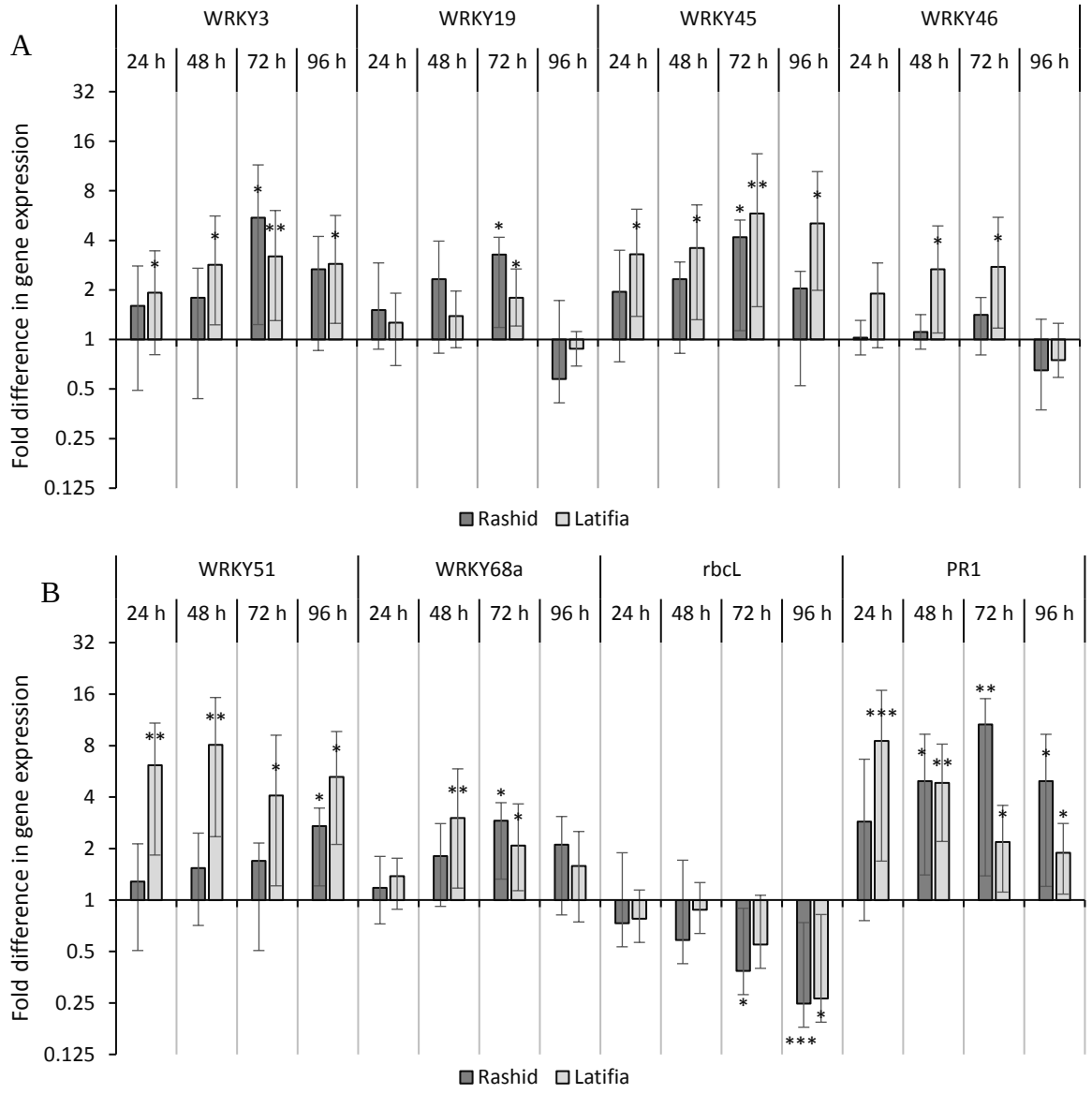


Figure 5.2 Fold changes in expression of (A) *WRKY3, WRKY19, WRKY45* and *WRKY46* and (B) *WRKY51, WRKY68a, rbcL* and *PR1* genes in infected leaves of Rashid and Latifia cultivars at 24, 48, 72 and 96 h after infection compared with time zero (0 h) as control, determined by qPCR. Confidence intervals used to comparison between values of fold changes within each gene and wheat cultivar (t-test, P <0.05*, 0.01**, n= 9) is statistically significant.

5.5.2 qPCR-based quantification of *B. sorokiniana* infection

RT-qPCR analysis of mRNA transcript levels of *B. sog1rokiniana* in overtime infected leaves is useful for estimation of pathogen prevalence in infected leaves, especially in the early stages of infection. *Coch, BipITS* and *ITS1-ITS4* primer pairs (Chapter 3) would provide a reproducible scale where qPCR data could be directly mapped to fungal load. Such a scale could provide rapid and reliable pathogen quantification in the study of early stages of infection. Encouraging results obtained in Chapter 3 showed increased expression with increasing spot blotch prevalence in infected leaves. Fold differences in abundance of RNA measured with *Coch*, *BipITS* and *ITS1-ITS4* primers relative to endogenous wheat control (*eIF4A*) was measured in

Rashid and Latifia cultivars. Wheat at 24 hpi, which was assessed as having very low levels of *Bipolaris sorokiniana* symptoms, was used as the reference condition in comparison to the rest of the infection time series (48, 72 and 96 hpi). Relative expression measured with all three primer pairs increased over time in infected Rashid wheat comparison to 24 hpi (Figure 5.3 A). The fold changes with Coch primers were 5.5-fold and 7.7-fold with significant increases in relative expression (CI, $P < 0.001$) at 72 hpi and 96 hpi, respectively, but only 1.2-fold at 48 hpi. With *BipITS* primer, the most highly significant increase was at 96 hpi (CI, $P < 0.001$), whilst at 48 hpi and 72 hpi the increase was significant at $P < 0.05$ and $P < 0.01$, respectively. With the *ITS1-ITS4* primer pair, the most significant increase in expression (CI, $P < 0.01$) was by 3.6-fold at 96 hpi. The increases at 48 and 72 hpi were also significant (CI, $P < 0.05$), by 2.3-fold and 3.2-fold, respectively.

Relative gene expression measured with all three primer pairs was lower in Latifia, the more resistant cultivar, than in Rashid, the more susceptible cultivar, but increased with increasing spot blotch prevalence over time (Figure 5.3 B). The *Coch* primer pair gave the highest increase in relative expression (CI, $P < 0.001$) at 96 hpi; the increase at 72 hpi, by 3.2-fold, was also significant (CI, $P <0.05$), but the change at 48 hpi was only 1.8-fold. Measurement with *BipITS* primers also showed a time-dependent response with significant increases in relative expression at 96 hpi (CI, $P < 0.01$), 2.9-fold, only 1.8-fold increase (CI, $P < 0.05$) at 72 hpi, and low and no difference at 48 hpi, 1.4-fold. Measurement with *ITS1-ITS4* showed significant increases in relative expression (CI, $P < 0.05$) at 96 hpi and 72 hpi, but the difference at 48 hpi was not statistically significant.

These findings show that there is a clear trend increasing in *Coch, BipITS* and *ITS1-ITS4* expression levels in both wheats over time after spot blotch infection, which displayed to less pathogen prevalence level at 24 h. The susceptible Rashid showed double increased in *Coch, BipITS* and *ITS1-ITS4* expression levels compared to resistant Latifia at same time infection.

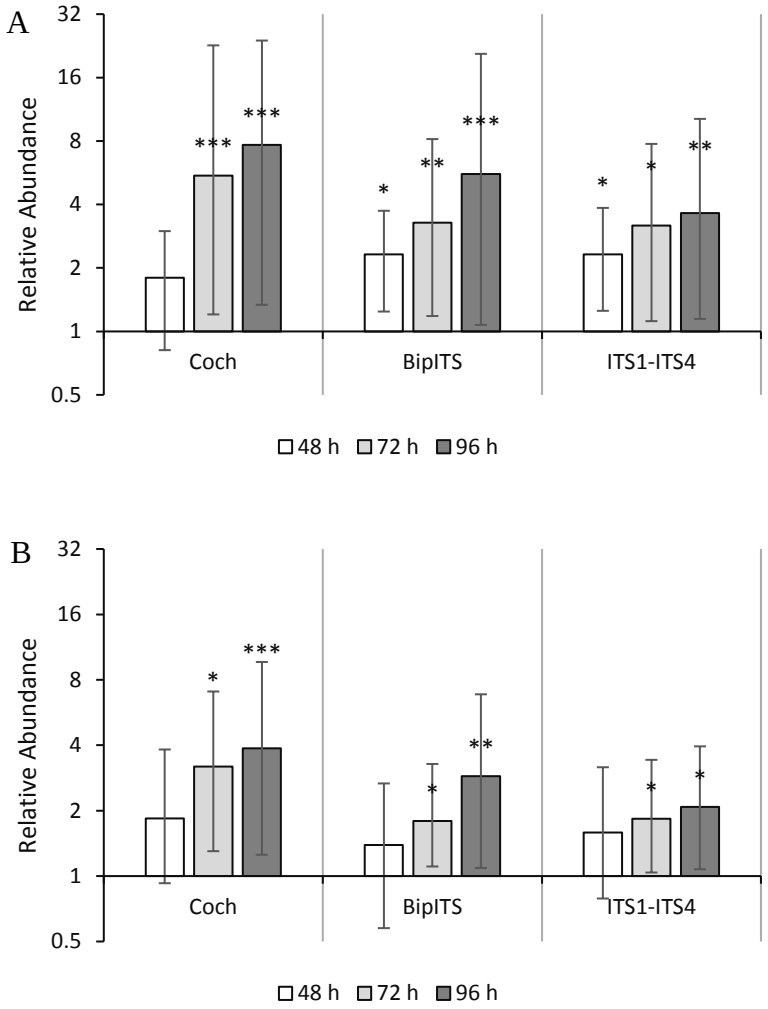


Figure 5.3. Relative abundance of *Bipolaris sorokiniana ITS* RNA measured with *Coch*, *BipITS* and *ITS1-ITS4* primers in infected (A) Rashid and (B) Latifia wheat leaves at 48, 72 and 96 hours after infection compared to 24 h determined by qPCR.
Fold changes in gene expression were calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). *eIF4A* was used as endogenous control. Data are presented as means of three biological replicates, each with three technical replicates. Error bars (confidence intervals) that do not cross the x-axis indicate that the difference is statistically significant as a t-test ($P < 0.05$*, 0.01**, $n = 9$) within each gene.

5.5.3 Physiological measurements

Chlorophyll content index was measured at 18 days old Rashid and Latifia control plants and in infected by spot blotch after four days of infection (Figure 5.4). Chlorophyll content index decreased steadily in infected Rashid and Latifia plants and was significantly (ANOVA, Tukey, $P < 0.05$) lower at 48, 72 and 96 hpi than at 0 h for both Rashid and Latifia. There was no significant change in uninfected plants of both cultivars (ANOVA, Tukey, $P > 0.05$). However, Rashid showed accelerate trend of decreasing in chlorophyll content compared to Latifia cultivar.

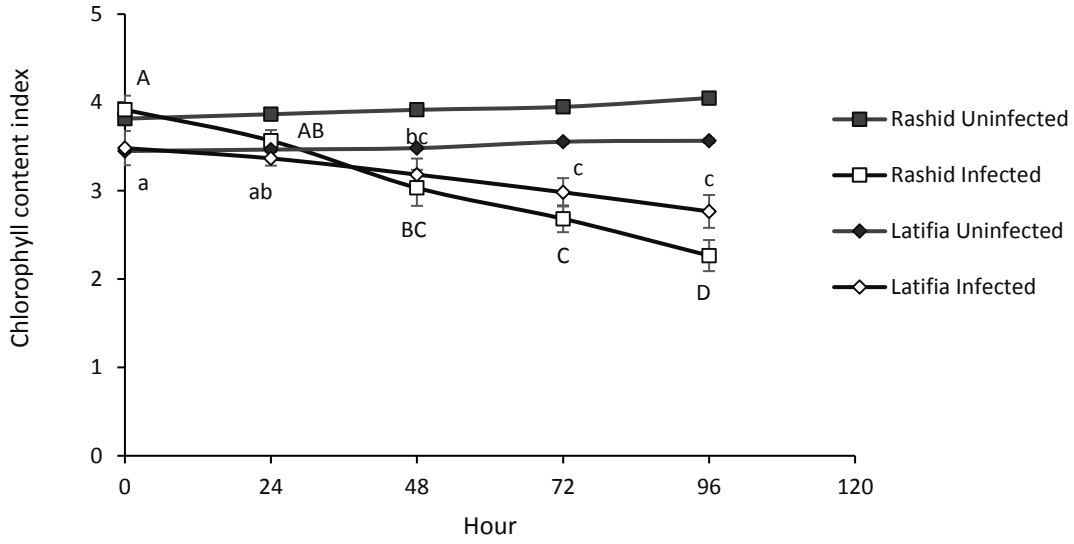


Figure 5.4. Chlorophyll content index (CCI) in infected and non-infected Rashid and Latifia plants at 0, 24, 48, 72 and 96 hours after infection).
Different letters represent significant differences (ANOVA, one way, Tukey $P < 0.05$, n= 9), error bars represent ± 1 standard deviation.

Detached leaves of Rashid and Latifia plants infected by *B. sorokiniana* at 14 days old were incubated at high humidity for four days starting directly after infection by spot blotch, and 24, 48, 72 and 96 h after infection. On both Rashid and Latifia, the numbers of spores were highest when the incubation at high humidity began on the day of infection (0 hpi) (Figure 5.5). Numbers of spores in both wheat then decreased, with significantly lower numbers (ANOVA, Tukey, $P < 0.05$) at subsequent time points up to 72 hpi 45000 and 22500 spores/ml in Rashid and Latifia, respectively. But number of spores washes from Rashid only started significant increasing (ANOVA, Tukey, $P < 0.5$) again by 96 hpi by 58000 spores/ml. Numbers of spores on Latifia were significantly lower (ANOVA, Tukey, $P < 0.5$) at subsequent time points, with the lowest number of spores at 96 hpi (only 18250 spores/ml). The number of spores at all-time points on Rashid was higher than on the more resistant cultivar Latifia.

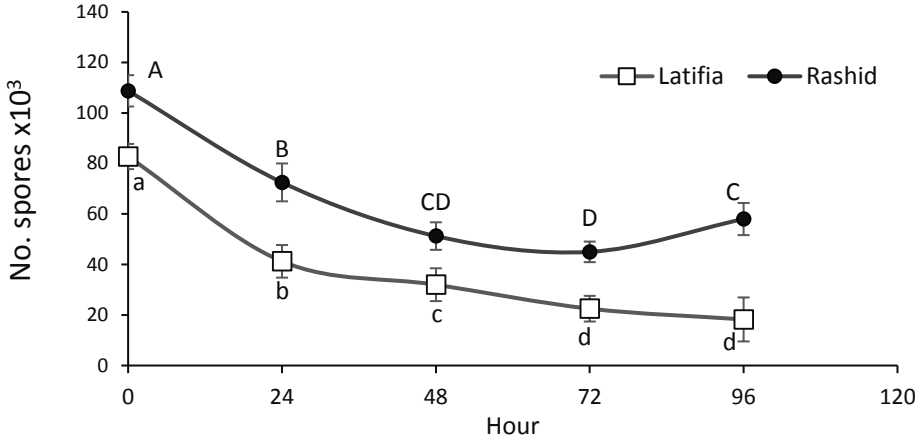


Figure 5.5. Number of spores $\times 10^3$ on infected leaves of Latifia and Rashid wheat cultivars at 0, 24, 48, 72 and 96 h after infection.
For each cultivar, different letters represent significant differences (ANOVA, one way, Tukey $P < 0.05$, $n = 9$), error bars represent ± 1 standard deviation.

5.5.4 Quantitative nature of the wheat germ agglutinin (WGA) chitin assay

To determine whether or not the binding of WGA to chitin particles is quantitative, a homogenate of uninfected wheat leaf tissue homogenate was used to serially dilute a suspension of ground-up, purified chitin particles. Reactions were carried out in triplicate. A linear dependence of fluorescence on chitin content (R^2= 0.989) was obtained when 0 to 200 µg of chitin particles was added to 80 mg of leaf tissue homogenate (Figure 5.6).

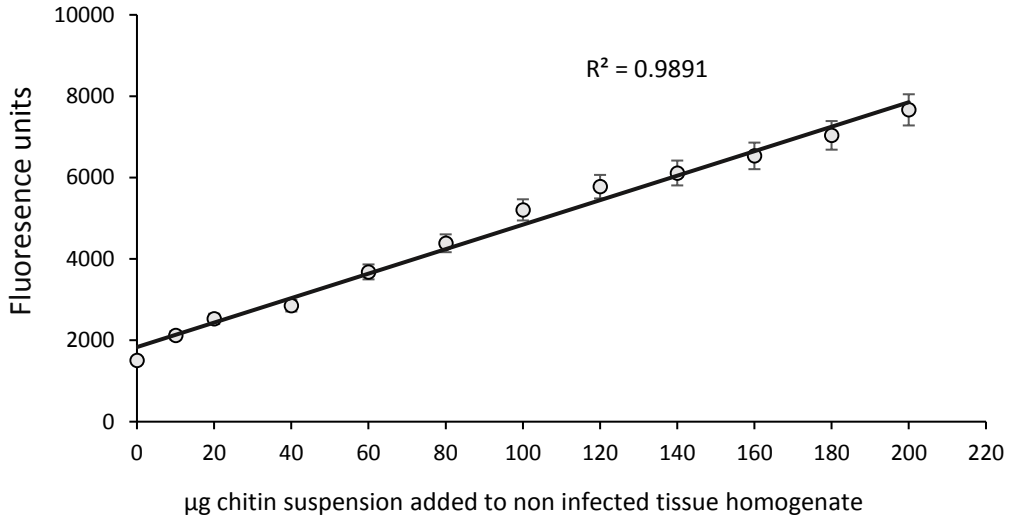


Figure 5.6. Chitin standard curve. A suspension of chitin particles was added to uninfected wheat leaf tissue homogenate and processed via the WGA assay.
Each data point represents the average fluorescence from six technical replicates (Ayliffe *et al.*, 2014), error bars represent ± 1 standard deviation.

5.5.5 Quantification of fungal biomass by WGA assay

Chitin measurement by wheat germ agglutinin WGA conjugate assay was used to estimate fungal biomass in infected wheat leaves. The WGA assay showed increases in chitin content over time after infection in both Rashid and Latifia cultivars (Figure 5.7). Differences among most of the time points were significant (ANOVA, Tukey, $P < 0.05$). The highest fluorescence units of chitin contents were recorded at 96 hpi (70700 and 44890 units for Rashid and Latifia, respectively). Chitin content in susceptible Rashid was higher than in resistant Latifia.

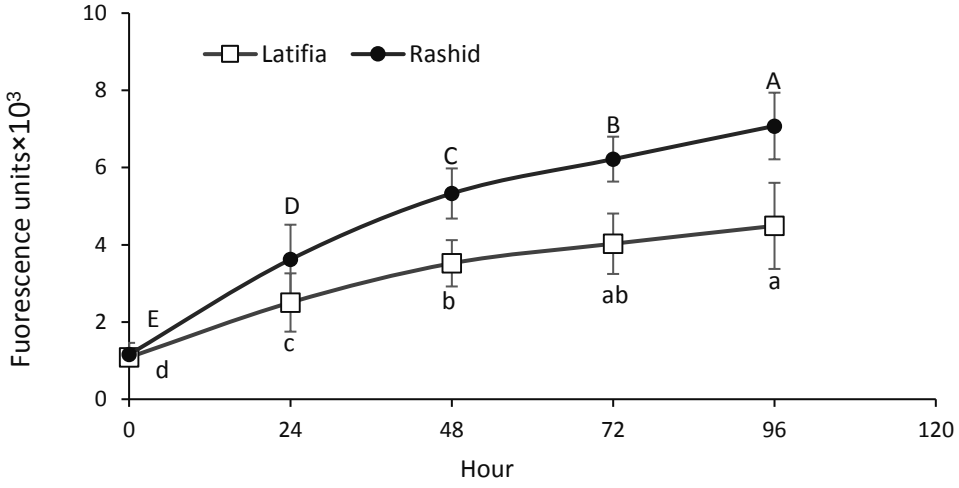


Figure 5.7. Chitin content (fluorescence unit) in infected leaves of Latifia and Rashid wheat cultivars at 0, 24, 48, 72 and 96 h after infection).
Different letters represent significant differences between values for each cultivar (ANOVA, one way, Tukey, $P < 0.05$, $n = 9$), error bars represent ± 1 standard deviation.

5.5.6 Microscopic analysis of spot blotch infection site areas by WGA assay

Microscopic analysis by wheat germ agglutinin WGA conjugate was used to examine growth of *B. sorokiniana* over time in infected Rashid and Latifia leaf tissues. The infection spread with time in both wheat cultivars. At every time point after 0 hours, fungal spread and disease damage were greater in Rashid than Latifia. The microscopic analysis suggested that in Rashid the pathogen switched from a biotrophic to necrotrophic phase between 72 hpi and 96 hpi (Figure 5.8), but no such change was observed in Latifia. The necrotrophic growth phase is characterised by invasion of mesophyll tissue and host cell death. Dead cells appeared decolourised in transmitted light and yellow auto fluorescing in UV light in Rashid wheat at 96 hpi.

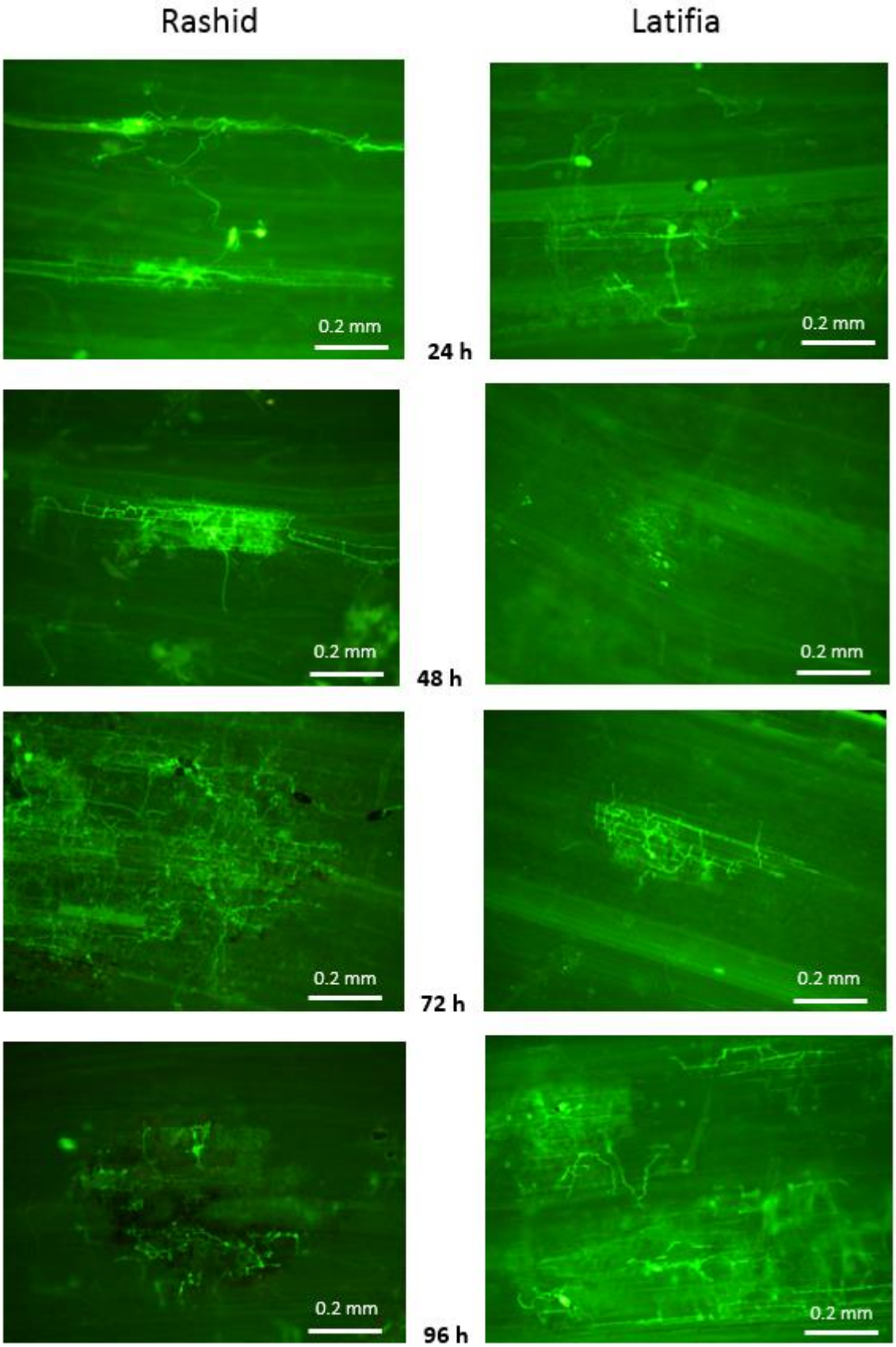


Figure 5.8. Microscopic analysis of *Bipolaris sorokiniana* growth in infected leaves of Rashid and Latifia at 24, 48, 72 and 96 hpi. WGA staining of infected wheat leaf tissue viewed under blue-light excitation at 10× magnification. Stained filamentous fungal hyphae can be seen. Scale bar = 0.2 mm.

5.6 Discussion

Understanding the responses of WRKY TF genes during interactions between fungal pathogens and host plants at the early infection stage, especially in economically important host-pathogen interactions such as wheat spot blotch, is considered a crucial target that could lead to exploiting these genes for crop improvement by manipulating their functions by transgenic methods or genome editing. The conclusions obtained in this field are still limited, especially in wheat. According to the research so far, the molecular mechanisms of the early interactions of pathogens and plants are likely to involve protein phosphorylation, ion fluxes, SA, JA, reactive oxygen species (ROS) and other signalling (Shen *et al.*, 2017; Wasternack, 2017). For a precise understanding of the changes involved in disease progression, samples should be taken at multiple time points. The present study analysed the fungal load on the plant at 24 hpi and continued at 48 hpi, 72 hpi and the end of the biotrophic and entry into the necrotrophic phase at 96 hpi. It will be necessary to adjust conditions to ensure progression of the disease to the necrotrophic stage. This will make it possible to determine whether nitrogen availability does affect the severity of spot blotch symptoms during the biotrophic or necrotrophic phase. In wheat infected with *Zymoseptoria tritici* Rudd *et al.* (2015) found differential expression of 686 wheat genes at 24 hpi, including genes for WRKY transcription factors and other defence associated genes. However, only 87 genes were differentially expressed at 96 hpi, indicating that whilst wheat may detect *Z. tritici* very early in the infection, changes in the transcriptome during the symptomless phase of growth (up to approximately 9 dpi for *Zymoseptoria tritici*) are minimal. Transcription factor (TF) families may alter expression over time after infection, with different groups of TFs active at different stages. Thus, these data make it possible to connect metabolic processes, signalling pathways, and specific TF activity, which will contribute to the development of network models of processes senescence (Breeze *et al.*, 2011).

Expression of six WRKY TF genes, in addition to the *rbcL* and *PR1* genes, was examined by qPCR. Changes in gene expression in the plants as a response to a pathogen over time after infection are not surprising. From Figure 5.1, there were differential changes in the expression of a range of WRKY genes in two wheat cultivars (Rashid and Latifia). In the more susceptible cultivar Rashid, five out of six WRKY genes showed the highest up-regulation of expression at a relatively late stage of the period studied, 72 hpi. Expression of all six WRKY genes tested showed no significant up-regulation until 48 hpi, and changes were less than 2-fold. Expression of *TaWRKY3* was strongly up-regulated at 72 hpi in Rashid and Latifia, but dropped in Rashid at 96 hpi so that the difference from time zero was only 2.7-fold and not significant. Relative expression of *WRKY3* increased significantly at 48 hpi in Latifia wheat. In both cultivars, the

response of *WRKY3* was quite similar to the response patterns of *WRKY45* and *WRKY68a* over time from infection. These results indicate that the late response of these genes in Rashid may be related to the high susceptibility of Rashid to spot blotch. Ding *et al.* (2014) found that *TaWRKY68* has different functions at different plant developmental stages and might have a "hub gene function in wheat responses to various biotic stresses".

Furthermore, there was a convergence in the responses of these genes to spot blotch infection with the *PR1* pathogenesis-related protein gene. In Rashid, the *PR1* gene showed the highest up-regulation of expression, more than 10-fold at 72 hpi and 5-fold for 48 hpi and 96 hpi. That this response of *PR1* gene expression was slightly late, at 48 hpi, may be because the amount of pathogenesis-related protein significantly increases at this time after infection and it plays a main role in defence against fungal pathogens. Yadav *et al.* (2016) suggested that increases in amounts of pathogenesis-related proteins lead to an increase in cell wall degradation, as PR proteins include chitinases and β-1,3-glucanases, which act on major structural components of the cell walls of pathogenic fungi. In Rashid, relative expression of *WRKY3*, *19*, *45*, *46* and *68a* decreased at 96 hpi. Thus, these genes do not show the same up-regulation during the necrotrophic phase as during the biotrophic phase. That this change from biotrophic to necrotrophic phase occurred between 72 hpi and 96 hpi is clearly suggested by microscopic analysis (section 5.5.6). Meanwhile, expression of *WRKY19* and *WRKY46* was down-regulated at 96 hpi, although the difference from time zero was not significant. This change in the expression of WRKY genes towards down-regulation is associated with the transition from the biotrophic to the necrotrophic phase of the infection cycle. It is likely that the suppression of *WRKY19* and *WRKY46* expression is via pathogen effectors. Many pathogens are able to alter host gene expression by producing effectors, which are secreted intracellularly. Effectors can enable host plants to recognise the pathogen presence, as explained by the Guard Hypothesis (Bent and Mackey, 2007). Host R gene products may recognise the effect of an effector on a host molecule or instead recognise an effector directly and activate a strong resistance reaction inhibiting pathogen infection (Rajamuthiah and Mylonakis, 2014). Therefore, it is likely that the suppression of *WRKY19* and *WRKY46* expression occurred via effectors, suggesting that WRKY genes may play an important role in the defence of wheat against *B. sorokiniana*. However, the changes in relative expression of *WRKY46* in Rashid are small compared to the other genes, and so whether there is biological significance to these changes should be questioned. Only *WRKY51* showed significant up-regulation in the late time course of infection at 96 hpi. This suggests that *WRKY51* may be important in the defence response against spot blotch in the necrotrophic phase in susceptible plants.

In the more resistant cultivar Latifia, relative expression of WRKY TF genes increased earlier and to higher levels than in cultivar Rashid (Figure 5.2). Relative expression of *WRKY51* and *WRKY68a* was highest at 48 h, with 8.1-fold and 3.0-fold up-regulation, respectively. Also, expression of *WRKY51* was significantly and similarly up-regulated for all time points. Shen *et al.*(2017) pointed to PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), which leads to a rapid disease response in the process of long-term coevolution with a pathogen. Relative expression was greatest at 72 hpi for *WRKY3, WRKY19, WRKY45* and *WRKY46*, with 3.2, 1.8, 5.8 and 2.8-fold up-regulation respectively, but those changes were only slightly higher than their expression at 48 hpi. In addition, relative expression of *WRKY51* was high at 24 hpi, with 6.2-fold up-regulation.

Pathogenesis-related protein gene *PR1* showed the highest up-regulation of expression (CI, $P < 0.001$), with early accumulation of transcripts at 24 hpi in Latifia, whereas in Rashid *PR1* expression was induced slightly later, with a maximum at 72 hpi. The results are supported by previous work conducted with Sahu *et al.* (2016), who observed that in wheat infected with *B. sorokiniana* there was up-regulation of expression of different PR genes at 8 hours post-infection in a resistant wheat cultivar. In addition, Yangmai resistance cultivar and these genes were clearly up-regulated at 48 h after spot blotch infection, whereas no significant elicitation was recorded in the expression of most of the PR genes in the susceptible Sonalika wheat cultivar. Also, Soh *et al.* (2012) found that induced expression of the *PR* gene was more highly maintained at 24 h after infection in a resistant than in susceptible plants. In rice inoculated with *B. sorokiniana*, which is not a rice pathogen, transcripts for pathogenesis-related proteins, including (*PR-1*) and peroxidase, accumulated by 12 h, with maximum levels at 24 h except for peroxidase (Manandhar *et al.*, 1999). In a study by Campbell *et al.* (2012) indicated to the earlier expression of all seven PR genes after 12 h pathogen attack in resistant compared to susceptible wheat cultivars.

The early accumulation of PR protein and peroxidase transcripts in response to *B. sorokiniana* occurred in parallel with the appearance of hypersensitive lesions within 24 h and decreased only after 72 to 96 hpi. There was no sign that necrosis led to production of signals that caused prolonged induction of these transcripts (Manandhar *et al.*, 1999). Some scholars have found that early responses to pathogens are triggered by elicitors. For example in barley, the reaction to *Puccinia graminic* 1 (*Rpg1*) protein for resistance to *Puccinia graminis* is phosphorylated within 5 minutes (Nirmala *et al.*, 2011), whilst changes in transcription in wheat with the *Lr57* gene for resistance to leaf rust are detectable 12 h after infection with *Puccinia triticina* (Yadav *et al.*, 2016).

Furthermore, relative expression of *rbcL* was suppressed at late stages, 72 hpi and 96 hpi, in Rashid, whereas in Latifia, relative expression of the *rbcL* gene decreased later, with significantly lower expression than at zero time only at 96 hpi. These results are consistent with (Jantasuriyarat *et al.,* 2005), who found that *rbcL* gene expression was suppressed in both susceptible and resistant interactions. Nevertheless, it has been reported that Rubisco gene expression changes in a coordinated manner during leaf development and is reduced by leaf senescence with the progress of leaf age (Suzuki and Makino, 2013).

Relative abundance of the internal transcribed spacer (ITS) of ribosomal RNA, measured with *Coch, BipITS* and *ITS1-ITS4* primer pairs, increased rapidly as disease severity increased for both Rashid and Latifia cultivars. Relative abundance in susceptible wheat (Rashid) was approximately double that in resistant wheat (Latifia) at late times after infection. At the earlier stage of infection there was less difference between the two cultivars. It can be concluded that abundance of *ITS* represents a reliable, quantitative method of assessing pathogen growth during the biotrophic phase of infection. In order to produce an absolute measure of fungal load, it will be necessary to analyse *ITS* abundance against known masses of fungus, for example by quantitation of chitin content in leaves (wheat germ agglutinin WGA conjugate method), which was supported by the results of this method. Therefore, I present quantitative PCR as a precise method for assessing the fungal load and molecular events at an earlier stage of the biotrophic phase than was previously possible.

Furthermore, it was necessary to use a suitable endogenous control to normalise expression of pathogen RNA to the total amount of cDNA present (Figure 5.3). Thus, pathogen RNA is displayed relative to wheat endogenous control gene (*eIF4A*), which showed the highest stability under different condition. Measurement of chitin content using the WGA assay also enabled detection of the pathogen in leaves during the biotrophic phase. WGA conjugates have been used extensively for resolution of fungal infection structures (Ayliffe *et al.,* 2011). The assay showed a significant increase in the quantity of fungal biomass as disease severity increased over time that was significantly higher in Rashid than Latifia.

Microscopic analysis (WGA) was also used to examine visually the presence and spread of the pathogen over time in infected leaves. Microscopic analysis clearly showed the distinct successive growth phases characterised by cuticle and cell wall penetration, followed by the development of hyphae within living epidermal host cells (biotrophic phase) and growth into the mesophyll, accompanied by epidermal and mesophyll cell death (necrotrophic phase). Similarly, infected barley seedlings developed oval dark brown necrotic lesions on leaf blades

within 2 days after inoculation with *B. sorokiniana* (Kumar *et al.*, 2002). At every time point except time zero, Rashid showed greater spread of fungal growth than Latifia. Microscopic analysis observations suggested that in Rashid the pathogen switched from biotrophic to necrotrophic phase between 72 hpi and 96 hpi (Figure 5.8), but no such change was observed in Latifia.

In addition to the many advantages of obtaining a quantitative measure of pathogen growth, these methods also make it possible to detect the presence of the pathogen early in the biotrophic phase. Methods which rely on the observation of symptoms (Simon *et al.*, 2003; Domiciano *et al.*, 2014) cannot be used during the symptomless biotrophic phase, and a similar problem is encountered when spore washes are used to assess the fungal load present in an infected sample before conidia are produced. Therefore, the data suggest that observed increases in spot blotch severity seen over time after inoculation are only present in the necrotrophic phase of the infection, representing an important finding in developing our understanding of spot blotch progression in the early stages of infection.

5.7 Conclusions

Relative expression of WRKY TF genes was up-regulated earlier in more resistant (Latifia) than more susceptible (Rashid) wheat. *WRKY51* showed the highest relative expression at 48 hpi in Latifia, with 8.1-fold up-regulation. Relative abundance of *ITS* RNA increased as disease severity increased over time. The relative abundance was higher (approximately double) in susceptible wheat (Rashid) than in resistant wheat (Latifia) at 96 h after infection.

WGA assay showed a significantly increase in fungal biomass in infected wheat as disease severity increased over time that was significantly higher in Rashid than Latifia. Also, microscopic analysis with WGA showed visually that the spread of *B. sorokiniana* was greatest at four days after infection, there was greater fungal growth in Rashid than in Latifia. Both methods showed ability to detect pathogen presence at early stages during the biotrophic phase; the quantitative method produces an absolute measure of fungal load in relation to development of disease severity. Based on these results, the WRKY transcription factor genes that are involved in the early interaction between pathogen and host could be target genes for improvement of crops by manipulating their functions by using transgenic methods or genome editing. Furthermore, quantitative PCR measuring the abundance of the *B. sorokiniana ITS* sequence and WGA assay should be considered excellent candidates capable of detecting spot blotch presence even in the earlier biotrophic phase, when fungal load is low.

6 The interaction between nitrate supply and severity of spot blotch in wheat TILLING lines

6.1 Abstract

Chemical mutagenesis agents like ethyl methane sulphonate (EMS) induce point mutations efficiently. Use of the TILLING method can provide a high density of mutations in a gene of interest and so provide insights into protein function. TILLING lines with functional mutations in the *WRKY19* and *WRKY68a* genes and the parental wild-type Cadenza were examined under nutrient stress and *B. sorokiniana* infection. TILLING seed was obtained from KWS. Seedlings of TILLING mutants were grown in sand watered with Hoagland's solution modified to contain 0.75 mM, 3.75 mM or 7.5 mM nitrate. Fourteen-day-old seedlings were inoculated with *Bipolaris sorokiniana* and incubated for four days. The TILLING lines exhibited different patterns of response to infection at the three nitrogen levels. The *W19H334* and *W68aQ245* mutants had higher chitin content with reduced nitrogen input, whereas the opposite response was found in the *W68aV213* mutant and the wild-type. Microscopic analysis with WGA of line *W68aQ245* grown with severely reduced nitrogen showed a greater spread of the pathogen, associated with greater damage in infected tissue than with high nitrogen input. Expression of *WRKY68a* was up-regulated at severely and moderately reduced nitrogen levels in uninfected and infected Cadenza (wild type), whereas expression of *WRKY19* was up-regulated only at reduced nitrogen levels in uninfected Cadenza. In conclusion, TILLING line *W68aQ245* exhibited high defence against *B. sorokiniana* at the high nitrogen level, whereas wild-type plants grown with high nitrogen availability have high susceptibility to spot blotch, which accompanied by an increase in plant growth (growth *vs* defence). Because the mutant lines may contain other mutations, they will need to be backcrossed and screened before it can be categorically stated that the WRKY TF genes are involved in disease resistance. Furthermore, rapid screening techniques such as the WGA assay should be considered to be excellent candidates for the detection of fungi in wheat leaves.

Keywords; TILLING line, wheat, reduced nitrogen, *Bipolaris sorokiniana*, gene expression analysis, disease severity assessment.

6.2 Introduction

The gene redundancy associated with the polyploidy of wheat (*Triticum aestivum* L.) makes it challenging to identify desirable genetic changes based on phenotypic screening. A modified TILLING (Targeting Induced Local Lesions IN Genomes) method has been developed in response to this problem. The approach combines chemical mutagenesis with high-throughput genome-wide screening to find specific point mutations in gene of interest. This methodology has various advantages over other reverse genetics approaches. The mutagen EMS (ethyl methane sulphonate) produces a broad spectrum of mutations. These include truncations and missense mutations so that there is greater flexibility than with insertional mutagenesis or transgenesis (Henikoff *et al.*, 2004). Furthermore, the mutagenesis produces a high density of random point mutations in polyploid plants, so that it possible to obtain multiple alleles of a specific gene in a small population even when the genome is large (Sabetta *et al.*, 2011; Chen *et al.*, 2012). The TILLING method allows the directed identification of mutations in a specific gene in a low-budget small laboratory (Dong *et al.*, 2009).

Chemical mutagenesis agents like ethyl methane sulphonate (EMS) are favoured due to the ability to induce point mutations. The TILLING method can identify a high density of mutations in a gene of interest and it can provide in-depth insights into protein function in the desired gene domain. Using TILLING lines in bread wheat (hexaploid) is efficient, as its polyploid state makes it exhibit very high mutation frequencies with small numbers of treated plants (1 in 20–30 kb) (Slade *et al.*, 2005; Dong *et al.*, 2009). In contrast, the mutation frequency with EMS mutagenesis in the dicot Arabidopsis is 1 in 170 kb (Greene *et al.*, 2003). Among monocot plants, barley has produced 1 in 1000 kb (Caldwell *et al.*, 2004), and rice 1 in 300 to 600 kb (Till *et al.*, 2007). Furthermore, Krasileva *et al.* (2017) indicated that more than 10 million mutations in the protein-coding regions of 2,735 mutant lines of tetraploid and hexaploid wheats. Also, 2,705 and 5,351 mutations were detected as an average in tetraploid and hexaploid lines, respectively, which resulted in 35–40 mutations per kb in each population. The frequency of EMS mutations in the hexaploid genome of wheat is as much as 10 times higher than in the diploid genome of barley (Uauy *et al.*, 2017).

The TILLING method is an excellent tool for manipulation in functional genomics and crop improvement. In genetic studies in plants with large genomes, associating mutant phenotypes with gene sequence information is very time-consuming, especially when insertion mutagenesis is not practical as in wheat. Reverse genetics based on chemical mutagenesis provides the best solution to this problem. Although the DNA sequence of a gene may provide sufficient

information to infer function, it is necessary to test predictions phenotypically (Rothe, 2010). The TILLING method can offer the practical verification needed sequence-driven hypotheses (Henikoff *et al.*, 2004). Furthermore, TILLING can be applied to collections of genotypes (such as cultivars, collection of ecotypes, landraces or wild accessions), in an approach termed EcoTILLING (Comai *et al.*, 2004). The value of this method is demonstrated by the success of the Arabidopsis TILLING Project, which is a high-throughput service platform for functional gene analysis (Rothe, 2010). TILLING can also be applied in crop improvement, as TILLING mutants method can be used in conventional breeding programmes since they are non-transgenic and the new variations are stably inherited (Henikoff *et al.*, 2004; Uauy *et al.*, 2009). TILLING resources have been developed for many cereal crops. For example, in maize, pools of DNA samples were screened for mutations in 1-kb segments from 11 different genes, giving 17 independent induced mutations from population of 750 pollen-mutagenised maize plants.

The molecular mechanisms that cause variation in mutation yields in plants are unknown. Such knowledge would improve TILLING populations. There is some evidence that polyploidy, but not genome size, helps to confer tolerance to high mutation densities (Stadler, 1929; Tsai *et al.*, 2013). There are at least two possible explanations. Firstly, genetic redundancy may protect polyploidy plants from the damaging consequences of mutations. Secondly, polyploids may have greater physiological tolerance of genotoxic treatments or may be more susceptible to mutagenesis, possibly because of adaptive changes to DNA repair and genome maintenance mechanisms after polyploidisation (Tsai *et al.*, 2013). There are certain factors that should be considered when selecting the mutant line. Many independent lines will contain several point mutations in each gene of interest, so it may not be directly obvious which line rather than others to take forward. Lines will be different and the decision taken will depend on the aim of the study (Till *et al.*, 2004).

The wheat training website (STM, 2018) indicates that some types of mutations which are the most desirable should be considered when selecting TILLING mutants. Mutant lines with "stop gained" (premature termination codon) are generally the most desirable, as they will most likely result in a lack of function of the gene of interest. It is essential to consider the position of the stop codon in the protein as this might have implications for the phenotype. TILLING lines with homozygous mutations may be preferable since fewer seeds will need to be screened compared to heterozygous mutations, but generally, this method still has good distinct advantages. Mutations that are heterozygous will most likely still be segregating in the seed of M4 or M5 generation, so that a large number of seeds need to be screened to find homozygous mutations. However, it is essential to consider that some of the mutations that were

heterozygous in the M2 generation may have been fixed in the M4 or M5 seed, whereas other mutations may have been lost through genetic drift (STM, 2018).

6.3 Hypotheses

1- TILLING lines in which the WRKYGQK amino acid sequence is mutated have different levels of resistance to the hemibiotrophic pathogen *Bipolaris sorokiniana* compared to the wild-type Cadenza wheat cultivar.
2- WRKY TF genes are differently regulated according to reduced nitrogen input in Cadenza cultivar (wild-type).
3- The wheat germ agglutinin WGA conjugate method is an accurate, sensitive, simple, and non-subjective measurement of fungal growth in TILLING lines.

6.4 Aims

1- To investigate whether mutations in the WRKYGQK amino acid sequence change the effects of reducing nitrogen input in Cadenza wheat and its TILLING lines by using different physiological measurements.
2- To determine if Cadenza wheat grown with 0.75 mM and 3.75 mM nitrogen exhibits differential expression of *WRKY68a* and *WRKY19* compared to that grown with 7.5 mM nitrogen.
3- To investigate the quantification of fungal biomass and microscopic analysis of fungal development in infected leaves with *Bipolaris sorokiniana* of TILLING lines grown at different nitrogen levels by chitin measurements (wheat germ agglutinin WGA conjugate assay).

6.5 Results

6.5.1 Physiological measurements in infected and uninfected Cadenza and TILLING lines

Three-week-old control Cadenza plants and TILLING mutants (Table 6.1) were grown with three levels of nitrogen supply (0.75 mM, 3.75 mM and 7.5 mM nitrate) and infected with spot blotch (*Bipolaris sorokiniana*).

Table 6.1. List of TILLING lines modified in WRKYGQK amino acid sequences in Cadenza wheat used in this study.

TILLING name	WRKY name	Database gene	Consequence	Line-Cadenza	Notes
W19H334	WRKY19	Trase_2AS_0186B9E4F	H->Y	0757	Homozygous mutation within 2nd WRKY domain
W68aQ245	WRKY68a	Trase_2AL_15A7BB684	STOP gained	1237	Heterozygous mutation within WRKY domain
W68aV213	WRKY68a	Trase_2BL_6B75B32E3	V->M	1558	Homozygous mutation within WRKY domain

TILLING lines showed differences in growth between different nitrogen supply levels or between infected and uninfected plants: shoot heights, numbers of leaves and numbers of tillers were not significantly different (ANOVA, Tukey, $P > 0.05$) (Figure 6.1 A, B and C, respectively). The *W68aQ245* mutant had significantly (ANOVA, Tukey, $P < 0.05$) greater plant growth than the other mutants. Mean shoot height was 41.28 cm, number of leaves 4.48 and number of tillers 1.68 for *W68aQ245* line compared to means of 31.46 cm shoot height, 3.25 leaves and 1 tiller for *W68aV213* and 24.35 cm shoot height, 3 leaves and 1 tiller for *W19H334*. The *W68aQ245* mutant was not significantly different (ANOVA, Tukey $P > 0.05$) from the wild-type, except that it had a significantly greater average number of tillers. The *W19H334* mutant had a shorter plant shoot height and significant differences (ANOVA, $P < 0.05$) than the other TILLING lines and wild type wheat.

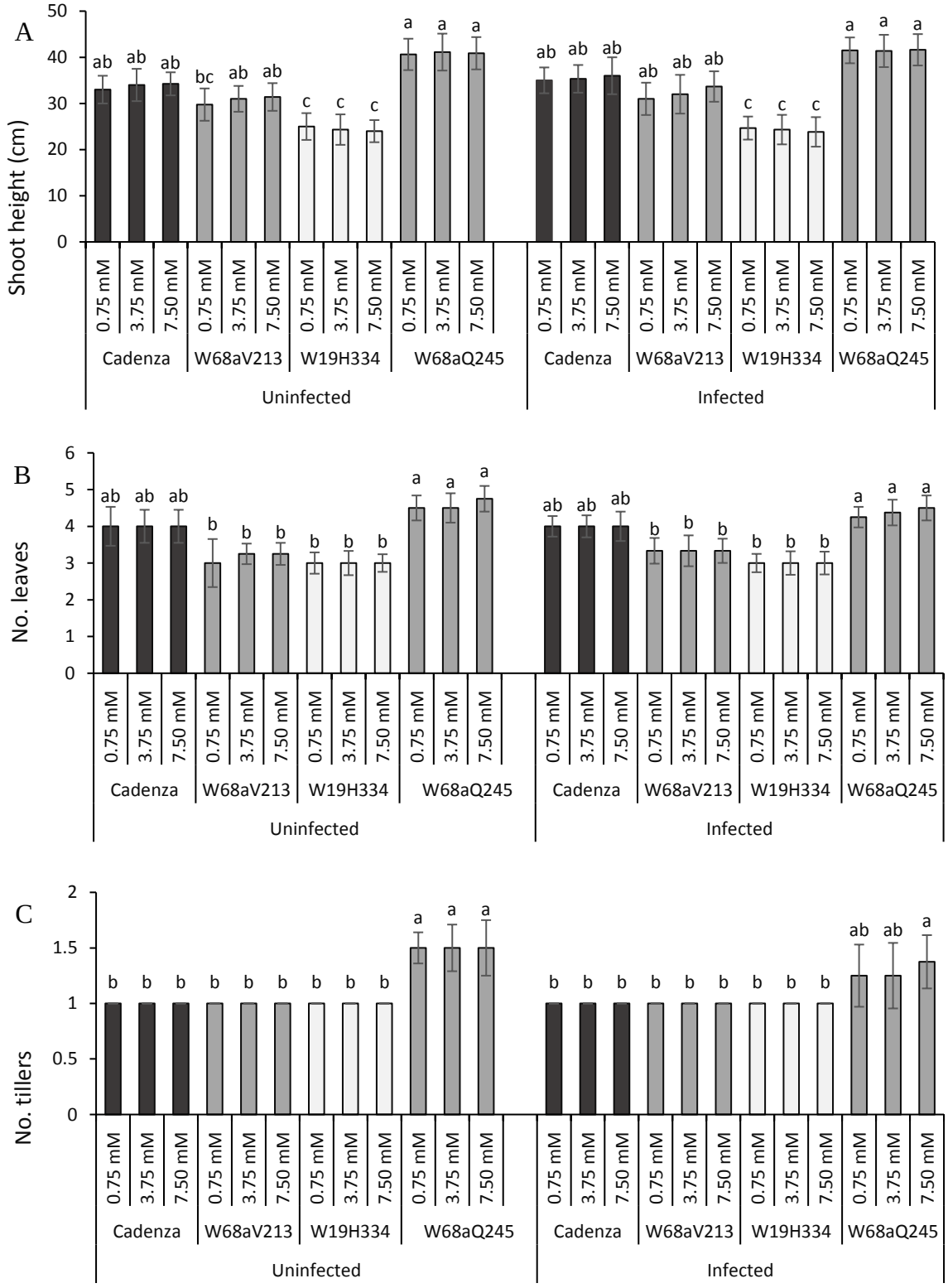


Figure 6.1. Effect of three levels of nitrogen supply (0.75, 3.75 and 7.5 mM nitrate) and infection with *Bipolaris sorokiniana* on (A) shoot height (B) number of leaves and (C) number of tillers in Cadenza and different TILLING lines mutated in WRKYGQK sequences.
Different letters represent significant differences (ANOVA, three way, Tukey, $P < 0.05$, $n = 8$).

As shown in Figure 6.2, differences were found in the chlorophyll content in leaves of wild-type and TILLING lines grown with reduced nitrogen availability. The chlorophyll content index value for uninfected Cadenza plants was significantly higher (ANOVA, Tukey, $P < 0.05$)

at 7.5 mM nitrogen (5.5 CCI) than at both reduced nitrogen levels (CCI 4.1 at 0.75 mM and 4.5 at 3.75 mM). The CCI in Cadenza infected by spot blotch severely and moderately reduced nitrogen was significantly higher (ANOVA, Tukey, $P < 0.05$) than in *W68aV213* and *W19H334* in same nitrogen concentration, but not different on *W68aQ245* mutant. The *W68aQ245* line had a significantly (ANOVA, Tukey, $P < 0.05$) lower chlorophyll content index (3.2 CCI) in the infected plants with severely reduced nitrogen (0.75 mM) than in the infected plants grown with moderate (3.9 CCI) and standard nitrogen levels (4.1 CCI). Also the CCI of infected *W68aQ245* line was significantly lower (ANOVA, Tukey, $P < 0.05$) than the CCI of uninfected plants at low nitrogen (0.75 mM). Leaves of the *W19H334* line had the lowest chlorophyll content (2.7 CCI and 3.3 CCI) with severely reduced nitrogen in infected and uninfected plants respectively, but this difference was only significant (ANOVA, Tukey, $P < 0.05$) compared to the standard nitrogen level (3.4 and 3.6 CCI in infected and uninfected plant, respectively). Overall, the values of chlorophyll content in Cadenza and the TILLING lines were higher at standard nitrogen availability than at severely and moderately reduced nitrogen levels for controls and plants infected with *Bipolaris sorokiniana*.

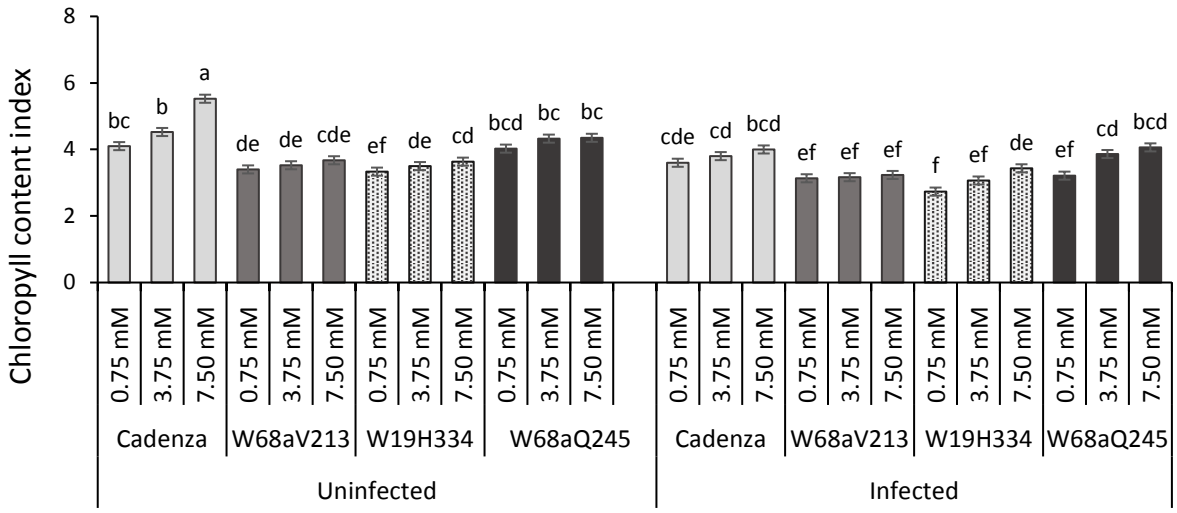


Figure 6.2. Effect of three levels of nitrogen supply (0.75, 3.75 and 7.5 mM nitrate) and infection with *Bipolaris sorokiniana* on chlorophyll content index (CCI) in Cadenza and TILLING lines mutated in WRKYGQK sequences.
Different letters represent significant differences (ANOVA, three way, Tukey, $P < 0.05$, $n = 8$), error bars represent ± 1 standard deviation.

There were differences in values of leaf area index at different nitrogen levels, but no significant differences between infected and uninfected plants (Figure 6.3). The *W68aQ245* mutant had significantly greater (ANOVA, Tukey, $P < 0.05$) leaf area (mean of six conditions, 318.5 LAI) than Cadenza and the other TILLING lines, but this did not differ between the three levels of nitrogen in the uninfected and infected plants. This mutant was also significantly different (ANOVA, Tukey, $P < 0.05$) from the wild-type and all three TILLING lines at all nitrogen

levels, except that the difference from Cadenza grown at the moderate nitrogen level (3.75 mM) was not significant (297.6 and 291.3 LAI in infected and control Cadenza, respectively). In Cadenza, the leaf area index was significantly (ANOVA, Tukey, $P < 0.05$) larger with moderately reduced nitrogen (291.3, 197.6) than with severely reduced nitrogen (262.3, 271.9). Whereas, leaf area index was not significant differences between the standard nitrogen level (279.1 and 287.2) and both nitrogen reduced stress in uninfected and infected Cadenza respectively.

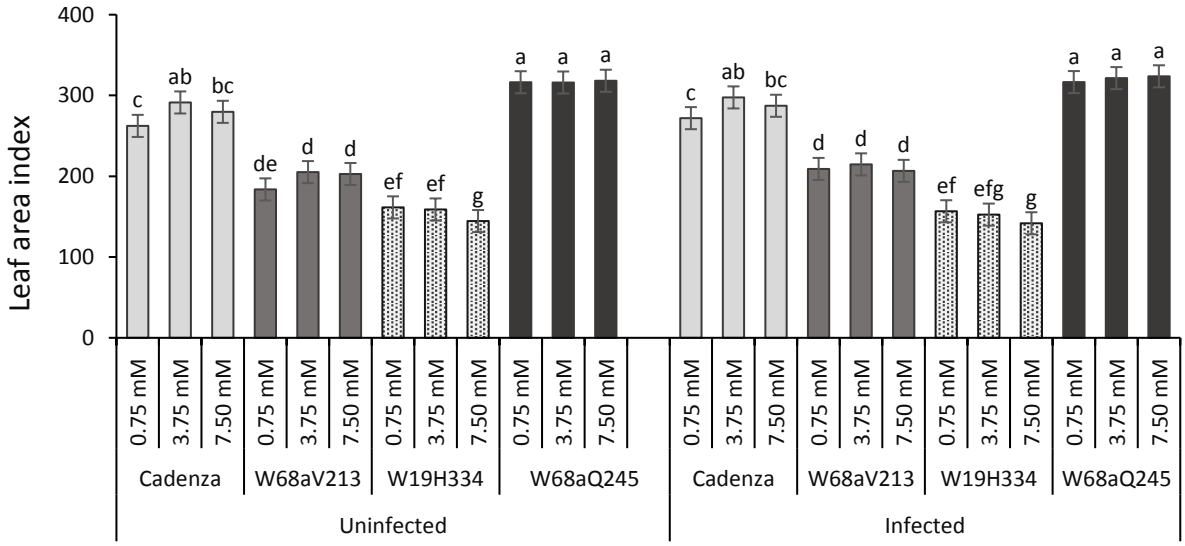


Figure 6.3. Effect of three levels of nitrogen supply (0.75, 3.75 and 7.5 mM nitrate) and infection with *Bipolaris sorokiniana* on leaf area index in Cadenza cultivar and different TILLING lines mutated in WRKYGQK sequences.
Different letters represent significant differences (ANOVA, three way, Tukey, $P < 0.05$, n= 8), error bars represent ± 1 standard deviation.

The *W19H334* mutant had the lowest leaf area index in uninfected and infected plants at the standard nitrogen level (144.5 and 141.76 respectively), and this was significantly lower (ANOVA, Tukey, $P < 0.05$) than with severely reduced nitrogen in infected and uninfected plants as well as lower than with moderately reduced nitrogen in uninfected plants. *W68aV213* line had low leaf area index values and no difference between the six conditions (mean of six conditions = 205.6); this was significantly lower than (ANOVA, Tukey, $P < 0.05$) the leaf area index in Cadenza and the *W68aQ245* mutant under all six conditions. The *W19H334* mutant showed the weakest growth based on the physiological measurements taken compared to the other mutants and the wild type used in this study. The values of leaf area index tended to be higher with moderately reduced nitrogen than with standard nitrogen and the severely reduced level in Cadenza and the *W68aV213* mutant. By contrast, the *W19H334* mutant had a low leaf area index with standard nitrogen level and increased leaf area index with reduced nitrogen input.

6.5.2 Disease severity and number of spores in infected wild-type and TILLING lines

Three week old infected Cadenza (wild type) and TILLING line plants grown at different levels of nitrogen (0.75, 3.75 and 7.5 mM nitrate) were screened for disease severity and spore production at four days after infection with *Bipolaris sorokiniana*. There were increases in disease severity and number of spores (Figure 6.4, A and B) in infected wild type and *W68aV213* plants when nitrogen input increased from 0.75 mM to 3.75 mM and 7.5 mM nitrate.

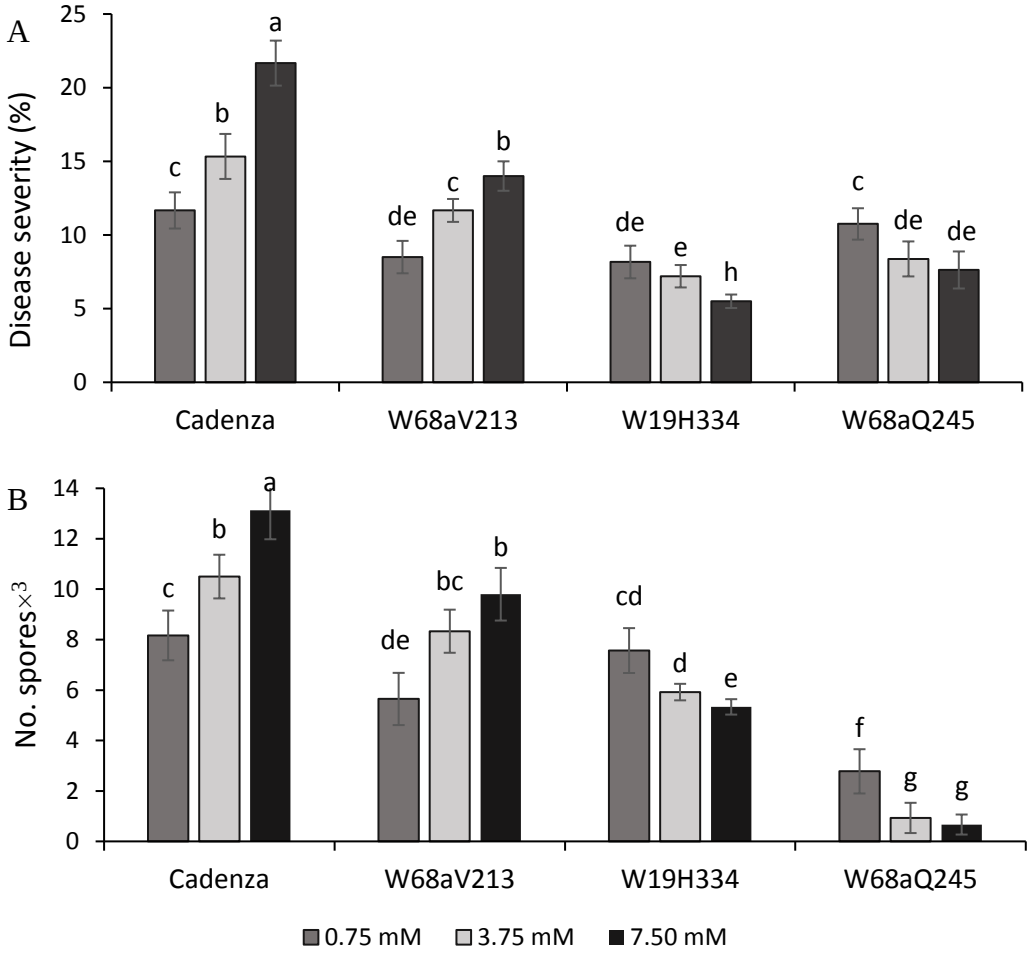


Figure 6.4. Effect of three levels of nitrogen supply (0.75, 3.75 and 7.5 mM nitrate) on (A) disease severity (%) and (B) number of spores×10^3 in plants infected with *Bipolaris sorokiniana* in Cadenza cultivar and TILLING lines mutated in WRKYGQK sequences.
Different letters represent significant differences (ANOVA, two way, Tukey, $P < 0.05$, $n = 8$), error bars represent ± 1 standard deviation.

Both wild type and *W68aV213* mutant plants showed higher percentage of disease scoring with significant difference (ANOVA, $P < 0.05$) at 7.5 mM than 0.75 mM and 3.75 mM nitrogen levels. Also, severe nitrogen stress showed significant ($P < 0.05$) more defence to spot blotch with lower disease severity scoring than in moderate nitrogen level. Whereas, in number of spores washed from infected leaves, there were a similar trend of Cadenza and *W68aV213* plants as shown in disease severity scoring except the differences between high nitrogen input

(7.5 mM) and moderate nitrogen level in *W68aV213* line was not significant (ANOVA, P > 0.05). There was a reverse pattern of change in TILLING lines (*W19H334* and *W68aQ245*) for disease severity and number of spores from infected leaves with reduced nitrogen levels compared to Cadenza and *W68aV213*. Lines *W19H334* and *W68aQ245* showed decreases in both values with increasing nitrogen availability. The *W19H334* mutant had significantly (ANOVA, Tukey, P < 0.05) higher defence against spot blotch infection at standard nitrogen level (disease severity 5.5 % and 5300 spores/ml) than at severely (disease severity 8.17 % and 7567 spores) and not significant with moderately (disease severity 7.2 % and 5920 spores) reduced nitrogen. Also, the *W68aQ245* mutant had the lowest number of spores only 660 spores/ml and disease severity score (7.63 %) at the standard nitrogen level, but the difference was only significant (ANOVA, Tukey, P < 0.05) compared to severely reduced nitrogen with a score of 10.75 % for disease severity and 2780 spores/ml.

Levels of chitin content (Fluorescence unit×10^3) by wheat germ agglutinin WGA conjugate assay in leaves of Cadenza and three TILLING lines four days after infection with *Bipolaris sorokiniana* at the three nitrogen levels are shown in Figure 6.5. Cadenza wheat had a high chitin content in infected leaves at the standard level of nitrogen (8102 units). This was significantly different (ANOVA, Tukey, P > 0.05) from chitin content in plants at severely and moderately reduced nitrogen levels (4990 and 5891 units, respectively) for Cadenza and all TILLING lines used in this study. The *W68aV213* mutant showed the same trend of response to *Bipolaris* infection as Cadenza, having significantly (ANOVA, Tukey, P < 0.05) higher chitin content (6406 units) at standard nitrogen level than at severely reduced (0.75 mM, 4703 units) and moderately reduced nitrogen levels (3.75 mM, 5223 units). In contrast, the *W19H334* and *W68aQ245* mutants had significantly (ANOVA, Tukey, P < 0.05) lower chitin content (4161 and 3476 units) in leaves at the standard nitrogen level than at moderate (5124 and 4278 units) and severely reduced nitrogen (5486 and 5358 units respectively).

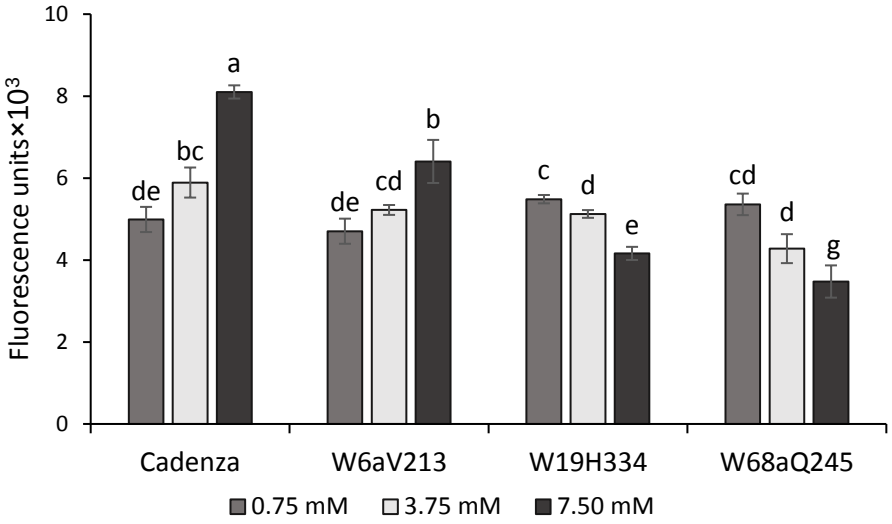


Figure 6.5. Effect of three levels of nitrogen supply (0.75, 3.75 and 7.5 mM nitrate) on chitin content (Fluorescence unit×10^3) in plants infected with *Bipolaris sorokiniana* in Cadenza cultivar and TILLING lines are mutated in WRKYGQK sequences.
Different letters represent significant differences (ANOVA, two way, Tukey, $P < 0.05$, $n = 8$), error bars represent ± 1 standard deviation.

6.5.3 Microscopic analysis of infected Cadenza wild-type and *W68aQ245* mutant

The results of microscopic analysis by wheat germ agglutinin WGA conjugate assay of wild-type wheat (Cadenza) and *WRKY68aQ245* mutant infected by spot blotch at three weeks old grown with reduced and standard nitrogen input are shown in Figure 6.6. The infection had different patterns of response to nitrogen supply, as the visualisation of the growth of *Bipolaris sorokiniana* showed a greater spread in the leaf tissue of the *W68aQ245* mutant with severely reduced nitrogen than with moderately reduced and standard nitrogen levels. In contrast, the visualisation of the growth of spot blotch in infected Cadenza showed a greater spread in leaf tissue at the standard nitrogen level of 7.5 mM. The growth of *B. sorokiniana* was slower in reduced nitrogen in Cadenza, with very low fungal presence at the severely reduced nitrogen level of 0.75 mM nitrate.

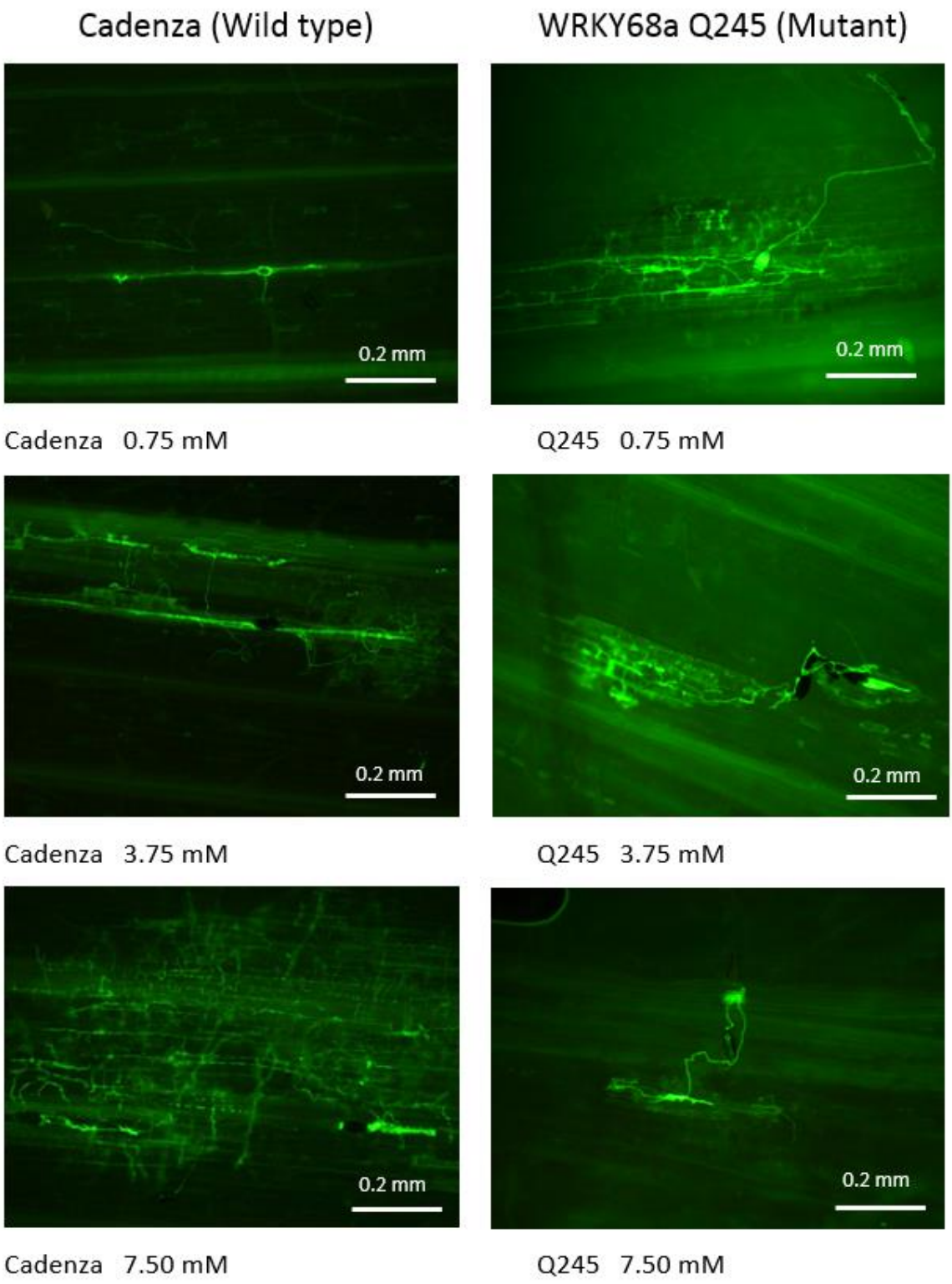


Figure 6.6. Microscopic analysis of the growth of *Bipolaris sorokiniana* in infected leaves of Cadenza and the *W68aQ245* TILLING line mutated in WRKYGQK sequences at three different nitrogen levels (0.75, 3.75 and 7.5 mM nitrate).
WGA staining of infected wheat leaf tissue viewed under blue-light excitation at 10× magnification. Bar = 0.2 mm.

6.5.4 Expression of *WRKY68a* and *WRKY19* in Cadenza cultivar under stress

The two WRKY TF genes that were mutated in the TILLING lines tested (*WRKY19* and *WRKY68a*) were investigated for differential expression in response to reduced nitrogen input (0.75 mM and 3.75 mM nitrate) compared to the standard nitrogen input of 7.5 mM at 3 week old Cadenza wheat (Figure 6.7 A). Both genes had positive fold differences in gene expression at both levels of reduced nitrogen availability, but only up-regulation of *WRKY68a* with severely reduced nitrogen was significant (CI, $P < 0.05$; 2.62-fold), whereas the change with moderately reduced nitrogen was not statistically significant (only 1.29-fold). Expression of *WRKY19* did not differ significantly (CI, $P > 0.05$) between 0.75 mM or 3.75 mM nitrogen and 7.5 mM nitrogen (1.66-fold and 2.03-fold, respectively). The effect of nitrogen supply on the expression of *WRKY19* and *WRKY68a* in plants infected with *Bipolaris sorokiniana* was analysed four days after infection at three-week-old plants with the infection still in the biotrophic phase (Figure 6.7 B). The plants scored differently for *B. sorokiniana* severity as low disease scores with severely reduced nitrogen, medium scores with moderate nitrogen and high scores with the standard nitrogen input according to the amount of leaf surface covered by symptoms of *B. sorokiniana* (see Figure 6.4 A and B).

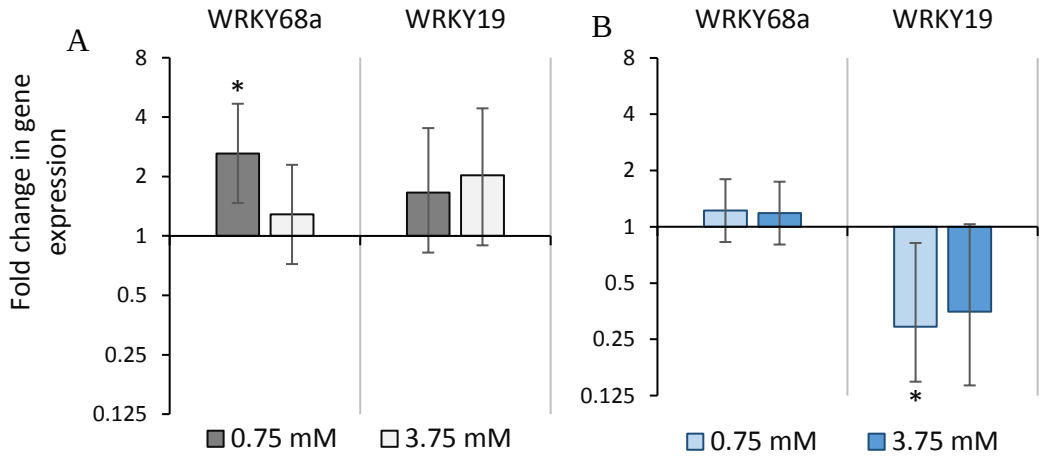


Figure 6.7. Expression of *WRKY68a* and *WRKY19* genes across the nitrogen treatments (A) and in infected leaves (B) in Cadenza. Expression at reduced nitrogen levels (0.75 mM, 3.75 mM) is displayed relative to 7.5 mM nitrate.
Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised against endogenous control wheat gene (*TaeIF4A*). Error bars represent confidence intervals, error bars that do not cross the x-axis indicate that the corresponding difference in means is statistically significant (t-test, $p < 0.05$*, $n = 9$) within each gene.

The combined stress analysis of the expression of *WRKY19* and *WRKY68a* is necessary to determine if an interaction existed between nitrogen and *B. sorokiniana* infection in determining the expression of these genes. Gene expression was analysed four days after

infection of three week old Cadenza wheat, when the disease is still in the biotrophic phase. The differential expression of the two WRKY genes was seen as a response to both reduced nitrogen and spot blotch infection when compared to expression in 3 week old uninfected plants grown with 7.5 mM nitrogen, as shown in (Figure 6.8). These results are in accordance with the differential expression seen in the single stress assays (Figure 6.7 A and B). Qualitatively, *WRKY19* and *WRKY68a* expression was down-regulated in infected plants compared to control Cadenza grown in 7.5 mM nitrogen. Relative expression of *WRKY19* was significantly (CI, $P < 0.05$ and $P < 0.01$) down-regulated with moderately reduced nitrogen (negative 6-fold) and severely reduced nitrogen level (negative 8-fold) respectively. For *WRKY68a* down-regulation was not significant.

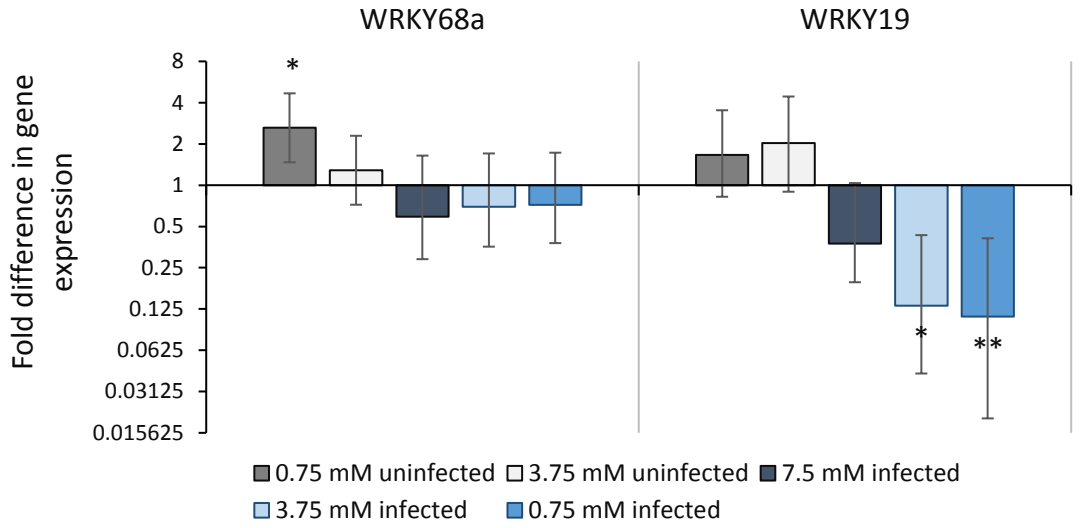


Figure 6.8. Relative expression of *WRKY68a* and *WRKY19* in uninfected and infected leaves of Cadenza wheat cultivar grown with 0.75, 3.75 and 7.5 mM nitrate, compared with the standard nitrogen level (7.5 mM) in uninfected plants.
Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised to endogenous control wheat gene (*TaeIF4A*). Error bars represent confidence intervals, error bars that do not cross the x-axis indicate that the corresponding difference in means is statistically significant (t-test, $P < 0.05$*, 0.01**, n= 9) within each gene.

The differences in gene expression are perhaps more clearly illustrated by analysing the response to spot blotch infection for plants grown with each nitrogen condition separately (Figure 6.9). By comparing how *WRKY* expression changes in response to infection when plants are grown with 7.5 mM, 3.75 mM and 0.75 mM nitrogen, it can be seen that there are changes in *WRKY68a* and *WRKY19* expression with severely and moderately reduced nitrogen levels but not with the standard nitrogen level. The expression of *WRKY68a* was significantly up-regulated (CI, $P < 0.01$ and $P < 0.05$) in plants grown with severely (5.68-fold) and moderately reduced nitrogen (3.78-fold) respectively. Up-regulation of *WRKY19* was only significant (CI,

P < 0.05) with severely reduced nitrogen (1.9-fold); the change was not statistically significant with moderately reduced nitrogen (1.51-fold). There were negative fold differences in expression of both genes at the standard nitrogen level (7.5 mM), but the differences were not significant. The results confirm that changes in gene expression in response to *Bipolaris sorokiniana* are dependent on nitrogen supply.

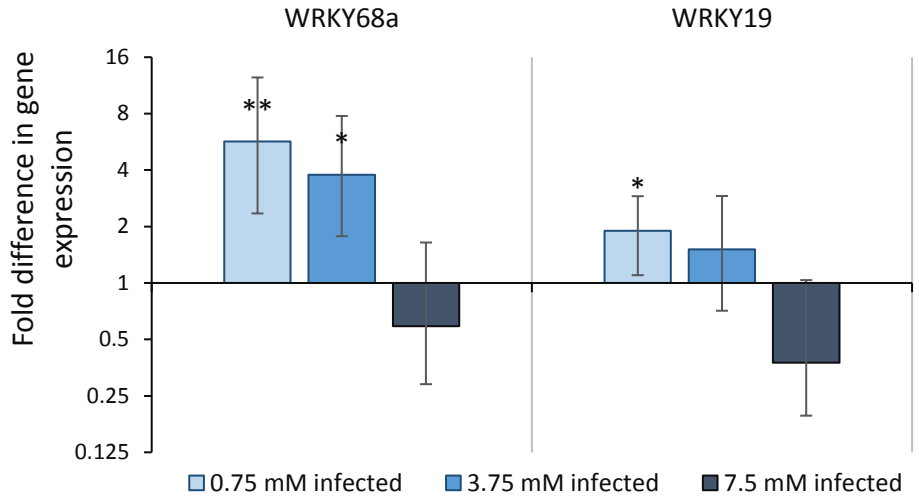


Figure 6.9. Fold differences in *WRKY68a* and *WRKY19* expression in infected leaves in Cadenza wheat cultivar at different levels of nitrogen availability (0.75, 3.75 and 7.5mM nitrate) relative to uninfected plants grown with the same nitrogen concentrations.
Gene expression was calculated based on the comparative threshold cycle method (Livak and Schmittgen, 2001). Relative levels of gene transcript were normalised for endogenous control wheat gene (*TaeIF4A*). Error bars represent confidence intervals, error bars that do not cross the x-axis indicate that the corresponding difference in means is statistically significant (t-test, P < 0.05*, 0.01**, n= 9) within each gene.

6.6 Discussion

The large genome of hexaploid wheat (*Triticum aestivum* L.) makes identifying desirable genetic changes based on phenotypic screening difficult due to gene redundancy. The TILLING method is a non-transgenic approach to functional genomics and crop improvement. The products are a mixture of knockout and missense mutations. Wheat TILLING lines that were identified as containing mutations within the WRKYGQK amino acid sequence showed differences in response to reduced nitrogen input and spot blotch infection compared to the wild-type Cadenza cultivar. Line *W68aQ245*, in which a stop codon replaces a glutamine codon (Q245 point mutant), had significantly greater mean shoot height, number of leaves, number of tillers and leaf area index than the wild type. In both control Cadenza plants and line *W68aQ245*, these values did not differ between the three levels of nitrogen or between uninfected and infected plants. Also, the leaf area index of the *W68aQ245* mutant was significantly greater than those of Cadenza and the other TILLING lines, except for Cadenza

grown at the moderate nitrogen level. Seeds with the heterozygous mutation *W68aQ245* were obtained from segregation in the M4 generation to investigate homozygous mutations that are more stable. As mentioned in the introduction, the gain of stop codon is the most desirable consequence in the selection of TILLING mutants, as it will most likely result in change of function of the protein, with implications for the phenotype. Gottwald *et al*. (2009) pointed out that phenotypic differences among mutants at the same position in gene may be the result of modifying loci. However, we could use this as rationale for trying to identify the genes that *WRKY68* regulates.

W68aQ245 can be compared with *W68aV213*, in which the amino acid valine-213 is replaced with methionine in the homozygous mutation. Plants of the *W68aV213* line were relatively small and had low level of both leaf area index and chlorophyll content index values. This could be due to the change in the binding of the *WRKY68a* protein to DNA that is related to the change in growth. Mutant *W19H334*, which has mutation from histidine to tyrosine (H->Y) in H334 point mutant (homozygous mutation), had the smallest plants, with 24.35 cm mean shoot height, three leaves and one tiller, but only mean shoot height was significantly different from wild-type (Figure 6.1, A, B and C, respectively). The *W19H334* mutant also had low leaf area index and chlorophyll content values, especially when compared with standard nitrogen availability. Overall, the *W19H334* line had the weakest growth based on the physiological. However, the *W68aQ245* mutation (stop gained) enhanced the growth of plants compared to the wild type and the other mutants.

The values of chlorophyll content in Cadenza and TILLING lines were higher with standard nitrogen availability than with severely and moderately reduced nitrogen for controls and plants infected with *Bipolaris sorokiniana*. There were significant differences between infected Cadenza and the two homozygous lines (*W68aV213* and *W19H334*) with severely and moderately reduced nitrogen, and not significant differences in infected Cadenza and *W68aQ245* line only with severely reduced nitrogen. The *W68aQ245* line (heterozygous) had significantly lower chlorophyll content index in infected plants grown with severely reduced nitrogen (0.75 mM) than infected plants grown with moderate and standard nitrogen supply and uninfected plants. The *W19H334* line (homozygous) had the lowest chlorophyll content with severely reduced nitrogen in infected and uninfected plants. From the results, leaf senescence in mutant plants was more than in the wild-type Cadenza at reduced nitrogen input. Campbell *et al*. (2012) investigated a semi-dominant mutation that caused broad-spectrum disease resistance in wheat and found that heterozygous lines had earlier disease development at 8 dpi than homozygous lines at 16 dpi. However, it is important to consider that some of the mutations

that were heterozygous in the M2 generation may have been fixed in the M4 or M5 seed, whereas other mutations may have been lost through genetic drift (STM, 2018).

Cadenza wild-type and TILLING lines had different responses to spot blotch infection at different levels of nitrogen. There were increases in disease severity, number of spores (Figure 6.4 A and B) and chitin content (Figure 6.5) in infected wild-type plants and the *W68aV213* line with increasing nitrogen input from 0.75 to 3.75 and 7.5 mM. There were different patterns of changes in TILLING mutants *W19H334* and *W68aQ245*: disease severity, number of spores and chitin content in leaves decreased with increasing nitrogen levels, in contrast to the wild type and the *W68aV213* line, which both had the highest susceptibility to spot blotch at high nitrogen input. Growth of *B. sorokiniana* observed by WGA staining is consistent with chitin content and disease severity. In the wild type Cadenza, visualisation of the growth of *B. sorokiniana* by microscopic analysis with WGA showed a greater pathogen spread at the standard nitrogen level of 7.5 mM than at 0.75 mM and 3.75 mM nitrogen (Figure 6.6). In contrast, *W68aQ245* mutant plants had a large visible spread of the pathogen in the leaf tissue at severely reduced nitrogen (0.75 mM), and the amount of visible fungus increased with reduced nitrogen input from the standard nitrogen level of 7.5 mM to 3.75 mM and 0.75 mM nitrate.

Overall, from these findings, the TILLING lines with STOP in Q245 mutant point within the WRKY domain of *WRKY68a* and the mutation in H334 within the second WRKY domain of *WRKY19* domain had a different pattern of response to spot blotch infection under reduced nitrogen supply. For the wild-type and the *W68aV213* mutant, the response corresponds to the growth against defence paradigm. Although nitrogen supply had little effect on growth in this study, plants grown at high nitrogen had the highest chlorophyll content and the most severe disease. However, the *W19H334* and *W68aQ245* mutants may uncouple this paradigm, as they have the least disease and no reduction in chlorophyll content with the highest nitrogen supply. A possible explanation is the loss-of-function mutation that disrupts a wild-type negative regulator of resistance that functions at high nitrogen supply and is necessary for suppression of pathogen invasion (Dangl and Jones, 2001). Another possibility is the loss of function event that creates a new functional resistance allele; an example is the resistant MNR220 mutant in wheat (Campbell *et al.*, 2012), which has broad-spectrum resistance to several pathogens. However, the challenge of defining the effect of the mutation is not easy, as there are thousands of mutations in the TILLING line plants. Thus, further work is needed by using back-cross and screening approaches before we can categorically state that the WRKY genes are involved in disease resistance and the apparent uncoupling of the growth against defence paradigm. In a

study by Krasileva *et al.* (2017) demonstrated that wheat TILLING resource for diploid pasta wheat and hexaploid bread wheat containing 2,735 lines with more than 10 million EMS-induced mutations.

If *WKRY68a* and *WRKY19* suppress disease resistance at high nitrogen, their expression might be up-regulated at high nitrogen. Contrary to this suggestion, expression of *WRKY68a* and *WRKY19* in the Cadenza cultivar was up-regulated at both severely and moderately reduced nitrogen availability, although the difference was only significant (CI, $P < 0.05$) in *WRKY68a* with severely reduced nitrogen. Wu *et al*. (2008) found that *TaWRKY68a* was up-regulated in response to abiotic stress (20% Polyethylene glycol (PEG) treatment) after 4 hours. However, the expression of *WRKY19* did not significantly change in the reduced nitrogen conditions of 3.75 mM and 0.75 mM compared to 7.5 mM nitrogen, whereas in infected plants, the expression of *WRKY68a* showed no differences in up-regulation at both 3.75 mM and 0.75 mM concentrations. However, expression of *WRKY19* was down-regulated in infected plants at both severely reduced and moderate nitrogen levels; this was significant with severely reduced nitrogen. Niu *et al*. (2012) found that over-expression of *TaWRKY19* enhanced tolerance to salt, drought and freezing stress in transgenic Arabidopsis plants.

The expression of the *WRKY68a* and *WRKY19* genes under the combined stresses of reduced nitrogen availability and *B. sorokiniana* fungal infection relative to uninfected plants at 7.5 mM nitrogen was then analysed. Expression of both genes was up-regulated when plants were grown with reduced nitrogen, but down-regulated in plants infected with *B. sorokiniana* (Figure 6.8). Analysis of how WRKY gene expression changes in response to infection in plants grown with the same nitrogen level showed that *WRKY68a* and *WRKY19* expression was up-regulated at both severely and moderately reduced but not at standard nitrogen levels. Lai *et al*. (2008) over-expressed *AtWRKY3* in transgenic Arabidopsis plants and found that it had a positive role in plant resistance to necrotrophic pathogens but a negative effect on the resistance of the plants to biotrophic pathogens.

These results from the use of the TILLING method indicate that direct phenotyping is applicable when a single-copy gene confers a detectable phenotype of interest. Dong *et al*. (2009) suggested that this method may be sufficient for the targeting of desired phenotypes, and is also useful in targeting mutations and genes without detectable phenotypes, as well as allowing efficient screening and mutation detection, leaving more time for the study of candidate mutants. Since some TILLING lines have altered patterns of response to spot blotch infection at reduced nitrogen availability, they may be useful for wheat breeding. For more

reliable results, further self-fertilisation and hybridisation with a wild-type plant would reduce undesirable mutations. Furthermore, information provided from this study may be useful for both functional genomics and practical breeding programmes. Large-scale progeny testing and molecular analysis of the disease resistance associated with the *W68aQ245* and *W19H334* mutations may lead to a better understanding of the regulation of defence response networks in wheat.

Overall, a more extensive series of TILLING lines generating an allelic series of mutation, including a knockout in the desired gene, could be tested for their resistance response to *B. sorokiniana*. Such lines could provide accessible resources to obtain useful genetic variation in wheat, which suits a small laboratory and budget.

6.7 Conclusions

Three week old seedlings of *W19H334* and *W68aQ245* mutants showed higher defence against *B. sorokiniana* infection when nitrogen input was increased from 0.75 mM to 3.75 mM and 7.5 mM nitrate compared to the wild type and the *W68aV213* mutant. Infected leaves of lines *W19H334* and *W68aQ245* had high chitin content, number of spores and disease severity when nitrogen input was reduced. This response was inverse compared to line *W68aV213* and wild-type (Cadenza) plants. Microscopic analysis of line *W68Q245* with WGA method showed a high visible spread of the pathogen associated with a high amount of damage in infected tissue when nitrogen input was reduced. This result was contrary to the results with wild-type plants, in which visible spread of the pathogen increased with nitrogen supply. Also, the *W68Q245* mutant showed higher plant growth than the wild type and all the other TILLING lines tested. The expression of *WRKY68a* was up-regulated at severely and moderately reduced nitrogen availability in uninfected and infected Cadenza wheat plants. Expression of the *WRKY19* gene was only up-regulated at reduced nitrogen levels. In conclusion, TILLING mutants can have different responses to fungi compared to wild type plants. They provide accessible resources to obtain genetic variation in wheat defence against spot blotch attack and may change growth of the mutant plants. The TILLING line *W68aQ245* had enhanced defence against *B. sorokiniana* at the optimum nitrogen level, where the wild type plants suffer from high susceptibility to spot blotch, with no decrease in plant growth and an increase in chlorophyll content, suggesting a change in the relationship between growth and defence. However, the TILLING lines will need more investigation by backcrossing and screening before we can categorically state that the WRKY genes are involved in disease resistance.

7 *Micromonospora luteifusca* strain induces plant defence against spot blotch damage in different wheat cultivars

7.1 Abstract

It has been reported that bacteria used to control plant pathogens can stimulate plant immunity. This study aims to investigate the potential of *Micromonospora luteifusca* as a biocontrol agent and examine its antifungal properties towards the wheat pathogen *Bipolaris sorokiniana*. In addition, the effects of treatment with *M. luteifusca* on the expression of WRKY transcription factor (TF) genes in wheat and *B. sorokiniana* genes were examined. Seedlings of three wheat cultivars (Rashid and Latifia from Iraq, and Cordiale from the UK) were grown in sand-perlite mixture watered with Hoagland's solution containing 7.5 mM nitrate. Rashid cultivar showed higher plant growth under the single effect of pathogen or under the combination of spot blotch infection and *Micromonospora*, mainly in plant roots, compared to control plants. These treatments did not significantly affect growth of Latifia and Cordiale. There were lower number of spores, lower chitin content and significant reduction of the disease symptoms, including higher chlorophyll content, when infected Rashid and Cordiale were treated with the bacteria. Expression of wheat *TaWRKY* genes was significant up-regulation with spot blotch infection in the presence of *Micromonospora* in Latifia and Cordiale. The *rbcL* (RuBisCO large subunit) gene showed significant down-regulation of expression in response to spot blotch challenge in the presence and absence of bacteria inoculation in Rashid wheat, whereas down-regulation was only significant in response to spot blotch challenge in Latifia and Cordiale. Expression of the *PR1* (pathogenesis-related protein 1) gene showed a dose-dependent response with significant up-regulation of expression in plants infected with spot blotch but non-significant in plants inoculated with bacteria in presence and absence of spot blotch for Rashid and Cordiale. Relative abundance of *B. sorokiniana* gene in infected plants was displayed to infected plant preinoculated with *Micromonospora* strain showed higher expressed when increasing spot blotch prevalence for *Coch* than *BipITS* and *ITS1-ITS4* region in susceptible Rashid. In conclusion, *M. luteifusca* should be considered as a potential plant probiotic candidate, due to its antifungal properties and its ability to induce defence mechanisms in wheat, especially relevant for the pathosystem wheat-*Bipolaris sorokiniana*.

Keywords: Actinobacterial endophytes, *Bipolaris sorokiniana, Micromonospora*, wheat, WRKY transcription factor genes, quantitative RT-PCR.

7.2 Introduction

Currently, one of the most pivotal topics of discussion is the harm resulting from excessive use of chemicals, including fertilisers, fungicides and other pesticides, in order to raise agricultural production. Some soil microorganisms are beneficial to plants as they promote nutrient uptake, produce plant growth-stimulating substances, and protect against attack by pathogens (Turner *et al.*, 2013; Carro and Nouioui, 2017). They can also improve tolerance of drought and soil contaminants and enhance other microbial interactions. Beneficial and non-pathogenic microorganisms include nitrogen-fixing bacteria and biological control organisms, which effectively reduce leaf infection by the fungal pathogen (Martinez-Hidalgo *et al.*, 2015).

Most known bacterial and fungal biocontrol agents have been isolated from soil or the rhizosphere. Some of these are commercially available. However, there has been varying success with the use of rhizobacteria as biocontrol agents (Franco *et al.*, 2007). Among such organisms, the genus *Micromonospora* has been shown to be a natural inhabitant of legumes (Trujillo *et al.*, 2010) and actinorhizal plants (Carro *et al.*, 2013), showing an innate capacity for plant growth promotion (Trujillo *et al.*, 2015; Carro *et al.*, 2018). Generally, the relative abundance of microbial eukaryotes found in legumes such as pea or in oat rhizosphere was five-fold higher than in plant-free soil or the rhizosphere of wheat. The pea rhizosphere was particularly enriched in fungi (Turner *et al.*, 2013).

Micromonospora is a widely-distributed actinobacterial genus. It inhabits soil and plant tissues, including root nodules of plants colonised by nitrogen-fixing bacteria. Some *Micromonospora* strains can promote plant growth, increase nutrient uptake, induce plant growth-stimulating substances, and enhance plant defences against pathogens (Turner *et al.*, 2013; Martinez-Hidalgo *et al.*, 2014). Others have antifungal activity (Martinez-Hidalgo *et al.*, 2015). A few actinobacteria have been observed that have the ability to reduce the harmful effects of pathogens in plants. They may inhibit pathogen growth via production of antibiotics, lytic enzymes or siderophores or induce plant defence mechanisms (Turner *et al.*, 2013; Martinez-Hidalgo *et al.*, 2014). Although *Micromonospora* to have potential as a novel inoculant, how it affects spot blotch disease has not been studied before. This study aims to assess the interaction among *Micromonospora*, the spot blotch pathogen *Bipolaris sorokiniana*, and wheat (*Triticum aestivum*) host plants. In this system, the bacteria will be tested as a potential plant probiotic: either directly inhibiting fungi or inducing plant defence mechanism through induced systemic resistance (ISR) (Taiz and Zeiger, 2010).

The signalling cascade in ISR involves jasmonic acid and ethylene. Signalling leads to the activation of protective mechanisms throughout the plant, so that the plant has enhanced preparedness to withstand attack by pathogens. In contrast to systemic acquired resistance (SAR) induced by initial pathogen infection, ISR does not involve salicylic acid as a signalling compound and does not induce the production of typical PR proteins. Some defensive responses are induced rapidly in ISR, but others only take places after infection by the pathogen, resulting in a more rapid and greater response. This defensive strategy reduces the direct investment of resources in defensive measures that could reduce growth and yield (Taiz and Zeiger, 2010).

Gene expression analyses are considered as a useful tool to show whether *Micromonospora* stimulates plants' capacity to activate defence mechanisms upon pathogen attack (Martinez-Hidalgo *et al.*, 2015). Non-pathogenic microorganisms that associate with roots associations cause major changes in the root transcriptome when they come into contact with plant roots, induced differently during the early and late stages of root colonisation (Conn and Franco, 2004). WRKY transcription factor genes function in controlling the establishment of arbuscular mycorrhizal fungi by regulating plant defence genes. The establishment of arbuscular mycorrhizal fungi seems to require a mild activation of defence responses that involves WRKY TF genes. Gallou *et al*. (2012) found that nine WRKY genes were up-regulated during the pre-stage of potato root colonisation with *Glomus* sp., but only one WRKY gene was up-regulated at late stages of colonisation. However, no regulation of WRKY genes was found at an early stage of root colonisation. Much more work is required to understand the role of WRKY TFs in beneficial plant-microbe interactions. This is particularly high efficiency because WRKY proteins are known to be involved in nutrient uptake and systemic resistance responses, which beneficial microbes can promote.

This study aimed to test the potential of a strain of *Micromonospora luteifusca* as a biocontrol agent, exploring its antifungal properties and its ability to boost plant defence mechanisms in the wheat-*B. sorokiniana* interaction.

7.3 Hypothesis

1- *Micromonospora luteifusca* may enhance plant growth and resistance to the hemibiotrophic pathogen *Bipolaris sorokiniana*.

2- WRKY TF genes are differentially regulated in plants under spot blotch challenge in the presence and absence of *Micromonospora*.

3- The wheat germ agglutinin WGA conjugate method provides an accurate, sensitive, simple, and non-subjective measurement of fungal growth.

7.4 Aims

1- To investigate the effect of inoculation of wheat roots with *Micromonospora luteifusca* on resistance responses to spot blotch by different physiological measurements.

2- To determine if wheat plants inoculated with *Micromonospora* exhibit differential expression of WRKY TFs in the presence and absence of spot blotch infection.

3- To investigate fungal biomass quantitatively and microscopic analysis in infected leaves by chitin measurements with wheat germ agglutinin-WGA conjugate.

7.5 Results

7.5.1 Preliminary experiment using two biocontrol bacteria against spot blotch stress

Previously, some studies have reported that biocontrol bacteria have antifungal activity against certain pathogenic fungi (Gad *et al.*, 2014). Cordiale wheat cultivar was planted in sand watered with modified Hoagland's solution. The roots were inoculated with *Pseudomonas aeruginosa* (P) and *Micromonospora luteifusca* separately or in combination (PM) or with neither (C = Control). *Micromonospora* treatment gave the lowest level of spot blotch infection, which was significantly different (ANOVA, Tukey, $P < 0.05$) from the control and *Pseudomonas aeruginosa* treatments, but not different from the combined *P. aeruginosa* and *M. luteifusca* treatment (Figure 7.1). This result showed that *Micromonospora luteifusca* can be considered as potentially having antifungal properties and the ability to induce defence mechanisms in wheat infected with *Bipolaris sorokiniana*.

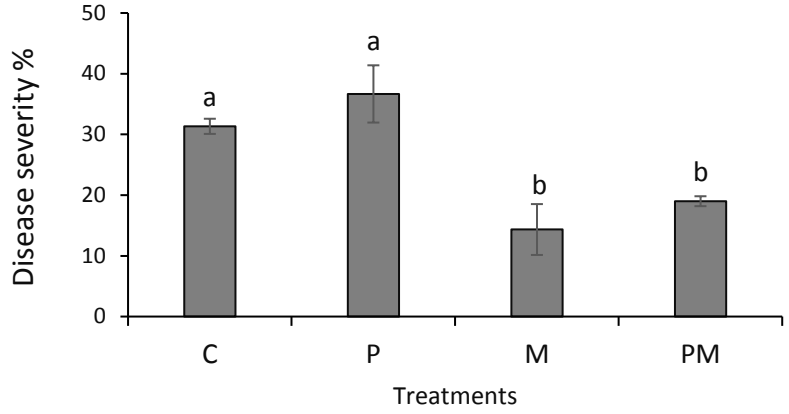


Figure 7.1. Disease severity (%) on Cordiale wheat infected with *Bipolaris sorokiniana* after inoculation with two candidate biocontrol bacteria.
P = *Pseudomonas aeruginosa* and M = *Micromonospora luteifusca*, PM = *P. aeruginosa* and *M. luteifusca* and C = Control. Different letters represent significant differences (ANOVA, one way, Tukey, $P < 0.05$, $n = 6$), error bars represent ± 1 standard deviation.

7.5.2 Diseases severity, number of spores in infected wheat cultivars

The preliminary study of disease severity (Figure 7.1) showed that inoculation with *Micromonospora luteifusca* strain CR21 reduced disease severity significantly compared to *Pseudomonas aeruginosa* treatment and the control, and there was no difference with the combination of *Pseudomonas aeruginosa* and *M. luteifusca*. Therefore, further experiments used *M. luteifusca*. In order to improve our understanding of how this bacterium affects wheat defence responses to spot blotch infection. Rashid, Latifia and Cordiale wheat cultivars were inoculated with *Micromonospora luteifusca* and watered with Hoagland's solution. Wheat plants were infected with *B. sorokiniana* at twenty-one days old and spot blotch severity was measured after four days. Disease severity was lower on wheat infected after inoculation with *Micromonospora* than on control wheat. The difference was significant in Rashid and Cordiale, but not in Latifia wheat cultivar (Figure 7.2). Disease severity was significantly higher on Rashid (ANOVA, Tukey, $P < 0.05$) than on Latifia and Cordiale with and without *Micromonospora*. Cordiale had the lowest disease severity in the presence of *Micromonospora*, whereas in the absence of bacteria it was not different from Latifia.

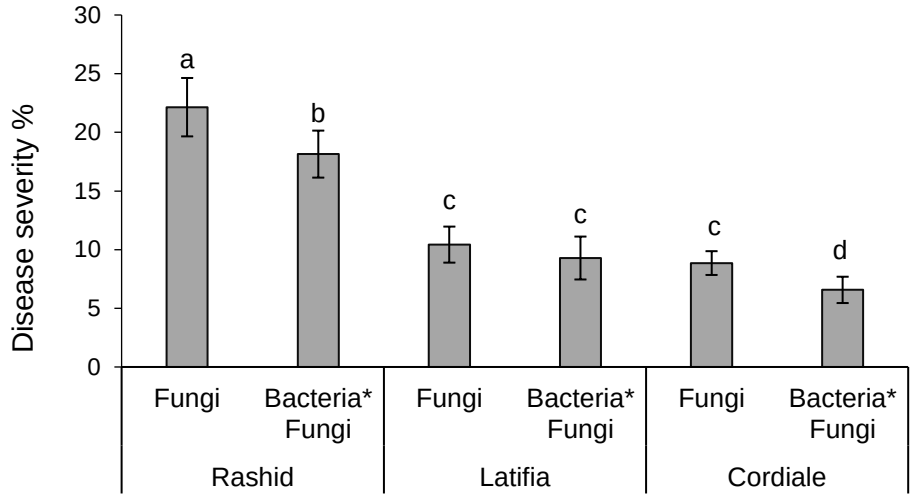


Figure 7.2. Disease severity in leaves of three wheat cultivars (Rashid, Latifia and Cordiale) infected with spot blotch in presence and absence of *Micromonospora luteifusca*.
Different letters represent significant differences (ANOVA, two way, Tukey, $P < 0.05$, n = 12 plants), error bars represent ± 1 standard deviation.

Numbers of spores were counted from leaves incubated for a further four days at high humidity. Cultivar Rashid had the highest number of spores, which was significantly (ANOVA, Tukey, $P < 0.05$) higher on untreated leaves (17129 spores/ml) than on leaves of plants treated with *Micromonospora* (14870 spores/ml) (Figure 7.3). Cordiale also had a significantly (ANOVA, Tukey, $P < 0.05$) lower spore count (3734 spores/ml) on leaves from plants treated with *Micromonospora* than on leaves from untreated plants 5795 spores/ml. However, Latifia showed no significant difference (ANOVA, Tukey, $P > 0.05$) between the two treatments, and no significant difference from Rashid treated with *Micromonospora*. Spore counts were significantly (ANOVA, Tukey, $P < 0.05$) lower on Cordiale than on Latifia in both treatments.

Quantitation of chitin at four days after infection was used to measure growth of the pathogen in infected wheat leaves. Treatment with *Micromonospora* significantly (ANOVA, Tukey, $P < 0.05$) lower in fluorescence units of chitin content in leaves of Rashid and Cordiale (14870 and 3734 units, respectively) than absence of bacteria 17129 and 5795 units, respectively, but not change in Latifia (Figure 7.4). Rashid had significantly higher chitin content (ANOVA, Tukey, $P < 0.05$) than Cordiale and Latifia. Cordiale wheat had the lowest chitin content in the presence of *Micromonospora*, this was significant (ANOVA, Tukey, $P < 0.05$) with absence of bacteria (5796 units).

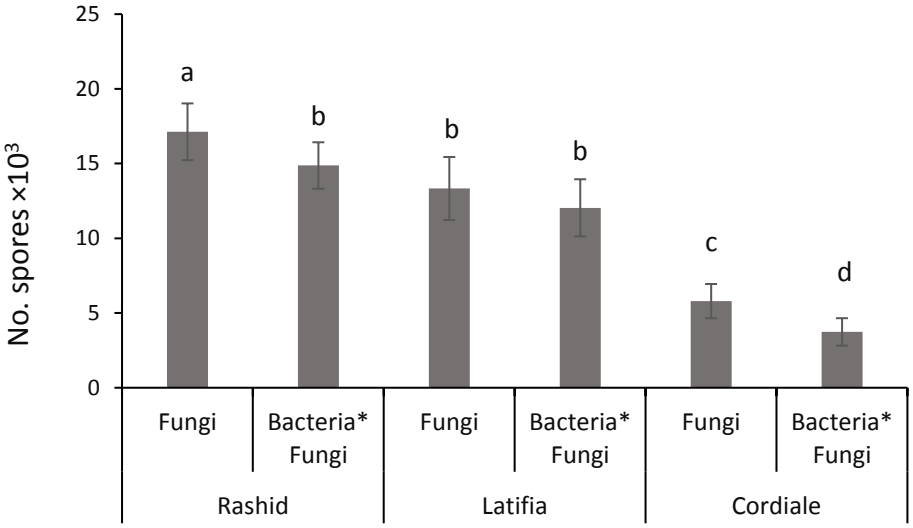


Figure 7.3. Number of spores $\times 10^3$ in infected leaves of three wheat cultivars (Rashid, Latifia and Cordiale) infected with spot blotch in presence and absence of *Micromonospora luteifusca*. Different letters represent significant differences (ANOVA, two way, Tukey $P < 0.05$, n= 12 Plants), error bars represent ± 1 standard deviation.

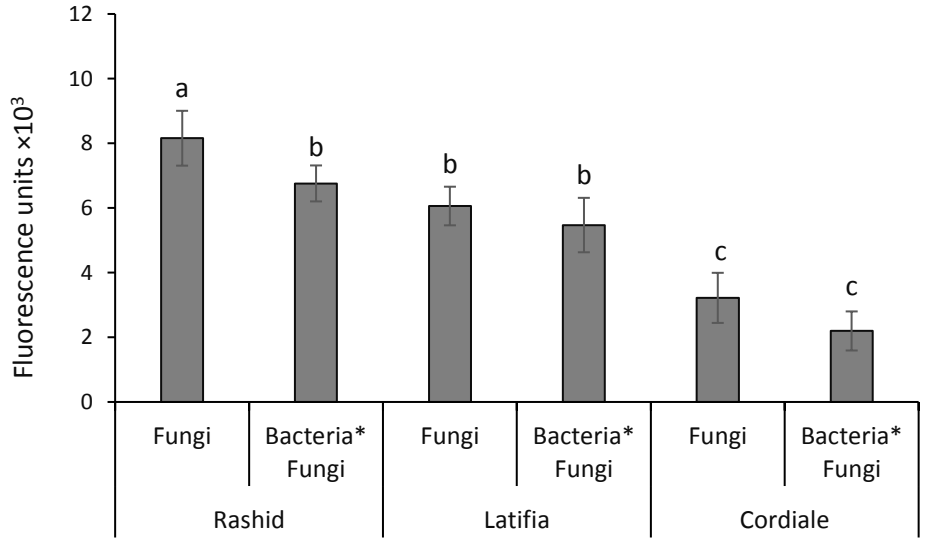


Figure 7.4. Chitin content (WGA assay) in leaves of three wheat cultivars (Rashid, Latifia and Cordiale) infected with spot blotch in presence and absence of *Micromonospora luteifusca*. Different letters represent significant differences (ANOVA, two way, Tukey, $P < 0.05$, n= 12 Plants), error bars represent ± 1 standard deviation.

Infected wheat leaves from plants with and without *Micromonospora* inoculation were stained with wheat germ agglutinin-WGA conjugate and examined by fluorescence microscopy four days after infection. All three wheat cultivars showed visibly decreased infection with *Micromonospora* inoculation. Rashid wheat had more and more widely spreading fungal hyphae than Latifia and Cordiale, consistent with the greater spot blotch disease severity (Figure

7.5). The microscopic analysis suggested that in Rashid the pathogen switched to the necrotrophic phase in the absence of bacteria, as a result of the appearance of dark spot blotches in spreading areas of fungus in the plant tissues, but no such change was observed in Latifia and Cordiale.

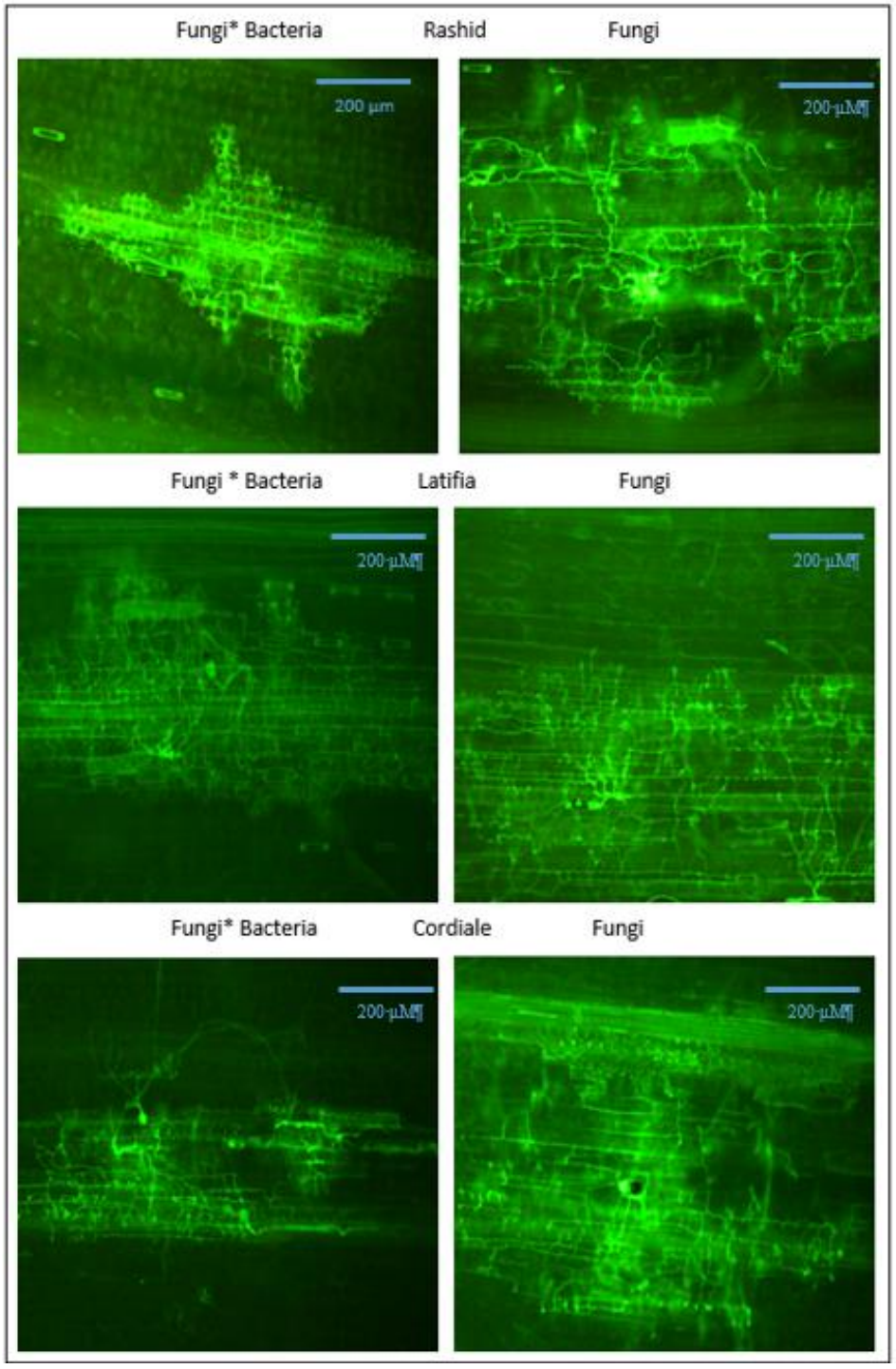


Figure 7.5. Analysis of *B. sorokiniana* growth in infected leaves of Rashid, Latifia and Cordiale in the presence and absence of *Micromonospora luteifusca*.
WGA staining of infected wheat leaf tissue was viewed by fluorescence microscopy under blue-light excitation at 10× objective magnification. Stained fungal hyphae can be seen.

7.5.3 Physiological measurement in infected and uninfected wheat

Twenty-five-day old wheat plants were treated with *B. sorokiniana* in presence and absence of *Micromonospora* inoculation and compared to non-infected plants as a control. Chlorophyll content index was significantly higher (ANOVA, Tukey, $P < 0.05$) in the uninfected plants with the presence of bacteria than in control plants and plants infected with spot blotch for all wheat cultivars tested (Figure 7.6 A). There were significant differences (ANOVA, Tukey, $P < 0.05$) in the presence and the absence of bacterial inoculation in infected plants and between plants treated with bacteria and control plants. Rashid showed the highest chlorophyll content and Cordiale had the lowest of chlorophyll content. Leaf area index was highest in Rashid wheat, with no difference among treatments. There were significant differences (ANOVA, Tukey, $P < 0.05$) between plants inoculated with bacteria and non-inoculated plants in infected Latifia and Cordiale (Figure 7.6 B).

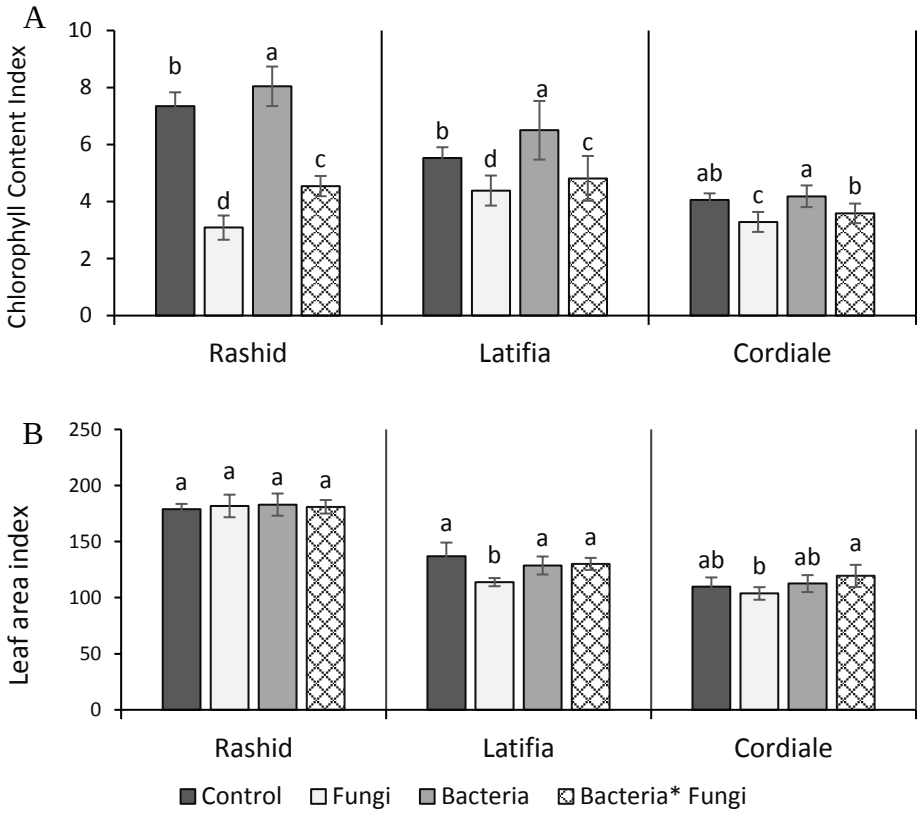


Figure 7.6. Chlorophyll content index (CCI) (A) and leaf area index (B) in Rashid, Latifia and Cordiale infected with *Bipolaris sorokiniana* and treated with *Micromonospora luteifusca* and with the two in combination, compared with control treatment.
Different letters represent significant differences (ANOVA one way, Tukey, $P < 0.05$, $n = 12$) within each wheat cultivar, error bars represent ± 1 standard deviation.

Average number of tillers and number of leaves was significantly (ANOVA, Tukey, $P < 0.05$) highest in Cordiale and there were no significant differences among treatments. The average number of tillers was lower in control than treated plants of cultivar Rashid (Figure 7.7 A and B). The average number of tillers (one tiller) and number of leaves (4 leaves) for each plant in Latifia, also Latifia showed significantly (ANOVA, Tukey, $P < 0.05$) lower than lower number of tillers and leaves than Rashid cultivar. Only Rashid showed significantly (ANOVA, Tukey, $P < 0.05$) lower of number of tillers (mean 2 tillers) in control plant than in *Micromonospora* (mean 2.43 leaves) or spot blotch (mean 2.57 leaves) treatments and their interactions (2.57 leaves).

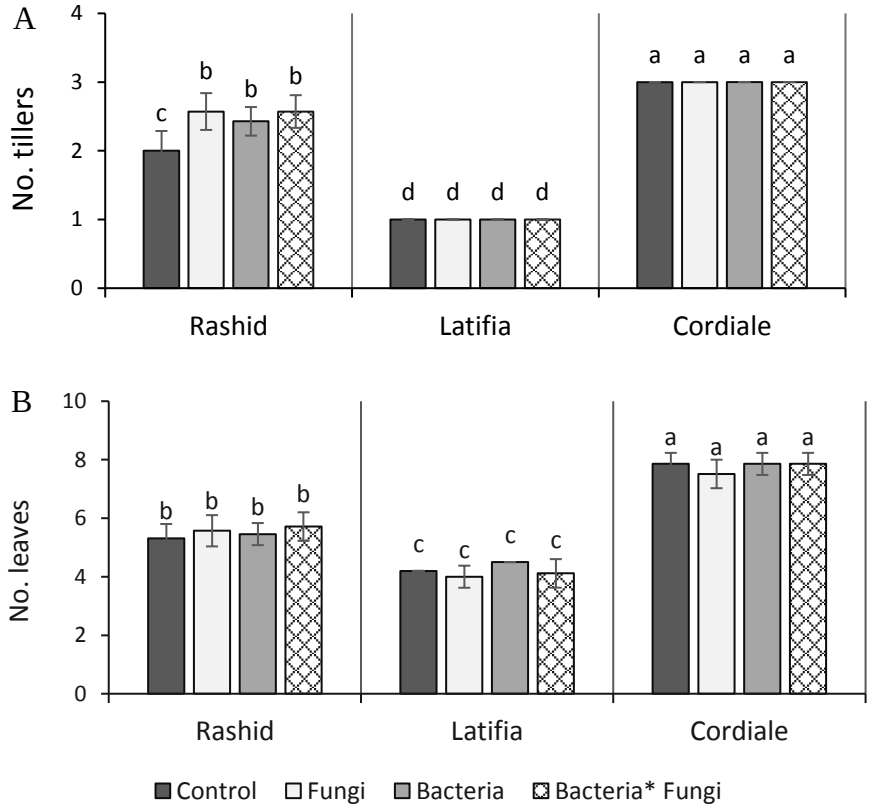


Figure 7.7. Number of tillers (A) and number of leaves (B) in Rashid, Latifia and Cordiale infected with *Bipolaris sorokiniana* and treated with *Micromonospora luteifusca* individually or in combination, compared with control.
Different letters represent significant differences (ANOVA, two way, Tukey, $P < 0.05$, $n = 12$) within each wheat cultivar, error bars represent ± 1 standard deviation.

7.5.4 Effect of *Micromonospora* on gene expression in infected wheat leaves

To investigate whether treatment with *Micromonospora luteifusca* affected gene expression in infected leaves, differences in expression of four WRKY transcription factor genes (*WRKY3*, *WRKY19*, *WRKY45*, and *WRKY68a*), the *rbcL* gene and the *PR1* (pathogenesis-related protein

1) gene in response to spot blotch infection were measured in the presence and absence of *Micromonospora* inoculation of the roots. These genes had previously been shown to produce desirably high amplification efficiency (see Chapter 4). Expression levels were compared to uninfected plants and relative levels of transcripts were normalised against wheat eukaryotic initiation factor 4A (*eIF4A*) mRNA. None of the genes showed significant differences in expression as a result of treatment with *Micromonospora* without infection, except that there was significantly less *WRKY19* RNA in treated Latifia plants (Figure 7.8 A, B and C).

In plants infected with *B. sorokiniana* and not treated with *Micromonospora*, no WRKY genes were up-regulated in Rashid, whereas *WRKY19* was significantly down-regulated in Latifia and *WRKY68a* was significantly up-regulated in Latifia and Cordiale. In plants infected with *B. sorokiniana* after treatment with *Micromonospora*, *WRKY68a* was the only gene that was significantly up-regulated in all three cultivars (3.1-fold in Rashid, 3.3-fold in Latifia, and 3.4-fold in Cordiale), while *WRKY45* was significantly up-regulated in Latifia (2.6-fold) and Cordiale (2.8-fold) and *WRKY3* was significantly up-regulated (4-fold) in Latifia. Expression of *PR1* was significantly up-regulated in all cultivars (6.3-fold in Rashid, 5.7-fold in Latifia, and 8.6-fold in Cordiale) when they were infected with *B. sorokiniana* and not treated with *Micromonospora*. After treatment with *Micromonospora* and infection with *B. sorokiniana*, Cordiale was the only cultivar in which *PR1* was significantly up-regulated (3.1-fold; $P < 0.05$). Expression of the *rbcL* gene was significantly lower in Latifia and Cordiale cultivars infected with *B. sorokiniana* and not treated with *Micromonospora* (2-fold in Cordiale). In Rashid, expression of the *rbcL* gene was down-regulated more than 3-fold both with and without *Micromonospora* inoculation ($P < 0.01$ and < 0.05 without and with *Micromonospora* inoculation, respectively), whereas in Latifia and Cordiale there was no significant difference in gene expression between infected plants treated with *Micromonospora* and uninfected plants.

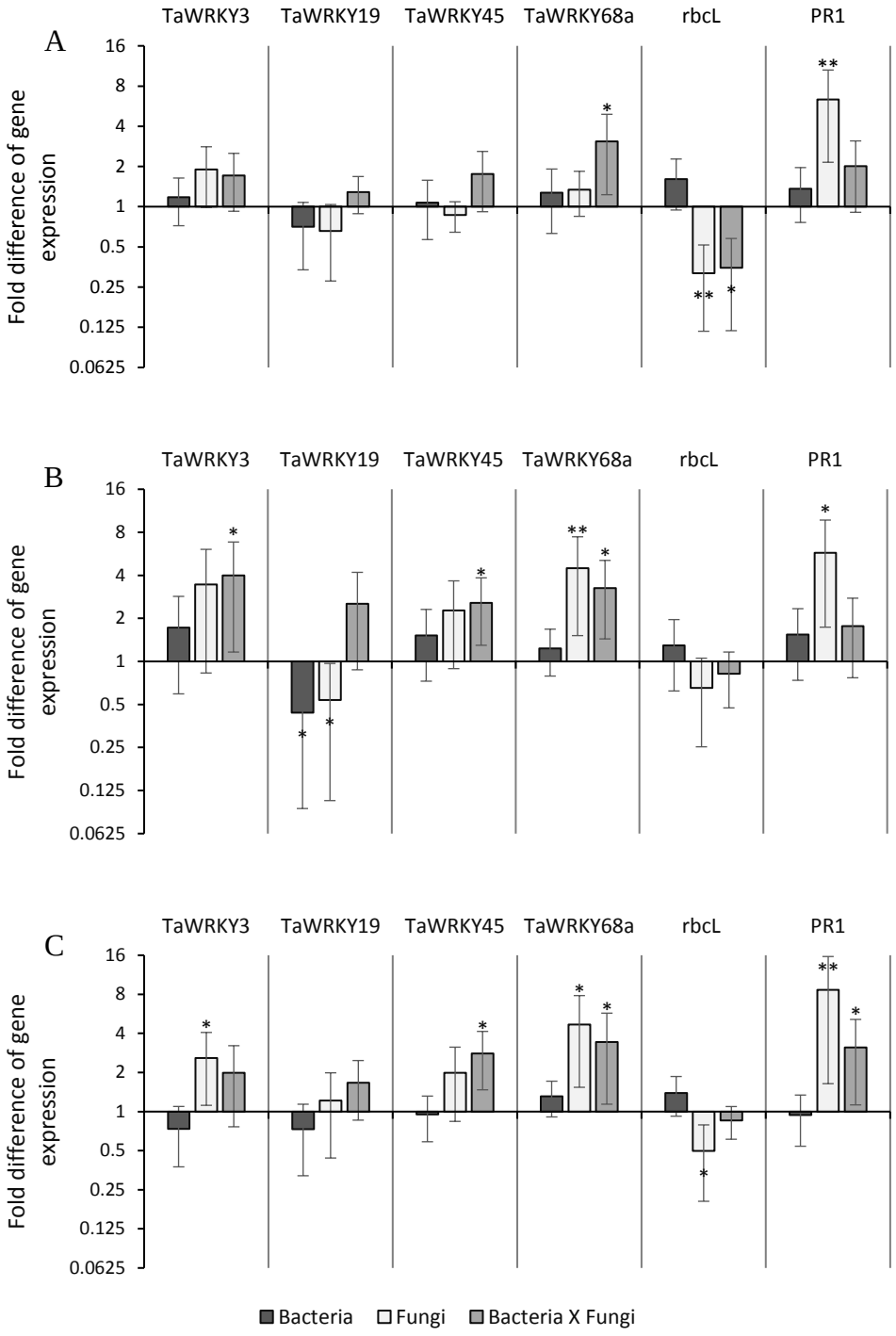


Figure 7.8. Fold differences in WRKY TFs, *rbcL* and *PR1* gene expression in wheat treated with *Micromonospora luteifusca* and infected with *B. sorokiniana* individually or in combination, compared with untreated control. (A) Rashid, (B) Latifia and (C) Cordiale.
Relative levels of transcripts were normalised against the *T. aestivum* eukaryotic initiation factor 4A (*TaeIF4A*) gene. Error bars (confidence intervals) that do not cross the x-axis indicate that the corresponding difference in means is statistically significant (t-test, P < 0.05*, 0.01**, n = 12) within each gene.

7.5.5 Effect of *Micromonospora* on *B. sorokiniana* gene expression

Expression of the internal transcribed sequence (ITS) of rRNA was tested with the non-specific primer pair *ITS1-ITS4* and two primer pairs specific to *B. sorokiniana*, *Coch* and *BipITS*. Gene expression was normalised relative to endogenous wheat control (eukaryotic initiation factor 4A, *eIF4A*). Wheat treated with *Micromonospora* before infection was used as the reference condition to identify differences in gene expression in infected plants. Relative abundance of the ITS region measured with all three primer pairs was higher in plants not inoculated with *Micromonospora* than in inoculated plants, associated with increased disease severity and increased sporulation. RNA abundance measured with *Coch* primers was significantly higher in plants not treated with *Micromonospora* than in treated plants, more than 15-fold in Rashid and 5-fold in Cordiale ($P < 0.001$ and $P < 0.01$, respectively) (Figure 7.9). Similarly, there were highly significant differences measured with *BipITS* and *ITS1-ITS4* primers in Rashid wheat (12.3 fold and 7.6 fold, respectively). The difference in expression in Latifia was lower but significant ($P < 0.05$) when measured with *Coch* (2.8-fold) and *BipITS* (3-fold) primers, but not significant with *ITS1-ITS4* primers. In Cordiale, there was significantly more *ITS* RNA measured with *BipITS* and *ITS1-ITS4* primers (CI, $P < 0.05$; more than 3-fold).

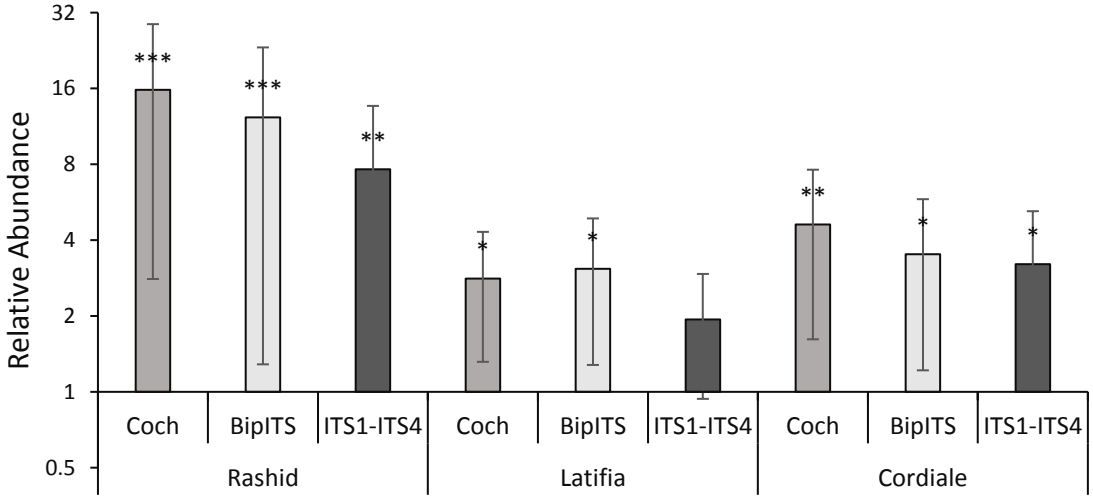


Figure 7.9 Abundance of RNA measured with *Coch*, *BipITS* and *ITS1-ITS4* primer pairs in Rashid, Latifia and Cordiale infected with *B. sorokiniana* in plants not treated with *Micromonospora luteifusca*, relative to expression in leaves of infected and treated plants.
Relative levels of transcripts were normalised against the *Triticum aestivum eIF4A* gene (Livak and Schmittgen, 2001). Error bars (confidence intervals) that do not cross the x-axis indicate that the corresponding difference in means is statistically significant (t-test, P <0.05*, 0.01**, 0.001***, n= 9) within each gene.

7.6 Discussion

Biotic interactions can improve agricultural productivity without costly and environmentally challenging inputs. Recently, strains of the actinobacterium were named *Micromonospora* that have been isolated from nodules of alfalfa plants have shown very good plant growth promoting activity (Martinez-Hidalgo *et al.,* 2014). They also enhance the ability of tomato plants to resist disease (Martinez-Hidalgo *et al.,* 2015). The potential of this genus as plant growth promoting bacteria has also been analysed from its genome sequences, which contains genes related to stimulation of growth and protection against pathogens, including genes for chitinases (Carro *et al.*, 2018). In this study, we investigated the potential of a different strain of *Micromonospora*, *Micromonospora luteifusca* (CR21) from roots of pea rhizosphere, as an agent for biological control of a wheat pathogen through its ability to boost plant defence mechanisms. Inoculation of roots with *Micromonospora* enhanced the resistance of leaves to infection by *B. sorokiniana*. Figure 7.2, Figure 7.3 Figure 7.4 showed that for two very different wheat cultivars, Cordiale (UK) and Rashid (Iraqi), but not for cultivar Latifia (also Iraqi) the severity of the symptoms, number of spores produced on leaves and chitin content in infected leaves were significantly lower in plants treated with *Micromonospora* than in plants infected in the absence of treatment with bacteria. This corresponds to the findings of Martínez-Hidalgo *et al.* (2015) that tomato plants inoculated with *Micromonospora* 24 h before challenge with the pathogen showed a reduction in the severity of disease caused by *Botrytis cinerea*. In both treatments, with and without *Micromonospora*, cultivar Rashid was more susceptible to spot blotch than Latifia and Cordiale, as found previously (section 7.5.2). In the absence of treatment with *Micromonospora*, disease severity in Cordiale and Latifia was not significantly different, whereas in the presence of *Micromonospora* disease severity was lower in Cordiale, so that Cordiale was the cultivar least susceptible to spot blotch in the presence of *Micromonospora*. Trends in the severity of symptoms broadly reflected the growth of the pathogen as assayed by chitin levels and sporulation. Microscopic analysis by the wheat germ agglutinin-WGA conjugate method reinforces the quantitative measurements, showing that Rashid wheat had more abundant and more highly spreading fungal hyphae than Latifia and Cordiale (Figure 7.5). The microscopic analysis also suggested that in Rashid the pathogen switched to the necrotrophic phase in the absence of bacteria, whereas no such change was observed in Latifia and Cordiale.

To understand expression response of wheat treated by *Micromonospora* strain by using several genes identified as being of interest based on previously tested. From finding in this study, a single treatment with *Micromonospora* did not significantly affect WRKY gene expression,

except that in Latifia there was significant suppression of *WRKY19* expression (Figure 7.8). This is consistent with previous observations (Martinez-Hidalgo *et al.*, 2015) that in the absence of pathogen attack, *Micromonospora* strains do not have a clear impact on major defence signalling pathways in the leaves. Infection of plants with *B. sorokiniana* caused up-regulation of *WRKY68a* in Latifia and Cordiale, cultivars with high resistance to the pathogen, irrespective of whether they were treated with *Micromonospora*. In Rashid, in contrast, this gene was only up-regulated in plants that were treated with *Micromonospora*. In Latifia and Cordiale treated with *Micromonospora*, there was also significant up-regulation of expression of *WRKY45*, whereas *WRKY3* was only significantly up-regulated in Latifia. Previously, Gallou *et al.* (2012) observed that nine WRKY genes are up-regulated during the pre-stage of potato root colonisation by *Glomus* sp. (before the fungus comes into contact with the root), whereas no regulation of WRKY genes was observed at the early stage of root colonisation and only one WRKY gene was up-regulated at the late stages of colonisation. Some actinomycetes prime defence pathways in plants, which can increase expression of defence genes and resistance to pathogens (Conn and Franco, 2004).

Expression of the *PR1* gene coding for pathogenesis-related protein-1 was strongly induced in all three wheat cultivars upon challenge with *B. sorokiniana*. The highest induction of expression was in Cordiale (8-fold), whereas induction was approximately 6-fold for Rashid and Latifia cultivars. Since Rashid was less resistant than Latifia and Cordiale, *PR1* gene expression does not seem to reflect disease resistance. The observation that treatment with *Micromonospora* attenuates the response of *PR1* expression to infection is consistent with the idea, first proposed for tomatoes (Martinez-Hidalgo *et al.*, 2015), that *Micromonospora* acts by activating induced systemic resistance. Induced systemic resistance is considered not to involve pathogenesis-related proteins (Hammerschmidt, 2007), and in wheat, treatment of plants with methyl jasmonate, a mediator of induced systemic resistance, attenuated the response of *PR1* gene expression to infection with *Fusarium graminearum* (Makandar *et al.*, 2011).

Analysis of the abundance of *rbcL* transcripts showed that *rbcL* gene expression was significantly down-regulated in Rashid and Cordiale under spot blotch challenge in the absence of bacteria, with the greatest down-regulation in Rashid. Suppression of *rbcL* expression was less in plants inoculated with *Micromonospora* than in uninoculated plants. Also, Afroz *et al.* (2009) have suggested that a high RubisCO activase activity may improve defence against pathogens because it increases the efficiency of carbon metabolism. Association of *Micromonospora* with plant roots enhanced fungus stress tolerance and reduced disease damage. This effect was observed in resistant Latifia compared to susceptible Rashid.

Velázquez *et al.* (2013) suggested that plant growth promotion is often achieved by both improved plant nutrition and enhanced stress tolerance, then reducing disease damages. However, treatment with *Micromonospora* did not significantly up-regulate *rbcL* expression in any of the three wheat cultivars. Jantasuriyarat *et al.* (2005) indicated that the transcription of photosynthetic genes, such as ribulose 1,5-diphosphate carboxylase, was suppressed in both the susceptible and resistant varieties in the interaction between a rice plant and rice blast fungus. It has been reported that Rubisco gene expression changes in a coordinated manner during leaf development. In young leaves and/or developing leaf tissues, it has been shown that Rubisco is actively synthesised due to the accumulation of the mRNAs of Rubisco genes (Suzuki and Makino, 2013).

Relative abundance of the internal transcribed sequence (ITS*)* of rRNA was tested with the non-specific primer pair *ITS1-ITS4* and two primer pairs specific to *Bipolaris sorokiniana* (*Coch* and *BipITS*). Expression was measured relative to endogenous wheat control (Eukaryotic Initiation Factor 4A, *eIF4A*). The expression of fungal genes in infected plants without *Micromonospora* treatment was compared to expression in infected plants treated with *Micromonospora* as the reference condition. Quantitative real-time PCR showed that relative abundance of the *ITS* region as measured with all three primer pairs was higher in the absence of *Micromonospora* inoculation, associated with increased disease severity and increased sporulation. Consequently, we can say that measurement of *ITS* RNA abundance would provide a reproducible scale where qPCR data could be directly mapped to absolute fungal load in infected plants in the presence and absence of *Micromonospora*. Such a scale could provide rapid, reliable disease quantification in future studies.

7.7 Conclusions

This study aimed to assess the potential of a novel agent for *Micromonospora luteifusca,* as a biological control agent of disease, exploring its antifungal properties against the wheat pathogen *Bipolaris sorokiniana.* In addition, the regulation of expression of WRKY TF genes was investigated. Neither treatment with *Micromonospora* nor infection substantially affected plant growth over the period of the experiment, although infected and inoculated Rashid plants had significantly more tillers than control plants. However, treatment with *Micromonospora* did significantly increase chlorophyll content in both uninfected and infected Rashid and Latifia and in infected Cordiale. Treatment with *Micromonospora* significantly reduced sporulation, chitin content and relative abundance of *B. sorokiniana ITS* RNA in infected Rashid and Cordiale.

Expression of WRKY TF genes was up-regulated more by spot blotch infection in Latifia and Cordiale than in Rashid. Treatment with *Micromonospora* had limited effect but did slightly increase up-regulation of *WRKY3* and *WRKY45* in one or more cultivars. Inoculation with *Micromonospora* substantially up-regulated expression of *WRKY68a* in infected Rashid, Latifia and Cordiale cultivars, this gene showed significant up-regulation with and without *Micromonospora*. Expression of the *rbcL* gene showed higher significant down-regulation in infected susceptible Rashid in the absence of bacteria than resistance Latifia and Cordiale cultivars. On the other hand, expression of *PR1* in Rashid and Cordiale was significantly up-regulated in infected plants that were untreated with bacteria but not in treated and infected plants.

In conclusion, *Micromonospora luteifusca* CR21 should be considered as a promising plant probiotic due to its antifungal properties and its ability to induce defence mechanisms in the wheat-*Bipolaris sorokiniana* pathosystem.

8 General discussion

8.1 Overview

The major objective of this study was to understand the relationship between nitrogen availability and resistance to the *Bipolaris sorokiniana* fungal pathogen in wheat. Previously, researchers have reported that a reduction in nitrogen input results in lower disease severity in infected plants in the field (Simon *et al.*, 2003; Loyce *et al.*, 2008). Abiotic stresses can reduce or increase susceptibility to biotic stress (pests or pathogens), and *vice versa* (Atkinson and Urwin, 2012). This study demonstrates that WRKY transcription factors, which have been shown to be involved in stress responses in a range of plants, show differential expression in response to both reduced nitrogen and fungal stressors. This may provide a basis for a molecular link that explains the observation of increased fungal susceptibility under increasing nitrogen input (Howard *et al.*, 1994). Furthermore, it is important to understand the molecular interactions of WRKY TFs after infection in wheat over time, especially those genes which are involved in the early interaction between the pathogen and the host (Shen *et al.*, 2017). This should help identify defence genes for crop improvement, which could potentially be exploited using transgenic methods or genome editing. In this project, TILLING lines were used to provide 'proof of concept' for the role of specific WRKY TFs. TILLING is a non-transgenic approach for functional genomics and crop improvement by changing the negative regulation of disease resistance that is necessary for pathogen invasion (Campbell *et al.*, 2012). Furthermore, it is considered as a cheap source for desirable genetic changes due to gene redundancy, especially in polyploid bread wheat, which exhibits very high mutation frequencies small numbers of treated plants (1 in 20–30 kb) (Dong *et al.*, 2009). Finally, the ability of *Micromonospora* strains as biocontrol agents to boost plant defence mechanisms was explored. The role of microbial communities in plant health and productivity has received substantial attention over recent years, including the exploitation of actinobacteria such as *Micromonospora* (Trujillo *et al.*, 2015). When these resistance resources are available to farmers, that can provide an effective and cheap means of enhancing crop resistance to pathogens (Gupta *et al.*, 2018).

8.2 Response of spot blotch to reduced nitrogen input

This study verified that reducing nitrogen availability resulted in reduced plant growth, with plants grown under severely reduced nitrogen showing significantly fewer leaves and tillers,

reduced chlorophyll content, reduced leaf area and shorter shoots. These results are consistent with Hinzman *et al.* (1986), who also found that reducing nitrogen input resulted in lower levels of both chlorophyll content and leaf area. It is possible that plants grown under higher nitrogen conditions may exhibit higher levels of spot blotch simply because the plants are larger. The finding of this study showed that the chlorophyll content in infected plants of both Rashid (susceptible) and Latifia (resistant) was significantly reduced under conditions of high nitrogen stress but not under moderate levels of nitrogen stress compared to plants grown under optimal conditions. Interestingly, the levels of chlorophyll in infected plants were lower in Rashid than in Latifia (Chapter 3). For both cultivars, this decline in chlorophyll content was accompanied by an apparent increase in leaf senescence based on increasing the suppression in transcription of *rbcL* under reduced nitrogen stress as shown in (Figure 4.11). Bielenberg *et al.* (2001) have previously demonstrated that reduced nitrogen results in increased leaf senescence along with reduced growth. In the present study, there was a positive correlation between size of plant (leaf area) and level of disease severity, and a negative correlation between chlorophyll content and disease severity. Thus the reduction in chlorophyll content appears to be a good indicator of disease severity.

8.3 Quantification of spot blotch in plants by quantitative real-time PCR

Expression of precursor ribosomal RNA measured with *BipITS, Coch* and *ITS1-ITS4* primer pairs was significantly up-regulated with increasing *Bipolaris sorokiniana* prevalence (fungal load), increasing with nitrogen supply from 3.75 mM and 7.5 mM in infected Rashid and Latifia wheat (Chapter 3). Quaedvlieg *et al.* (2013) demonstrated that the ITS genes increased in expression with increasing *Zymoseptoria tritici* prevalence by using quantitative real-time PCR. Both *B. sorokiniana* and *Z. tritici* are hemibiotrophic pathogens, and both fungi initially feed as biotrophs, and only later convert to necrotrophy (Gupta *et al*., 2018). Over the time series studied, gene expression measured with *BipITS, Coch* and *ITS1-ITS4* primers was associated with increased *B. sorokiniana* prevalence in cultivar Rashid but not in cultivar Latifia (Chapter 5). Thus, this method is precise in the detection of spot blotch in the early biotrophic phase and during the symptomless biotrophic phase. This is consistent with the findings of Sahu *et al*. (2016), who indicated that *B. sorokiniana* colonises host tissue during 24 hours and the hyphae then produce conidiophores that emerge out of the host, giving rise to a succession of conidia at four days after infection. Consequently, detection of *ITS* expression before visual symptoms appear in infected wheat could help in early discovery and timely action for disease management.

8.4 Resistance sources of wheat to spot blotch

Breeding for disease resistance is an environmentally sound and cost-effective means of managing spot blotch attack. Cultivars displaying a relatively high level of resistance have been developed and made available to growers. These tools are considered the cheapest and easiest resistance sources for wheat that may be provided. Globally, the major known sources of wheat resistance to spot blotch are available mainly from Brazil, China, India, Mexico (CIMMYT = International Maize and Wheat Improvement Centre), Nepal and Pakistan (Gupta *et al.*, 2018). Among seven wheat cultivars used in this study, Latifia showed the most resistance to spot blotch, followed by Tamoz3, while Rashid and Iraq cultivars were the least resistant to pathogen attack. In addition Dor29 (durum wheat) showed a high susceptibility to spot blotch infection (result not shown). Among cultivated wheats, tetraploid durum tends to be more susceptible than bread wheat, suggesting that the genes for spot blotch resistance at the hexaploid level are probably available mainly within the D genome, although the genetic studies do not support such a hypothesis (Results not shown). In study by Martinez *et al.* (2007) indicated that of the cultivars tested, modern durum wheat cultivars were more susceptible to leaf rust than bread wheat cultivars. The diversity study of seven wheat genotypes using 21 different marker genes showed that the relatively resistant cultivars Latifia and Tamoz3 were the most closely related. This is in contrast to the susceptible Rashid, which is most closely related to the Iraq and Aurok cultivars, which were both relatively susceptible to spot blotch. Diversity assays using marker genes may give early evidence of relationships among different wheat cultivars and their resistance to spot blotch and this method is cost-effective and highly efficient (see Chapter 3).

8.5 Validation of gene expression measurements

In comparative studies of gene expression, the selection of suitable control genes for normalisation of gene expression is vital but difficult. The expression of suitable endogenous control genes must stay at a highly similar level in all of the tissue samples being investigated and should show the least variation among treatments and against control sets in a given experiment (Joseph *et al.*, 2018). Combining results from RefFinder, geNorm, NormFinder, BestKeeper and ΔΔCq logarithms, similar patterns of expression in *eIF4A* and 28S ribosomal RNA was observed, suggesting that use of either normalisation gene will result in the same final trend being displayed. In contrast, 18S ribosomal RNA (*18S rRNA*) and elongation factor 1α (*EF1α*) showed the least consistency across various treatment conditions. Mascia *et al.*

(2010) found that 18S ribosomal RNA and elongation factor 1α demonstrated highly variable expression levels that should discourage their use for normalisation.

8.6 Effects of spot blotch and nitrogen stress on the expression of WRKY transcription factors

The main focus of this study was the investigation of the interaction between nitrogen supply, spot blotch disease severity and the regulation of WRKY TF gene expression in Rashid and Latifia cultivars. A secondary focus was to investigate the effects of *B. sorokiniana* infection on host WRKY TF gene expression over time after infection (0, 24, 48, 72 and 96 h) in susceptible Rashid and resistant Latifia cultivars. Finally, whether wheat plants (Rashid, Latifia and Cordiale) inoculated with *Micromonospora* exhibited differential expression of WRKY TFs in the presence and absence of spot blotch infection was also studied (Table 8.1). All seven WRKY TF genes used in this study showed up-regulation of expression at a reduced nitrogen supply of 0.75 mM compared to 7.5 mM, whilst no differential expression was seen at moderately reduced nitrogen concentrations (3.75 mM), suggesting that these genes play a role in plant responses to more severe stress levels. Bielenberg *et al.* (2001) previously suggested that reduced nitrogen results in increased leaf senescence, along with reduced growth. In the same vein Wu *et al.* (2008) demonstrated that wheat WRKY genes are involved in leaf senescence and responses to abiotic stresses (Chapter 4). *WRKY45* and *WRKY68a* showed up-regulation at low and medium nitrogen levels in the resistant cultivar Latifia, These changes in expression in response on spot blotch infection. In contrast, in the susceptible cultivar Rashid, expression of *WRKY45* and *WRKY68a* was down-regulated in response to reduced nitrogen, this suggests that the way in which *WRKY45* and *WRKY68a* expression changes in response to reduced nitrogen. Shimono *et al.* (2007) and Bahrini *et al.* (2011b) found that *WRKY45* is important for BTH (benzothiazole)-induced systemic resistance and that constitutive over-expression of the *WRKY45* gene confers enhanced resistance to blast in transgenic rice and *Fusarium graminearum* in transgenic wheat.

In the susceptible cultivar Rashid, expression of all six WRKY genes showed no difference, and low expression was observed until 48 hours after spot blotch infection compared to 24 h after infection (Chapter 5). In contrast, in the resistant cultivar Latifia, all six WRKY TF genes showed early and high expression levels after spot blotch infection. A number of studies have shown that WRKY proteins specifically recognise W-box sequences and are necessary for the inducible expression of genes containing W-box sequences (Rushton *et al.*, 1996; Rushton *et*

al., 2010; Wang *et al.*, 2013; Wen *et al.*, 2014; Hwang *et al.*, 2016). These results suggest that controlling the switch from the biotrophic to the necrotrophic stage of infection, especially in susceptible wheat (Rashid), may be key in controlling spot blotch development, and nitrogen may play a role in the timing and success of this switch. These results were clearly confirmed by microscopic analysis (WGA) in section 5.5.6. This analysis results showed that in cultivar Rashid the pathogen switched from a biotrophic to necrotrophic phase between 72 and 96 hpi

Spot blotch-resistant plants, Latifia and Cordiale, that were preinoculated with *Micromonospora* on the roots showed significant up-regulation of *WRKY45* and *WRKY68a* expression. However, Rashid wheat showed significant up-regulation of only the *WRKY68a* gene after spot blotch infection in the presence of bacteria compared to other treatments (Chapter 7). This is consistent with previous results of Gallou *et al.* (2012) who found no regulation of WRKY TFs have been observed at an early stage of root colonisation. Also, in a study by Martinez-Hidalgo *et al.* (2015) indicated that in the absence of pathogen attack, the bacteria had no clear impact on these major defence signalling pathways when used JA-marker genes in the leaves.

8.7 Role of the Pathogenesis-related protein - PR1

PR protein accumulation is one of the best-characterised plant defence responses (Yadav *et al*., 2016). Much evidence is available about the high response of the *PR1* (pathogenesis-related resistance protein 1) gene to abiotic stress, as was indicated in studies carried out in maize (Xie *et al.*, 2010), and rice (Jain and Kumar, 2015). In Rashid and Latifia, *PR1* was significant more up-regulated in response to infection at standard nitrogen level than at moderate and severely reduced nitrogen in infected plants. Expression of *PR1* was significantly up-regulated in infected Latifia than Rashid only under severe nitrogen stress (Chapter 4).

PR1 showed the highest up-regulation of expression with early accumulation of transcripts 24 hours after pathogen infection in Latifia (see Chapter 5). In resistant Latifia, *PR1* occurring earlier at 24 h, may kill *B. sorokiniana* and thereby arrest release of fungal elicitors (Manandhar *et al.*, 1999) sooner than in susceptible Rashid at 72 hpi. Campbell *et al.* (2012) indicated that the expression in the PR genes in the resistant wheat was activated and responded to the pathogen earlier after infection than in the susceptible wheat. Sahu *et al.* (2016) demonstrated that induced expression of the *PR10* gene was highly maintained from 24 h after spot blotch infection in a resistant wheat cultivar but not in a susceptible cultivar. Also, in another study Manandhar *et al.* (1999) found that in rice inoculated with *B. sorokiniana* (a non-pathogen of

rice), transcripts of several pathogenesis-related protein genes including *PR1*, as well as peroxidase, accumulated by 12 h with maximum levels at 24 h except for peroxidase.

8.8 Role of the Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)

Expression of the RuBisCO has been used to identify leaf senescence. In uninfected plants, the *rbcL* gene showed significantly lower expression with severely reduced nitrogen (high senescence) than with moderate levels compared to the standard nitrogen level in Rashid cultivar. This result further validates the suggestion of Noodén (2012) who demonstrated that senescence could also be characterised by declines in Rubisco, associated declines in net photosynthesis, increased proteolysis, altered fine organelle structure, loss of RNA and DNA, and increased ion leakage. Also Dann and Pell (1989), which indicated that RuBisCO is degraded during leaf senescence (see Chapter 4). In infected leaves, expression of *rbcL* was down-regulated compared to uninfected leaves with standard nitrogen but not with low nitrogen. This indicates that there was less senescence in Rashid and Latifia wheat with reduced nitrogen due to lower fungal damage than at high nitrogen levels. This result is consistent with the results of Jantasuriyarat *et al.* (2005) who found that in the interaction between rice plants and the rice blast fungus *Magnaporthe grisea*, the transcription of photosynthetic genes, such as those for RubisCO and chlorophyll a/b-binding protein, was suppressed in both susceptible and resistant interactions.

Relative expression of *rbcL* decreased with increase in infection over time: expression was lowest at 96 hpi compared to 24 hpi in Rashid and Latifia cultivars (see Chapter 5). It has been previously reported that there are coordinated changes in Rubisco gene expression during leaf development and Rubisco is reduced by leaf senescence with the progress of leaf age (Suzuki and Makino, 2013).

Inoculation of plant roots with *Micromonospora luteifusca* reduced disease damage. This effect was clearly observed in resistant Latifia compared to susceptible Rashid (see Chapter 7). The *rbcL* gene showed up-regulation of expression in control plants of three wheat cultivars inoculated with *Micromonospora*. These results suggested that pre-inoculation of plant roots with bacteria delayed progress of leaves to the senescent phase. In young leaves and/or developing leaf tissues, Rubisco is actively synthesised due to the accumulation of the mRNAs of Rubisco genes (Suzuki and Makino, 2013).

Table 8.1. Summary of expression of WRKY TF (W), *rbcL* and *PR1* gene tested in infected Rashid (R) and Latifia (L) were displayed to uninfected plant grown in the same nitrogen concentration, over time after infection and in addition Cordiale (C) in the interaction between treatment with biocontrol bacteria and pathogen.

No.	Gene name	Wheat name	Nitrogen concentrations mM			Over time infection (hour)				Biocontrol × Pathogen		
			0.75	3.75	7.5	24	48	72	96	B	F	B×F
1	W3	R	-	+	+	+	+	+*	+	+	+	+
		L	+*	+	+	+	+*	+**	+*	+	+	+*
		C								-	+*	+
2	W19	R	-*	-	-	+	+	+*	-	-	-	+
		L	+	-*	-	+	+	+*	-	-*	-*	+
		C								-	+	+*
3	W45	R	-*	-	-	+	+	+*	+	+	-	+
		L	+*	+	+	+*	+*	+**	+*	-	+	+*
		C										
4	W46	R	-**	-*	-	+	+	+	-			
		L	-	-*	-	+	+*	+*	-			
		C										
5	W51	R	*	+*	+*	+	+	+	+*			
		L	+	+	+	+**	+**	+*	+*			
		C										
6	W68a	R	-	-	+	+	+	+*	+	+	+	+*
		L	+**	+	-	+	+**	+*	+	+	+**	+*
		C								+	+*	+*
7	W85	R	-	-*	-							
		L	+	-*	-							
		C										
8	rbcL	R	-	-	-*	-	-	-*	-***	+	-**	-*
		L	-	-	-*	-	-	-	-*	+	-	-
		C								+	-*	-
9	PR1	R	+	+	+	+	+*	+**	+*	+	+**	+
		L	+**	+	+	+***	+**	+*	+*	+	+*	+
		C								-	+**	+*

+ Upregulation,- downregulation, B *Micromonospora*, F *Bipolaris sorokiniana*, B×F interaction and *, **, *** significantly (CI, t-test P < 0.05, 0.01, 0.001) respectively

8.9 *Micromonospra* enhanced plant defence against spot blotch

After inoculation of roots with *Micromonospora luteifusca*, Rashid and Cordiale showed a lower number of spores, chitin content and significant reduction in disease symptoms with high chlorophyll content in plants infected with *B. sorokiniana*. Plant development and growth can be improved by some soil microorganisms, which are necessary for nutrient uptake, produce plant growth-stimulating substances, and prevent pathogen attacks (Turner *et al.*, 2013; Velázquez *et al.*, 2013; Martinez-Hidalgo *et al.*, 2014; Martinez-Hidalgo *et al.*, 2015).

8.10 Quantification of chitin content as a measure of fungal load

Microscopic analysis showed that in cultivar Rashid the pathogen switched from a biotrophic to necrotrophic phase between 72 hpi and 96 hpi, but no such change was observed in Latifia cultivar (see Chapter 5) with the WGA assay (wheat germ agglutinin-WGA conjugate) suggested by Ayliffe *et al.* (2013). The necrotrophic growth phase is characterised by invasion of mesophyll tissue and host cell death. Dead cells appear decolourised in transmitted light and yellow auto-fluorescing in UV light in Rashid wheat at 96 hpi. In TILLING lines, the *WRKY68Q245* mutant showed greater visibility of pathogen growths spread with a high chitin content in infected tissue with severely reduced nitrogen than with moderate and high nitrogen levels. This result was contrary to the wild-type response in which increasing nitrogen input increased the spread of spot blotch (see Chapter 6). Additionally, three wheat cultivars experienced increased infection in the absence of *Micromonospora luteifusca* inoculation. Rashid wheat showed much greater visible fungal spread and damage from spot blotch than Latifia and Cordiale cultivars, both of which were observed as more resistant to spot blotch attack (see Chapter 7). These results are consistent with results of other methods used in this study such as chitin content (WGA), number of spores counted from wash leaves and disease severity scoring.

8.11 Conclusion and proposed future studies

The current project has sought to answer questions fundamental to the link between reducing nitrogen availability and resistance to *Bipolaris sorokiniana*, with a view to improving agricultural production of crops and reaching global food security. It has raised as many questions as it has tried to answer, leaving significant scope for future work.

If the link between nitrogen and spot blotch is to be fully explored, then the infection must be allowed to progress to the necrotrophic stage to confirm decreased spot blotch severity with low nitrogen. Rashid cultivar (susceptible wheat) reached the necrotrophic phase after four days of infection, but Latifia cultivar (resistant wheat) did not. Therefore, future work should focus on growth during the biotrophic phase, the switch to necrotrophy, and growth during the necrotrophic phase, especially in resistant wheat (Latifia). In addition to spot blotch effect, WRKY gene expression should also be monitored. Again, this will provide a more comprehensive view of how transcription changes with stages of the infection, which can be translated to a better understanding of the nature of the interaction between pathogen and host

during the different stages, as well as elucidating which WRKY proteins play a pivotal role in affecting the progression of the disease (see Chapter 4).

An alternative approach would be to elucidate the effect of up or down-regulation of specific WRKY genes, both in terms of the transcriptome and the phenotypic effects on the plant. The results of qPCR would provide more confidence to further investigation of these WRKY genes, especially *WRKY19* and *WRKY68a*, across other an intensive method. These findings suggest WRKY gene may play a role for controlling the switch from the biotrophic to the necrotrophic stage of infection, especially in susceptible wheat (Rashid), then they could have a key in controlling spot blotch; furthermore, nitrogen may play a role in the timing and success of this switch. Further investigation is needed to look at how nitrogen concentration affects disease severity either side of the change, especially in late growth stages, where wheat cultivars exhibited high susceptibility to spot blotch see (Chapter 5). Thus, phytohormones use could clearly explain of wheat response against spot blotch over time infection or under reduced nitrogen. Wen *et al*. (2014) found that there was a strong correlation between promoter cis-elements and the phytohormones (SA, ABA and JA)-induced WRKY gene expression.

TILLING lines had different patterns of response to infection at three levels of nitrogen. Plants of the *W68aQ245* mutant were bigger than wild-type and *W68aV213* plants were small. Whatever the approach, TILLING lines can contribute to identification of the role played by WRKY genes which regulate the link between spot blotch and nitrogen, especially in the context of increased spot blotch tolerance in *W68aQ245* at high nitrogen availability compared to wild type, thus providing an insight into how wheat tolerance to spot blotch can be improved. This will also allow WRKY genes to be identified as key molecular markers, where a simple analysis of gene expression levels may provide important information regarding spot blotch resistance (Chapter 6).

From the results of this study, *B. sorokiniana* triggers a strong induction of WRKY TF gene expression in the infected leaves, when plants are preinoculated with *Micromonospora* in the roots. In the plants that showed high resistance to spot blotch with low disease symptoms, Latifia and Cordiale wheat showed significant up-regulation of expression of *WRKY45* and *WRKY68a*, whereas *WRKY3* was significantly up-regulated only in Latifia. For fully understanding of the role of *Micromonospora* in protecting wheat against spot blotch infection, further experiments may be used at different developmental stages of wheat (see Chapter 7).

The WGA assay and quantifying *ITS* expression in the plant–pathogen interaction may provide reliable and robust methods for quantifying fungal load and progression of numerous plant diseases at early stages of infection when there are no symptoms. Rubisco (*rbcL*) gene expression changes in a coordinated manner during leaf development or with increasing fungal load on plants, and is an excellent tool to measure leaf senescence under stress and *rbcL* can be considered as a marker of cell degradation. Pathogenesis-related protein (*PR1*) accumulation is one of the best-characterised plant defence responses and may provide a precise method to quantify early resistant responses of plants with the highest expression accompanied by earlier accumulation of transcripts after pathogen infection in resistant plants than in susceptible plants. Furthermore, quantitative method of *Coch*, *ITS1-ITS4* and *BipITS* regions to increase in RNA abundance as disease severity increased by qPCR can be used for assessing pathogen load during the biotrophic phase of infection.

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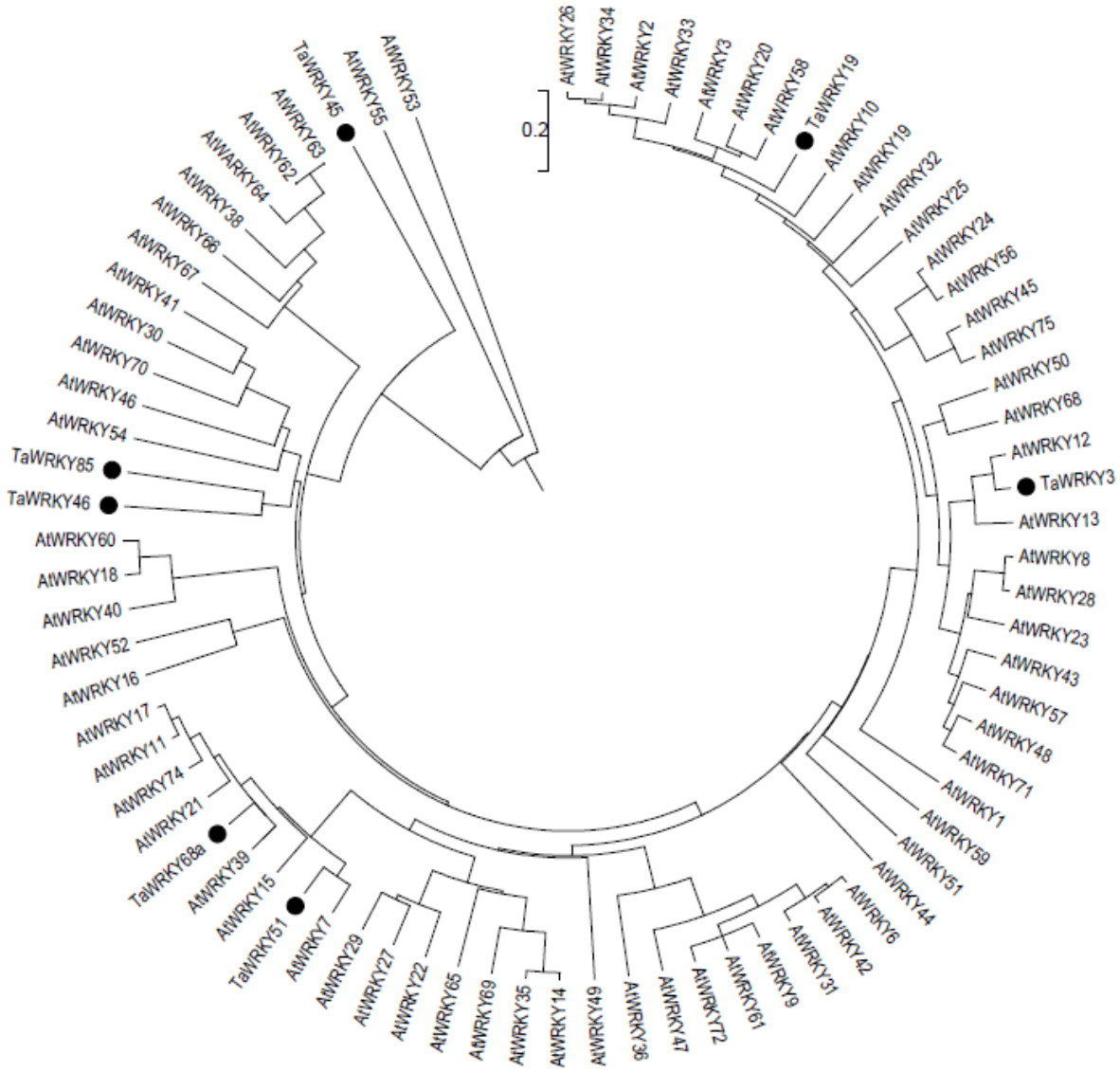
Appendix A Neighbour-joining cladogram of the WRKY domains of Arabidopsis and wheat WRKY gene of interesting in this study.

WRKY TFs cluster first with proteins from the same species, making identifying one to one relationships difficult between species, scale bar (0.2) is marked.



Appendix B Oligo name and sequences of genes used in this study

Appendix B 1 Oligo name and sequences of wheat *Triticum aestivum WRKY* genes.

	Oligo Name	Genbank No.		Sequence 5' to 3'
1	TaWRKY3	EU665432.1	F	GTGCTGGACGACGGATACAA
			R	TAGCTCCTGGGATGAAGGCT
2	TaWRKY19	EU665430.1	F	AGGGAAGCATACGCATGACGTGC
			R	GGCGAGATCGTTCAGAATGGCTG
3	TaWRKY45	EF397613.1	F	CATGAGGAGCTTGGAGGACG
			R	AGGCCTTTGAGTGCTTGGAG
4	TaWRKY46	EF368365.1	F	CGAGCACAACCAACCAACAG
			R	ACGGTGATGTGTCTGTCCAC
5	TaWRKY51	JX277054.1	F	TCCATGCTCATCGTGACCT
			R	ACGACGGTAATGGCAAAGA
6	TaWRKY68a	EF368360.1	F	TGTCCGGGCACAAGAGAAA
			R	CTTCTCTTGGAGCAGTGGCA
7	TaWRKY85	LS992087.1	F	TCTTTCGCTCGATACGTCTGAC
			R	ATGAGGACGCAGCGAAATAC

Appendix B 2 Oligo name and sequences of reference genes in wheat.

	Oligo Name	Genbank No.		Sequence 5' to 3'
1	TaeIF4A	Z21510.1	F	GGTTCATGCATGTGTTGGAG
			R	AACGACATGCACTCCACTTG
2	Ta28S rRNA	AY049041.1	F	ATTGTCAGGTGGGGAGTTTG
			R	ACCCTTTTGTTCCACACGAG
3	TaEF1α	M90077.1	F	ACCTGAAGAAGGTCGGCTACAA
			R	ATCTGGTCAAGCGCCTCAAG
4	Ta18S rRNA	AH001810.2	F	CCGTTCTTAGTTGGTGGAGC
			R	GGATGGCTCCGCATAGCTAGT
6	rbcL	LT576864.1	F	ATGTCACCACAAACAGAAACTAA
			R	AAACGGTCTCTCCAGCGCAT
7	TaPR1	KR351308.1	F	ACTCAACGCATATCGGCTGC
			R	CCTCTGCCCAATGTAGTTGC
8	TaPR1 from TcLr1	HQ848391.1	F	ATCAACGACTGCAAGCTCCA
			R	GACGCCGAGGTTATTGTTGC
9	TaDRP	DQ205351.2	F	CTGGCACGCAGTTACAAGGAC
			R	TCCATACGCCGTACTTCTGC

Appendix B 3 Oligo name and sequences of *Brachypodium distachyon* genes.

	Oligo Name	Genbank No.		Sequence 5' to 3'
1	BdWRKY3	XM_003562717.3	F	TGAGGGAAGGGGGAGATGAG
			R	GCCACCTATAGCCATCGTCC
3	BdWRKY12	XM_014896045.1	F	GAGCGGAGAAGGGGAAGATG
			R	TAGCTCCTGGGATGAAGGCT
4	BdWRKY51	XM_003579337.4	F	TGCCACTGCTCCAAGAGAAG
			R	CACCCTCTTACTGTGCTGCA
5	BdWRKY55	XM_010239644.2	F	TGCTCGACTTCACGAAAGCT
			R	GCGTAGTCACAGACATGCCT
5	BdWRKY70	XM_010233230.2	F	GAAGGACCCAGCAATCCTCC
			R	GAAATAGGCCCTTGGGTGCT
6	BdWRKYA2	XM_014898779.1	F	GAGGAGTACTGTCGCTCTGC
			R	GAGTACACCGCCGAGATGAG
7	BdPR1	XM_003572835.3	F	ACGGTACGACTACTGGAGCAA
			R	GCCGCATGCCGACGTAGTTA
8	BdPR5	XM_003563416.3	F	ACGGCGTACTCGAAGCTGTT
			R	CTACTGAAACACGGTGCCTC
9	BdWRKY40	XM_003570693.3	F	CTAATTAATCCCCATGCTCC
			R	TGATCGTCAATCGACCCACT
10	BdWR3	XM_003575233.3	F	CCTTCTTCTTCTTCATCGAG
			R	CCGAGTATGCCTTGCAATAG
11	BdEF1α	XM_003581641.3	F	AAGCGTGGGTTTGTTGCATC
			R	TGTCGATCTTGGTCACCAGC
12	BdUBC18	XM_003578990.3	F	TATCCCATGGAGGCACCTCA
			R	AGACAGCAGGGACAAGATGC

Appendix B 4 Oligo name and sequences of *Bipolaris sorokiniana* genes.

	Genbank No.	Oligo Name	Sequence 5' to 3'
1	HF934937.1	Coch -F	GCGCACTTGTTGTTTCCTGGGC
		Coch-R	AAACCAGTAGGCCGGCTGCC
2	HF934937.1	BipITS-F	ATATGAAGGCCTGGCTTCGC
		BipITS-R	TATGGTTTGGTCCTGGTGGC
3	FJ609737.1-Ta KY985464.1-Sb	ITS1-F	TCCGTAGGTGAACCTGCGG
		ITS4-R	TCCTCCGCTTATTGATATGC
4	JX256386.1	Bs28S rRNA_F	CCGAGCGCGTAATGAAAGTG
		Bs28S rRNA-R	GCCTCCACCAGAGTTTCCTC
5	U42479.1	Bs18S rRNA-F	ACACTGACAGAGCCAACGAG
		Bs18S rRNA-R	CTCTATCCCCAGCACGACAG
6	KU870644.1	B.soro-GPD-F	ATCGAGCCCCACTACGCTGTA
		B.soro-GPD-R	TGACGGTCAGGTTGTTGCCGT

Appendix B 5 Amino acid sequences

Appendix B 5 1 TaWRKY19

M A A G Q W S G I G D G G G L W A P P A L D S L F P D D Q P S P A A S A L G F F G
G S L A Q L P S P P P L C G T A L L G Y P Q D N F D V F H E R D L A Q L A A Q V A Q
K K E L R E K Q G A G L H H K I G P Q L A F S K Y S I L D Q V D N S S S F S L A T S
V L T P Q H V S S S V G A A L M Q G R T L P S H T G S G S V N T G P T G V L Q A L Q
D S S T T L D S I N T G S T G V L E A L Q G S S I T L D R P A D D G Y N W R K Y G Q
K A V K G G K Y P R S Y Y K C T L N C P A R K N V E H S A D R R I I K I I Y R G Q H
C H E P P S K R F K D C G D L L N E L N D F D D A K E P S T K S Q L G C Q G Y Y G
K P I T P N G M M T D V L L P T K E E G D E Q L S S L S D I R E G D G E I R T V D G
D D G D A D A N E R N A P G Q K I I V S T T S D A D L L D D G Y R W R K Y G Q K V
V R G N P H P R S Y Y K C T Y Q G C D V K K H I E R S S E E P H A V I T T Y E G K H
T H D V P E S R N R S Q A T G Q H H C K E Q T Y S E Q S A A S F C S S S E K R K Y G
T A I L N D L A F Sto **Stop**

Appendix B 5 2 TaWRKY68a

R A K I C R V T G D L T P A T M A V D P M G C Y T P R R A D D Q L A I Q E A A T A
G L R S L E L L V S S L S G A A P S K A P Q Q H L Q Q P F G E I A D Q A V S K F R K
V I S I L D R T G H A R F R R G P V Q S P T P P P P A P V A P P P P P P R P L A V V E P
A R P A P L T A V A P V S V A A P V P L P Q P Q S L T L D F T K P N L T M S G A T S
V T S T S F F L S V T A G E G S V S K G R S L V S A G K P P L S G H K R K P C A G A
H S E A N T T G S R C H C S K R R K N R V K T T V R V P A V S A K I A D I P P D E Y
S W R K Y G Q K P I K V S P Y P R G Y Y K C S T V R G C P A R K H V E R A L D D P
A M L V V T Y E G E H R H S P G P M P M Q M A P S P V P I P M P M G A P V A V A S
V S A G N G H V Stop L S

Appendix C Genomic DNA extraction- fungi

All the fungus from a culture plate containing *B. sorokiniana* was scraped off into a 2.0 ml screw cap tube which contained 0.5 g of fine glass beads (≤106 μm) and 0.5 ml CTAB extraction buffer (equal volumes of 10% CTAB in 0.7 M sodium chloride and 0.24 M potassium phosphate buffer were mixed, pH 8.0). 0.5 ml phenol/chloroform/isoamyl alcohol was added to the mixture, then vortexed for 30 s after which the fungal mycelium was lysed at 5.5 m s^{-1} for 30 s using a FastPrep FP120 cell disrupter. The lysed sample was placed on ice for 2 min before it was centrifuged at maximum speed (14,000×*g*) for 5 min. The upper aqueous layer was transferred to a new 2.0 ml screw cap tube and an equal volume of chloroform/isoamyl alcohol (24:1 w/v) was added to remove phenol. Then tube centrifuged at 14,000×*g* for 5 min. The volume of the upper layer of the tube was transferred to a new 1.5 Eppendorf tube after which DNA was precipitated using twice the volume of the extracted upper layer of 30% PEG6000 (polyethylene glycol 6000) [9.35 g NaCl and 30 g PEG6000 made up to 100 ml with SDW], incubating it for 2 h at -20 °C. After two hours, the sample was centrifuged for 10 min at 14,000 *g* and the supernatant was discarded. 200 μl of 70% (v/v) ice-cold ethanol was used to wash the pellet. This was centrifuged at 14,000×*g* for 5 min, after which the supernatant was discarded. At room temperature, the pellet was air-dried then suspended in 50 μl SDW after which it was stored at -20 °C. Agarose gel electrophoresis was used to detect the presence of genomic DNA. This protocol adapted from (Aada, 2013)

Appendix D Genome DNA extraction- plant

Add 1 mL TRIzol (Invitrogen) and held at room temperature for 5 minutes. A 200 µL aliquot of chloroform was added to each extract with shaking for 15 seconds, and then incubate at room temperature for 3 minutes, and the resulting mixture was centrifuged (15 min 12000×*g* at 4 ºC). The aqueous layer was transferred to a clean tube, DNA precipitated by the addition of 300 µL from 100% ethanol per 1 ml TRIzol used, and mix samples by inversion. Leave samples at room temperature for 3 minutes, then centrifuged at 2000×*g* for 5 minutes at 4 °C. Remove the supernatant to waste and retain for protein isolation. Wash the DNA pellet, with 1 ml of 0.1 M sodium citrate in 10% ethanol per 1 ml of TRIzol, and mix for 30 minutes at 4 °C. Centrifuge samples at 2000×*g* for 5 minutes at 4 °C. Two washes are usually sufficient, however for large containing >200 µg of DNA an additional wash may be necessary. Following the wash step, add 1.5 ml of 75% ethanol per 1 ml TRIzol used. Mix for 20 minutes at room temperature, then centrifuge samples at 2000×*g* for 5 minutes at 4 °C. Air-dry the pellet for 15 minutes. Resuspend the pellet in 8 mM NaOH,and centrifuged at 12000×*g* for 10 minutes to remove any insoluble materials, and then transfer the supernatant to another tube. This protocol adapted from bioline-TRisure product munual. This protocol adapted from TRIzol (Invitrogen).

https://www.bioline.com/us/downloads/dl/file/id/953/trisure_product_manual.pdf

Appendix E Internal control gene analysis

Firstly calculates the pairwise variation of each candidate reference gene with all other tested genes in order to subsequently determine the stability value (*M*) for each reference gene as the average of this pairwise variation. The gene showing the lowest *M* value would be the most stably expressed. On the other hand, geNorm calculates the minimal number of reference genes for accurate normalisation from the pairwise variation between two sequential normalisation factors containing an increasing number of genes (pairwise variation, *V*). It proposes a 0.15 cut-off value in such a way that a Vn/n+1 pairwise variation below 0.15 indicates that n genes are sufficient for normalisation and the gene n+1 should not be included.
For every combination of two internal control genes *j* and *k*, an array A_{jk} of *m* elements are calculated which consist of $\log_2$-transformed expression ratios a_{ij}/a_{ik} (Equation 1). We define the pairwise variation V_{jk} for the control genes *j* and *k* as the standard deviation (*st.dev* or SD) of the elements (Equation 2). The gene-stability measure M_j for control gene *j* is the arithmetic mean of all pairwise variation *Vjk* (Equation 3). The acceptable *Mj* value is <0.15 (Vandesompele *et al.*, 2002). The value of *Mj* represents the geNorm gene stability measure *M* for a particular reference gene *j* (Hellemans et al., 2007).

$(\forall\, j,k \in [1,n] \text{ and } j \neq k)$:

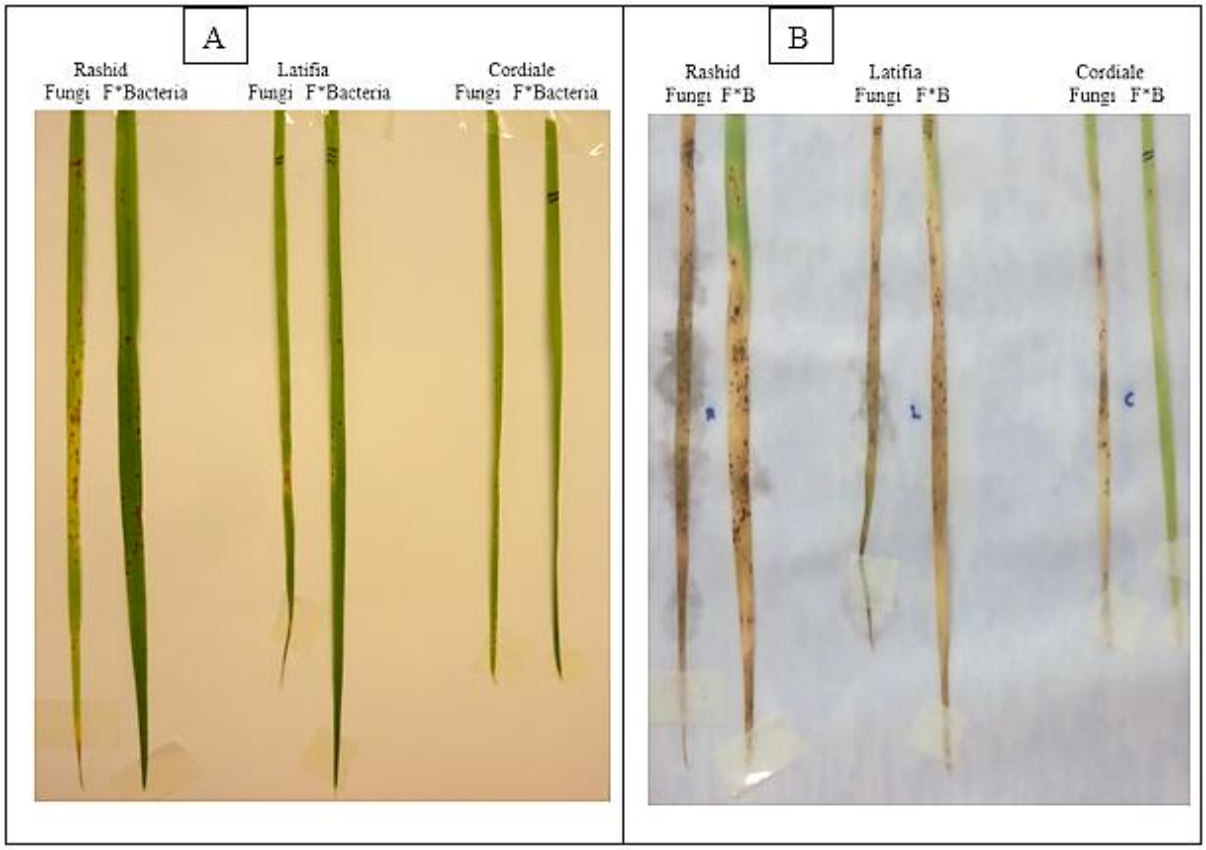
$$A_{jk} = \left\{\log_2\left(\frac{a_{1j}}{a_{1k}}\right), \log_2\left(\frac{a_{2j}}{a_{2k}}\right), \ldots, \log_2\left(\frac{a_{mj}}{a_{mk}}\right)\right\} = \left\{\log_2\left(\frac{a_{ij}}{a_{ik}}\right)\right\}_{i=1\to m} \quad (1)$$

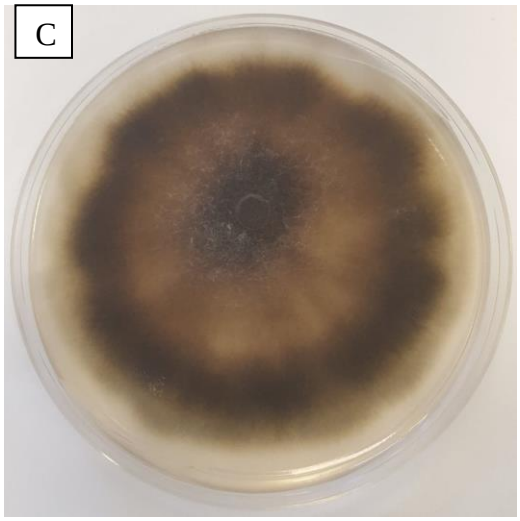
$$V_{jk} = st.dev\,(A_{jk}) \quad (2)$$

$$M_j = \frac{\sum_{k=1}^{n} V_{jk}}{n-1} \quad (3)$$

Appendix F Spot blotch Infection in three wheat cultivars (Rashid, Latifia and Cordiale) and *Bipolaris sorokiniana* on agar plate.

Three wheat infected at 21 days old in the present (F*B) and absence (F) of *Micromonospora luteifusca*. (A) Infected leaves four-day post infection at 25 day old. (B) Further four-day post infection in humidity condition at 29 days old. (C) *Bipolaris sorokiniana* incubated at 14 days old.





Appendix G Infected leaves of Rashid, Latifia and Cordiale wheat cultivars at 10 dpi.

Three infected wheat cultivars after six days incubation by spot blotch spores for the plants under high humidity condition at 25 days plants old, four-day post infection. Under XT-400 Stereo zoom microscope, 10×objective magnification.