Inter-relationships between *Bipolaris sorokiniana* isolates involved in spot blotch, common root rot and black point in winter cereals

A dissertation submitted by

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Abstract

Foliar spot blotch (SB) and common root rot (CRR) are important cereal diseases caused by the fungal pathogen *Bipolaris sorokiniana* (teleomorph Cochliobolus sativus), and the relationship between the causal agents responsible for the two diseases is poorly understood. This thesis investigated the population structure of *B. sorokiniana* in the Australian grain belt to determine the relationship between SB and CRR. We examined 126 single spore isolates of *B. sorokiniana* from basal and aerial tissue of wheat and barley, through amplified fragment length polymorphism analysis. Cluster analysis revealed two populations of the pathogen; one that consisted primarily of wheat CRR, and the other that consisted primarily of barley SB isolates. This indicated the isolates were possibly tissue and/or host specific. Genetic divergence among isolates according to geographic location was also evident. We further investigated the extent to which genetic differentiation occurs in Australian isolates during the infection process by the analysis of isolates from a single inoculated plant in the field, and inoculated wheat and barley genotypes. The question addressed was whether genetic divergence occurs among isolates during the infection process according to the host genotype. In the current investigation, genetic variation was evident among isolates from single lesions, even in controlled glasshouse conditions. Genetic variation was observed among isolates sampled from different genotypes after inoculation with the same original isolate. This indicates that although the haploid pathogen is considered to reproduce by asexual means only, genetic differentiation does occur in isolates at low levels even with a single infection event.

We determined whether the virulence of *B. sorokiniana* could be predicted based on the genetic variability identified in the AFLP analyses. A selection from each host and tissue source (barley SB, wheat SB, barley CRR and wheat CRR) was selected for phenotypic testing on leaf tissue, then on root tissue of wheat and barley differential sets. The spot blotch phenotype testing revealed both host and tissue specificity of isolates. The phenotypic common root rot assessment suggested the possibility of tissue specificity, but the screening method requires further development.

The most effective means for control of fungal pathogens is by use of genetic resistance when available. Two major quantitative trait loci (QTL) associated with spot blotch resistance have been identified on barley chromosomes 7H and 3H in a number of mapping studies. The QTL on chromosome 7H has been fine mapped, however fine mapping of the QTL located on chromosome 3H has proven difficult due to a low level of polymorphism in the region. Fine mapping of the 3H region is necessary to identify new markers closely linked to the QTL of interest for use in breeding programmes, and also potential identification of candidate genes. This was attempted in an ND24260 x Flagship doubled haploid (DH) population of 334 lines, which had previously been mapped using DArT markers. Seven PCR-based markers based on EST sequence data from an unrelated mapping study were screened across the population, as well as eight PCR-based markers from a second mapping study, which were designed based on single nucleotide polymorphism (SNP) data. Identification of markers more closely linked to the resistance region was unsuccessful, as none of the fifteen markers were found to be polymorphic. However

high resolution melt analysis may be used in future as a more thorough method for identifying polymorphism.

A third disease historically attributed to infection by *B. sorokiniana* is the grain defect black point, which occurs in both wheat and barley. The defect is undesirable in barley as it is believed to be associated with fungal contamination detrimental to beer production. Black point in wheat is well-studied, but little has been done to determine the cause of the defect in barley. Additionally, many of the studies investigating black point of wheat have produced contradictory results. Although this symptom has been attributed to *B. sorokiniana* as well as other pathogens, this association has been questioned in two aspects. On one hand, symptoms may appear even in the absence of detectable levels of the pathogen, and in many instances *B. sorokiniana*- colonised wheat grains did not exhibit BP symptoms. In this work we examined whether BP affected and BP unaffected barley grains exhibited different levels of *B. sorokiniana* colonisation by comparing the amount of DNA recovered from barley grains as a measure of fungal biomass. This is the first time this issue has been addressed in this manner. There was no compelling evidence that colonisation of seeds by *B. sorokiniana* is a sufficient condition for the production of BP symptoms under favourable environmental conditions.

Certification of Dissertation

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

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Signature of Candidate Date

ENDORSEMENT

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Signature of Principal Supervisor Date

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Signature of Associate Supervisor Date

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Chapter 1: Literature Review and Scope of Project

1.1 Introduction

Wheat and barley grain both have high nutritional value, and as such are cultivated for human consumption all over the world. Wheat is ranked as one of the most commonly grown crops along with rice, with approximately 6.71 x 10^8 and 7.19 x 10^8 mega tons, respectively, produced annually worldwide (Food and Agriculture Organisation of the United Nations, 2013). In Australia, wheat and barley are the two most important grain crops produced, with wheat primarily being used for human consumption, and barley being used for malting and animal feed. Both wheat and barley are major exports for Australia, with a five year average production (up to the production year 2012-13) of 24 606 kt and 7 909 kt per year, respectively, and these exports contribute significantly to the global market (ABARES, 2014; Turner, 2011). The percentage of total agricultural area in Australia planted to wheat was approximately 3.17% and the area planted to barley was approximately 0.89% in the 2011-12 cropping season (ABS, 2011).

1.1.1 Occurrence and economic importance of barley in Australia

Barley (*Hordeum vulgare*) is the second most important crop after wheat in Australia (Murray and Brennan, 2010). The average area sown annually to barley in Australia is approximately 3.79 million ha, with an average annual value of $1.289 billion (Murray and Brennan, 2010). According to the Australian Bureau of Statistics Survey of Agricultural Commodities (ABS, 2011), Qld produced approximately 5% of Australia's barley in the year 2010-11 and NSW produced about 21% (see Table 1.1). The highest producer of the crop was South Australia at 28%. There are two grades (reflecting the main uses) of barley grain in Australia: malting and feed grades. The best quality grains grown in the current season, and belonging to an approved malting variety are accepted for malting (GTA, 2010). Barley that does not meet these requirements is downgraded to feed grade. Approximately 32% of the barley produced in Australia is selected for malting (ABARES, 2014).

1.1.2 Occurrence and economic importance of wheat in Australia

Wheat (*Triticum aestivum*) is the most widely grown and economically important crop in Australia, with an average area of 11.9 million ha planted per season and an average value of $4.68 billion per annum (Murray and Brennan, 2009). The total area sown to wheat Australia-wide in the season 2009-10 was 13.9 million ha, with a total production of 21.8 million tonnes (Murray and Brennan, 2009). The proportion of Australia's wheat produced in NSW (Table 1.1) was approximately 35%, second only to Western Australia, which produced 36% of Australia's total. Queensland produced just less than 8% of the total (ABS, 2011).Wheat is the main winter crop grown in southern and central Queensland and approximately 800 000 ha is planted annually (Turner, 2011).

The primary use of wheat is for milling of grains to make flour, which is used as a major ingredient in bread, flat breads, cakes, biscuits, noodles and pasta. Wheat is

divided into different grades according to a variety of parameters including milling characteristics, protein content/target, dough strength and extensibility. Lower quality grain is used for poultry and stock feed.

Table 1.1 Wheat and barley production (tonnes) in Australia 2010-11 by state (ABS, 2011).

State	Barley (tonnes)	% Total	Wheat (tonnes)	% Total
New South Wales	1 040 300	20.67	8 601 900	34.74
Victoria	1 189 300	23.63	2 642 100	10.67
Queensland	253 800	5.04	1 904 000	7.69
South Australia	1 409 200	28.00	2 585 500	10.44
Western Australia	1 117 300	22.20	9 004 100	36.37
Tasmania	22 200	0.44	19 800	0.08
Northern Territory	0	0	0	0
Australian Capital Territory	0	0	0	0
Total	**5 032 100**		**24 757 400**	

1.1.3 Importance of the fungal pathogen *Bipolaris sorokiniana*

With regards to root and crown diseases, *Bipolaris sorokiniana* Sacc. (Shoemaker) is the third most important fungal pathogen of both crops in Australia. The pathogen causes $30 million damage to wheat and $13 million to barley per year due to yield losses. This is exceeded only by *Fusarium pseudograminearum* and *Rhizoctonia solani*, which annually cause damage worth $79 million and $59 million to wheat, respectively (Murray and Brennan, 2009), and $18 million each to barley (Murray and Brennan, 2010). In Australia, the foliar form of the pathogen (spot blotch) causes a total of $1 million worth of damage annually to both crops (Murray and Brennan, 2009; Murray and Brennan, 2010).

The disease is somewhat difficult to control and inoculum can increase over time in optimum conditions (Burgess and Griffin, 1968). This is due to its large host range and ability to survive over summer on remaining stubble (i.e. crown and stem tissue remaining in the paddock after harvest). Plants reported to be alternate hosts of the disease include oilseed rape (*Brassica campestris*), soybean (*Glycine max*), lentil (*Lens culinaris*), pearl millet (*Pennisetum americanum*), sesame (*Sesamum indicum*), sorghum (*Sorghum bicolour*), mungbean (*Vigna mungo*) and maize (*Zea mays*) (Acharya et al., 2011).

1.2 Disease states of *B. sorokiniana*

Bipolaris sorokiniana causes foliar spot blotch (SB) and common root rot (CRR) of wheat and barley, and can also be isolated from the heads of plants. A number of researchers suggested that *B. sorokiniana* causes the grain defect black point in infected heads of grain (Kumar et al., 2002; Malaker and Mian, 2010; Monaco et al., 2004), however results have not been conclusive. These three disease states are discussed in the following sections.

1.2.1 Spot blotch

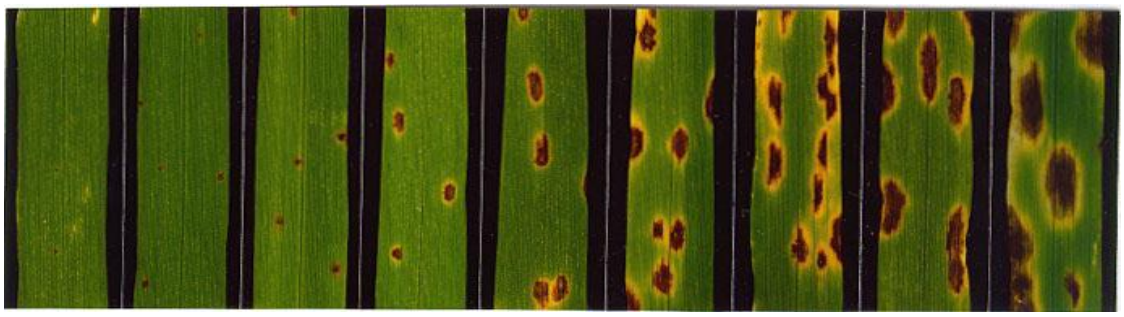
1.2.1.1 Occurrence and Favourable Environmental Conditions

SB of wheat is rare in Australia, but is a significant constraint in warmer, more humid wheat growing areas in Latin America, Africa, India, South-East Asia and China (Knight et al., 2010; Murray and Brennan, 2009; van Ginkel and Rajaram, 1997). The disease occurs virtually everywhere barley is grown, but in Australia is restricted to northern New South Wales and Queensland (Knight et al., 2010; Mathre, 1982) where the climate is warmer and more humid to enable proliferation of the pathogen (Kumar et al., 2002).

1.2.1.2 Symptoms and Glasshouse Disease Assessment

The fungus causes light to dark brown, round to oblong necrotic lesions, varying in size and degree of chlorosis. The host infection response varies depending on the virulence of the pathogen and the level of susceptibility of the host plant (Arabi and Jawhar, 2003; Duveiller and Altamirano, 2000).

Infection responses are generally rated on a scale of 1 to 9, from low to high susceptibility (Figure 1.1), according to the method of Fetch and Steffenson (1999). A rating of 1 is given to individuals with very small necrotic lesions (less than 2mm long) and no visible chlorosis, and a rating of 9 is allocated to individuals with large lesions (greater than 6mm in length) with expanding chlorosis. Other rating scales for disease assessment are also used, for example the number of lesions per leaf are counted, and an infection index is calculated, the area under disease progress curve, or AUDPC (Aggarwal et al. 2009).



1 2 3 4 5 6 7 8 9

Figure 1.1 Spot Blotch rating scale of Fetch and Steffenson (1999).

Once disease reaction has been assessed, pathotypes are designated according to the coded triplet nomenclature method of Limpert and Muller (1994). Firstly, plant genotypes are each allocated to a set of three arbitrarily ordered lines, and each genotype is designated a number according to whether it is susceptible or resistant to each isolate. If resistant, the number designated is always zero. If the first isolate of a triplet is susceptible, it is given a score of ‘1’, the second if susceptible is given a score of ‘2’, and the third if susceptible is given a score of ‘4’ (Table 1.2). The scores within each triplet are added together to give a total that is unique to one possible

combination of resistant and susceptible scores across the three lines in the triplet. For a differential set of twelve, this will produce a four digit pathotype. This enables ease of comparison between tested plant genotypes and isolates.

Table 1.2 The method of coded triplet nomenclature, as described by Limpert and Muller (1994), for the designation of pathotypes.

	Genotype A	Genotype B	Genotype C	Pathotype
Score →	**1**	**2**	**4**	
e.g.	S	S	R	3
	S	S	S	7
	R	S	R	2
	R	R	R	0

1.2.2 Common Root Rot

1.2.2.1 Occurrence and Favourable Environmental Conditions

Unlike SB, CRR is a significant and widespread disease of barley, wheat and other small grain cereals in Australia, and occurs worldwide everywhere cereals are grown (Arabi and Jawhar, 2001; Mathre, 1982). Inoculum of CRR survives in the soil in the form of mycelia and asexual conidia, which are found in the highest levels within the top 10cm of soil (Mathieson et al., 1990). Propagules can also survive on remaining stubble in between growing seasons. In Australia, CRR of wheat and barley occurs mainly in the northern and southern regions, but also occurs to a lesser extent in the west (Murray and Brennan, 2009; Murray and Brennan, 2010). The disease develops most profusely and causes the most damage to plants in higher temperatures (29^{o}C, Hill and Blunt, 1994), and water-stressed conditions (Duczek, 1989; Hill and Blunt, 1994; Ledingham et al., 1973).

1.2.2.2 Symptoms and Disease Assessment

The disease causes a brown to black discolouration of the subcrown internodes (SCIs, underground stem segments between the original seed and the crown of the plant) and roots (Arabi and Jawhar, 2001; Kokko et al., 1993; Mathre, 1982). Symptoms first appear as small brown oval necrotic lesions on the roots, lower leaf sheath and SCI. If the disease progresses sufficiently, these lesions can lengthen and coalesce until the whole SCI constricts and turns brown to nearly black.

Further effects of the disease include a reduction in the fresh weight, number of tillers, number of heads and the yield (Arabi and Jawhar, 2001; Duczek, 1989; Ledingham et al., 1973; Piening, 1973). If infection occurs early or is severe in adult plants, death may result from infection, however death due to CRR in adult plants is rare (Arabi and Jawhar, 2001; Mathre, 1982). When infection is only moderately severe, some plants may compensate for the reduction in tillers by increasing the number of grains per head and the grain size (Duczek, 1989; Piening, 1973).

The method currently used for assessing CRR reaction is visual rating of disease severity of field plants (Wildermuth, 1986). Most commonly the rating scale of Ledingham *et al.* (1973) is used, which categorises the percentage of discolouration

of the SCI, according to four levels (nil to severe). Currently the Queensland Department of Agriculture, Forestry and Fisheries (QDAFF) uses an eleven point rating scale which rates the percentage of SCI discolouration (0; 1-10; 11-20; 21-30; 31-40; 41-50; 51-60; 61-70; 71-80; 81-90; 91-100). A number of plants from each differential variety are rated, and the number of plants in each category is recorded. The rating of the level of SCI discolouration is directly related to yield losses, and hence is the preferred method (Kokko et al., 1993; Ledingham et al., 1973; Verma et al., 1976). An example of infected SCIs is shown in Figure 1.2.

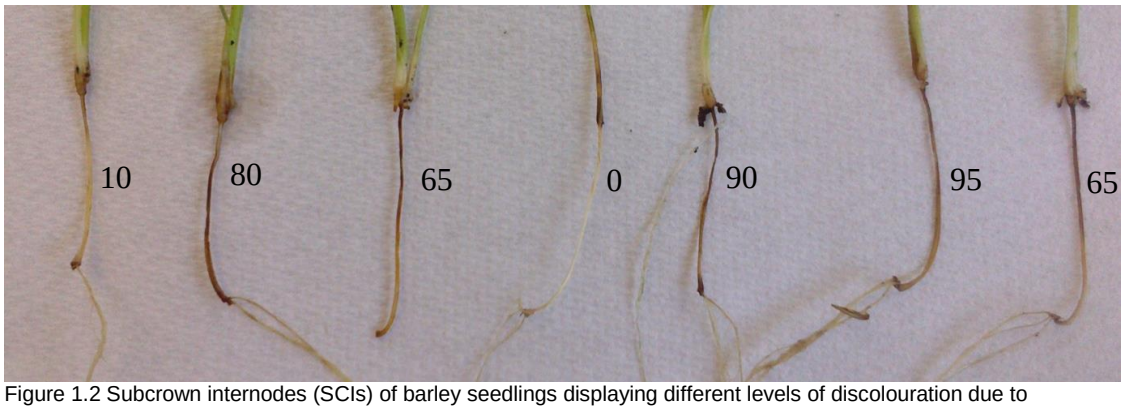


Figure 1.2 Subcrown internodes (SCIs) of barley seedlings displaying different levels of discolouration due to common root rot. The number to the right of each plant indicates the percentage of discolouration on the entire surface of the SCI tissue.

Various research groups have suggested a number of more rapid methods for testing CRR reaction (Arabi and Jawhar, 2001; Burgess and Griffin, 1968; Conner and Atkinson, 1989; Hill and Blunt, 1994; Kokko et al., 1993; Sheedy and Reen, 2010). The method proposed by Sheedy and Reen (2010) for testing specific isolates appears to be relatively rapid and least labour intensive. This group carried out a preliminary pot study in which they placed a layer of powdered inoculated grain just above the seed at planting. Disease severity ratings for this method correlated closest with the field data, as compared with inoculum evenly dispersed in the soil.

1.2.3 Black Point

1.2.3.1 Occurrence and Favourable Environmental Conditions

Black point (BP) occurs in all regions where wheat and barley are grown (Mak et al., 2006; Malaker and Mian, 2010; Monaco et al., 2004). In Australia, while BP occurs in Western Australia, South Australia, Victoria and Northern NSW, it is most severe in Queensland (March, 2008). It develops in conditions of environmental stress, such as high temperature and high relative humidity, heavy morning dews and most severely under irrigation (Maloy and Specht, 1988; Moschini et al., 2006). It occurs during the soft dough to hard dough stages of grain development (March, 2008).

Black point has historically been attributed to fungal infections, most frequently by *Alternaria alternata*, *Bipolaris sorokiniana*, and *Fusarium* spp. (Fernandez et al., 2000; Malaker and Mian, 2010; Maloy and Specht, 1988; March, 2008) and less commonly by species of the genera *Cladosporium*, *Curvularia*, *Chaetomium*, *Epicoccum* and *Stemphylium* (Malaker and Mian, 2010; Monaco et al., 2004;

Moschini et al., 2006; Williamson, 1997). However, associations of these fungi with black point are inconsistent, and they have been observed in grains not affected by black point (Maloy and Specht, 1988). Black point symptoms in wheat have also been induced *in vitro* without exposure to any fungus (Williamson, 1997).

1.2.3.2 Symptoms and Disease Assessment

Black point is a brown-black discolouration at the embryo end of the grain (Figure 1.3). Symptoms are expressed differently in wheat and barley, due to retention of the husk in barley, and a detached husk in wheat. In barley, the discolouration occurs primarily in the husk, and to a lesser extent in the germ aleurone tissue (March, 2008; March et al., 2007). In severe cases discolouration can extend along the ventral groove (Mak et al., 2006; Monaco et al., 2004) and can also result in grain shrivelling (Han et al., 2010). Discolouration of grains occurs randomly within a head so that not all grains in a single head are affected (Sulman et al., 2001a). There is some evidence that black pointed grain has a lower germination rate (Hudec 2007). Seed with black point is undesirable to the barley malting and wheat flour milling industries. When black point-affected wheat is used in milling, the discolouration can affect flour quality, give a greyish colour to bread, and undesirable black spots when milled for semolina (Lorenz, 1986; Maloy and Specht, 1988; Rees et al., 1984). Discoloured barley grains are undesirable in the barley malting industry, due to the assumption it is colonised with fungus, which can lead to beer gushing or fungal toxin release and staling of beer (Hadaway et al., 2005). If more than 5% of the grains display symptoms of black point, the load is downgraded to feed quality (Hadaway et al., 2005). Downgrading of barley due to black point is estimated to cause up to $10 million losses per year (Walker, 2012).

Figure 1.3 Black pointed barley seeds with husk, dorsal view (Photo: Maree Horne).

In a number of studies investigating black point of wheat or barley, various methods have been used to assess the severity of the defect (Conner and Davidson, 1988; Ellis et al., 1996; Fernandez et al., 2000; Lorenz, 1986; Moschini et al., 2006; Rees et al., 1984). These methods either assess the percent discolouration of individual seeds, or the percentage of seeds with discolouration in a sample. Grain sold for milling or malting in Australia is assessed on the percentage of discoloured seeds in a sample.

In Queensland, Western Australia and New South Wales, grain colour (or lightness) is measured using a colour meter (Fox et al., 2001).

1.3 Infection mechanisms and disease cycle

Fungal plant pathogens can be classified according to their modes of invasion and acquisition of nutrients. Biotrophs require a living host in order to survive and continue their life cycle. These pathogens usually obtain nutrients by means of haustoria: a determinate structure formed by the fungus inside host cells for the purpose of nutrient uptake (Hammerschmidt, 2006). Necrotrophs on the other hand kill host tissues by means of enzymes and/or toxins to achieve nutrient uptake. Hemibiotrophs combine the two strategies and begin with a relatively short biotrophic phase, followed by a necrotrophic phase. *Bipolaris sorokiniana* is a hemibiotroph, and is known to produce a number of toxins (discussed in detail in the 'Mycotoxin' section).

There are several steps in the infection of a host by *B. sorokiniana* in both leaf and root tissue. The first step is adhesion, which occurs within the first hour after hydration of the conidia (Apoga et al., 2001). An extracelluar matrix (ECM) surrounds the conidia and germlings, and enables the fungus to adhere strongly to the surface of the host plant after the initial adhesion. The ECM is also believed to form an ideal microenvironment for the fungus on the surface of the host (Apoga and Jansson, 2000). Production of the ECM is accompanied by germination of the conidium, which occurs between 4 and 6 hours after inoculation (Han et al., 2010). The fungus then grows and branches on the surface, and invades the cell by forming a specialised structure called an appressorium, which is the swelling of a hyphal tip pressed firmly against the tissue surface. The appressorium enables invasion by forming a penetration peg, an outgrowth at the base of the appressorium that forces through the cuticle and into the epidermal and parenchyma cells in leaf tissue, and the outer and inner cortex of root tissue (Figure 1.4). A study by Hammerschmidt (2006) found that appressoria rarely formed over leaf stomata, but primarily formed over the joints of cell walls of epidermal cells. Invasion of the tissue can occur intercellularly as well as intracellularly (Carlson et al., 1991b; Han et al., 2010).

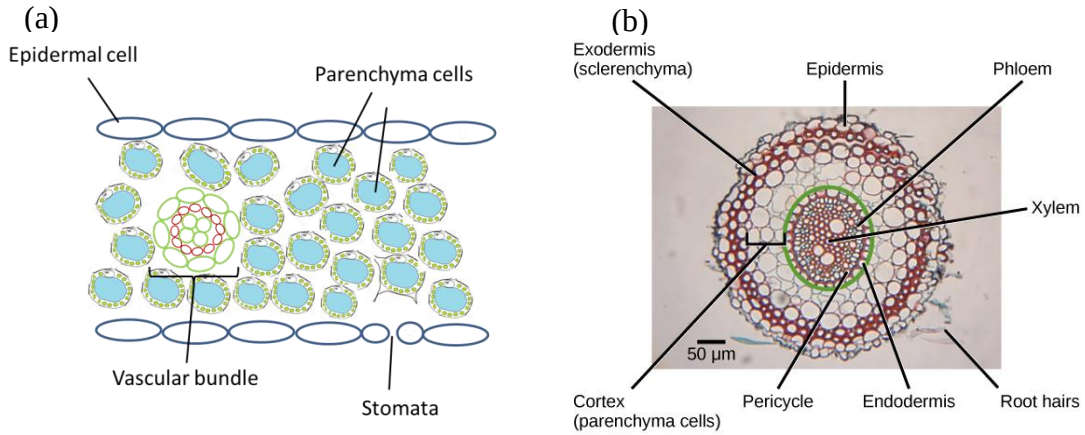


Figure 1.4 (a) Diagram of a leaf cross-section (picture by Maree Horne), and (b) Image of a stained wheat root cross-section under a light microscope (image from: http://cnx.org/content/m47405/latest/?collection=col11569/latest).

1.4 Toxins produced by *B. sorokiniana*

The production of mycotoxins (toxic secondary metabolites) enables easier invasion of the cells by prevention of the plant's natural defences. They interfere with cellular membranes, organelles and enzymes of host plants (Apoga et al., 2002). Phytotoxins (secondary metabolites toxic to plants) isolated from *B. sorokiniana* include prehelminthosporol, prehelminthosporolactone, helminthosporol, sterigmatocystin, averufin, versiconol, versiconol acetate and sorokinianin (Barnes et al., 1994; Cutler et al., 1982; Maes and Steyn, 1984; Marder et al., 2006; Nakajima et al., 1994).

Prehelminthosporol is the most active and abundant phytotoxin produced by *B. sorokiniana* (Apoga et al., 2002; Carlson et al., 1991a). It inhibits growth of wheat coleoptiles at high concentrations (Cutler et al., 1982). At the molecular level, the toxin interferes with proteins of the plasma membrane involved with nutrient and ion uptake (H^+-ATPase and Ca_2^+-ATPase) and protection of the cell against physical stress (1,3-β-glucan synthase) (Olbe et al., 1995).

1.5 Morphology

1.5.1 Asexual Stage

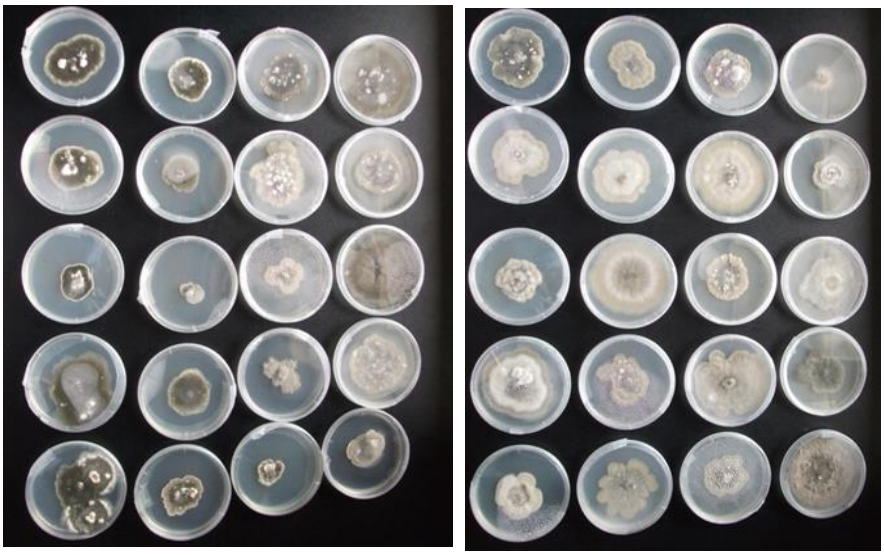


Figure 1.5 Single spore *B. sorokiniana* isolates sampled from different hosts and tissues and grown on potato dextrose agar for 14 days.

In culture, *B. sorokiniana* varies in morphology, from dark olive-brown suppressed mycelial growth to white, fluffy abundant growth (Figure 1.5) (Jaiswal et al., 2007). The anamorph, is the only stage observed under natural conditions (Kumar et al., 2002). Simple conidiophores (hyphae on which asexual spores, or conidia, are formed) are produced in fresh culture, and may be single or clustered. Conidiophores measure 6–10 x 110–220 µm with 6–11 septations (Mathre, 1982; Wu et al., 2011). The conidia, or asexual spores (Figure 1.6), are olive-brown and elliptical with 5 to 9 cells and measure 15–28 x 40–120 µm (Kumar et al., 2002). They arise from pores beneath each conidiophore septum and exhibit bipolar germination, or development of hyphae from each end of the spore.

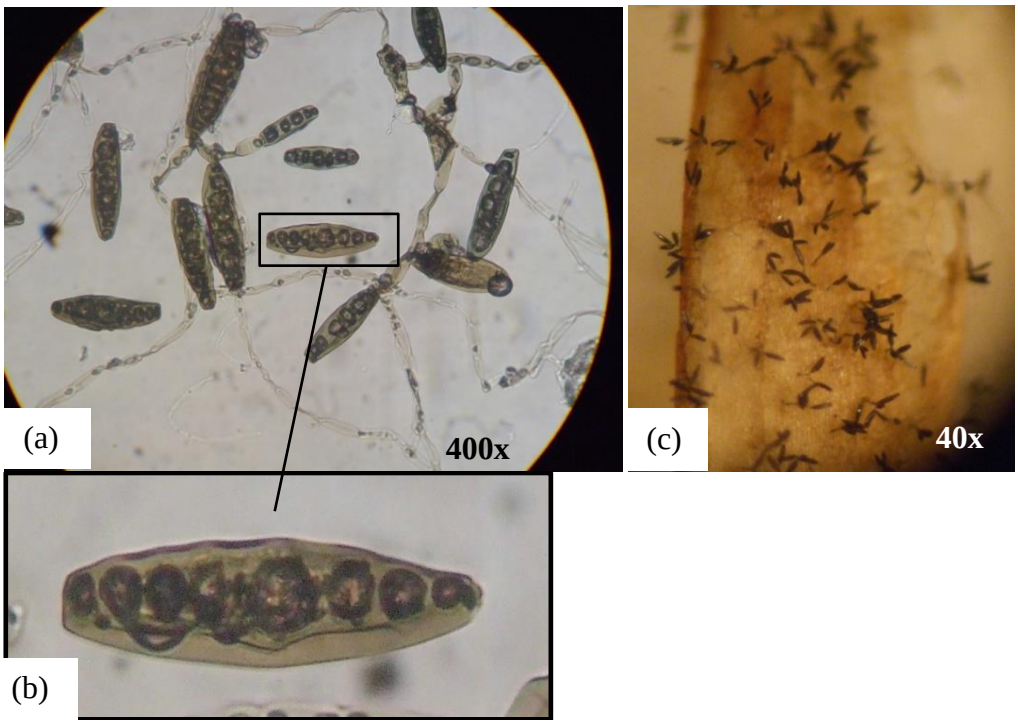


Figure 1.6 (a) Conidiophores of *B. sorokiniana*, (b) higher magnification of a single conidium, and (c) conidia on the husk of a barley seed (Photos: Maree Horne).

1.5.1.1 Parasexual Recombination

During normal fungal growth, separate hyphae belonging to the same individual undergo constant fusions (known as anastomosis) with adjacent hyphae (Glass et al., 2000), and thus often single hyphal cells contain multiple nuclei. This is the case with *B. sorokiniana*. Chand *et al*. (2003) found up to 6 nuclei within a single cell of the fungus in culture, with 3 or 4 nuclei occurring most frequently. Two genetically distinct hyphae may also fuse in this manner to form a heterokaryon (or a cell containing distinct nuclei). As a result, the asexual stage of fungal growth is not restricted merely to clonal propagation; recombination can occur by means of parasexual recombination, also known as somatic hybridisation. During this process, nuclear and cytoplasmic material is exchanged between two anastomosing hyphae (Burdon and Silk, 1997). This may then be followed by nuclear fusion and chromosomal recombination. However, the process of parasexual recombination has been found to be quite complex due to its control by *Het* genes, which prevent heterokaryon formation between two genetically distinct individuals. When the individuals differ at one or more of the *Het* loci, the result is vegetative incompatibility, and the hyphae are aborted (Glass et al., 2000). Alternatively, distinct hyphae may grow away from each other so that direct contact does not occur at all. Somatic hybridisation is thought to be rare in nature, and an *in vitro* study of the vegetative incompatibility of 35 Brazilian isolates of *B.sorokiniana* (Poloni et al., 2009) found that 18 out of 31 combinations were incompatible.

1.5.2 Sexual Stage and Mating Types

The sexual stage (or teleomorph, named *Cochliobolus sativus*) is very rarely observed in nature, but can be induced in culture (Zhong and Steffenson, 2001a). The pseudothecium, or fruiting body of the sexual stage, appears as a globose structure 300–400 μm in diameter with a pore opening to allow release of the sexual spores (or ascospores). The asci, which bear the ascospores, are clavate (club-shaped) and measure 20–35 x 120–250 μm. Ascospores are hyaline (translucent), filamentous, arranged spirally within the asci, and are 4–10 septate and 200–250 μm in length (Mathre, 1982).

Like the parasexual cycle of *B. sorokiniana*, the sexual stage of the pathogen is controlled by specific genes, which are known as mating-type (MAT) genes. There are two mating types: MAT-1 and MAT-2, designated A and a, which can be induced in the laboratory (Zhong and Steffenson, 2001a). A sexual cross must be between opposite mating types of *B. sorokiniana*.

1.6 Genetic Analysis of *B. sorokiniana*

1.6.1 Genome Sequencing

Several genomic regions have been sequenced in various isolates of *B. sorokiniana* by different research groups. For example, the region coding internal transcribed spacers (ITS) 1 and 2, the 5.8S and 2.8s subunits of ribosomal RNA and the glyceraldehyde-3-phosphate dehydrogenase (gpd) regions was sequenced in 93 isolates by Gurung *et al.* (2013). The Brn1 reductase gene (involved in melanin biosynthesis) was sequenced in four isolates of *B. sorokiniana* by Shimizu *et al.* (1998). The polyketide synthase (PKS1) gene has also been sequenced in a number of isolates, and these sequences are available on Genbank (Accession numbers KF769134 to KF769141). The whole genome and transcriptome of the North Dakota isolate ND90Pr has been sequenced at the North Dakota State University (led by Shaobin Zhong) as a part of the Fungal Genomics Program of the US Department of Energy Joint Genome Project, and the full sequence is now publicly available. The strain ND93-1 has also been fully sequenced by Condon *et al.* (2013). Previous research on *B. sorokiniana* by Zhong and Steffenson (2002) found 15 chromosome-sized DNA segments using contour-clamped homogeneous electric field (CHEF) electrophoresis. Several genes of *B. sorokiniana* have been fully or partially sequenced by a number of research groups worldwide. Sequence data for these regions is publicly available via Genbank.

1.6.2 Genetic Diversity

The genetic diversity of a fungal pathogen is frequently investigated in order to determine the most appropriate strategies for control, the likelihood of resistance breakdown and to understand historic and prospective gene flow across regions. Historically, population genetic studies have utilized RAPD or AFLP markers to compare significant differences or similarities between individuals and groups of individuals (Arabi and Jawhar, 2007b; Ghazvini and Tekauz, 2012; Knight et al., 2010; Leisova-Svobodova et al., 2011). Markers are often scored as presence or absence of bands, and comparisons are made using computer programmes such as

NTSYS, Arlequin, CLUMPP, Winboot and GenAlex. These programmes compare individuals diagrammatically and/or statistically.

Genetic diversity studies of *B. sorokiniana* populations have been undertaken in various countries, including Australia, Brazil, Canada, Czech Republic, USA, Pakistan, Poland, India and Syria (Aggarwal et al., 2010). These studies vary in the number of isolates surveyed, the molecular marker method used and the number of bands scored.

To date, very little research has investigated the genetic diversity among leaf and root isolates, and barley and wheat isolates. Many studies have compared isolates from leaves only, or leaves and kernels of either barley or wheat. Of the five studies that included isolates from roots, three contained six or fewer root isolates (Arabi and Jawhar, 2007b; Baturo, 2005; Ghazvini and Tekauz, 2012; Zhong and Steffenson, 2001b). Of the studies including wheat and barley isolates, only Knight *et al.* (2010) included a significant number of wheat isolates from a single tissue and statistically compared these to barley isolates from the same tissue type. A high degree of similarity among isolates was observed in four of the studies, which scored both polymorphic and monomorphic bands (Knight *et al.*, 2010; Leisova-Svobodova *et al.*, 2011; Müller *et al.*, 2005; Zhong and Steffenson, 2001b). Low similarity among isolates was observed in two other studies (Baturo, 2005; Jaiswal et al., 2007) where both monomorphic and polymorphic bands were scored, however these two studies used a less reliable molecular marker method and scored a minimal number of bands.

The most significant studies influencing the current population study include the papers of Knight *et al.* (2010), Leisova-Svobodova *et al.* (2011), de Oliveira *et al.* (2002), and a preliminary study (Knight et al., 2010) aimed chiefly to investigate the relationship between isolates sampled from SB and CRR infections of wheat and barley hosts. Isolates from the two infection types were found to cluster separately, and additionally, isolates formed distinct subgroups according to host of isolation. Since only the CRR isolates were sampled from both wheat and barley hosts, this raised the question of whether this host specificity exists among SB isolates.

From these previous investigations, it is clear that genetic diversity does exist among isolates in asexually reproducing populations of *B. sorokiniana*. During the course of the current study, a paper was published by a Czech group, Leisova-Svobodova *et al.* (2011) with observations of higher than expected levels of genetic variability among *B. sorokiniana* isolates. Genotyping of a collection of isolates from the field in different regions in the Czech Republic was undertaken, some of which were isolated from single leaves and single lesions. Amplified fragment length polymorphism (AFLP) genotyping, described in section 1.9.1, revealed genetic differences even among isolates from within the same lesion. It was suggested that since no single spore isolate from the same lesion was genetically identical to another, these differences could not be solely attributed to multiple infection sites on single lesions. The authors proposed the possibility of genetic recombination occurring on alternate host species between cropping seasons, and also of highly variable chromosomal rearrangements in *B. sorokiniana*, as established by Zhong and Steffenson (2007) and Zhong *et al.* (2002). The possibility of somatic crossing-over was also suggested, however, the author recognises that this phenomenon is rather rare.

de Oliveira *et al.*(2002) investigated somatic recombination of *B. sorokiniana* over two generations on different wheat genotypes. They used RAPD markers to determine the genotype and found a high level of variability was present in isolates from the original parental source and the host genotype.
Much like the Czech study, a high level of variability was present for isolates from the same original parental source and host genotype. However, only single isolates were taken from each host genotype, and it is unclear whether control isolates were included in the study to rule out variability between RAPD tests

Studies of the population diversity of *B. sorokiniana* can provide information essential for the effective and efficient control of its disease states. If isolates of the pathogen are non-specialised and are equally virulent in root and leaf tissue, both forms of the disease could be controlled somewhat effectively with the same control methods. However if distinct, the root and SB diseases will require control measures such as deployment of resistant cultivars, that are specific to each form of the disease.

1.7 Current control methods

One major limitation on the yields of both wheat and barley is fungal disease, which is most effectively controlled through breeding of genetically resistant crops. Further recommendations for the control of SB and CRR include stubble removal, crop rotation, seed treatment, biological control and the use of partially resistant varieties (Acharya et al., 2011; Bovill et al., 2010; Knudsen et al., 1995; Reis and Abrao, 1983; Steffenson, 1996; Wildermuth and McNamara, 1991), although many commercial varieties are susceptible to the disease (Queensland Department of Agriculture, Forestries and Fisheries 2013). The barley varieties Morex and Tolar possess differing resistances to the disease, and Morex has been used in crosses for mapping studies investigating the genomic regions associated with resistance (Bilgic et al., 2005). Additionally, SB may be controlled by use of triazole foliar fungicides, with the active ingredients such as propiconazole, tebuconazole and epoxyconazole (Acharya et al., 2011; Couture and Sutton, 1978; Duveiller et al., 2005; Sharma and Duveiller, 2007).

Stubble burning and removal have been commonly employed to reduce fungal propagules in the soil (Chan and Heenan, 2005; Valzano et al., 1997). However these methods are not advised as they cause soil degradation, a reduction in available nitrogen and carbon in the soil, and changes in the soil microbial populations, including nitrogen fixing bacteria (Chan and Heenan, 2005; Kirkby and Broad, 2006; Martin-Rueda et al., 2007; Reis and Abrao, 1983). A more effective and environmentally sustainable method of control is crop rotation. This method involves alternation of the primary crop (i.e. wheat or barley) with crops that are not susceptible to CRR and SB, which reduces pathogen load in soil, and hence disease severity in the following season (Wildermuth and McNamara, 1991; Wilson and Hamblin, 1990).

The most effective and economical means of control is by the use of adapted, genetically resistant cultivars, if they are available. These reduce the need for

expensive application of fungicides and other control methods that are less sustainable. Current research in this area is discussed in the following sections.

1.8 Resistance to the disease states of *B. sorokiniana* in wheat and barley

1.8.1 Barley and Wheat Genomes

Research into genomic regions associated with resistance to the various disease states of *B. sorokiniana* has been conducted for both wheat and barley. In order to understand the locations of these regions, the structure of the barley and wheat genomes must be understood.

The barley genome is diploid (2n = 2x = 14) and is approximately 5×10^9 bp in size (Kleinhofs and Han, 2002). It is fully sequenced, which in the past has proven difficult due to its high level of repetitive DNA, approximately 84% (The International Barley Genome Sequencing Consortium, 2012). The wheat genome, on the other hand, is hexaploid (2n = 6x = 42) and is approximately 16×10^9 bp in size (Arumuganathan and Earle, 1991). This genome is currently being sequenced, and 94 000 to 96 000 genes have been identified (Brenchley et al., 2012). This genome also contains a high level of repetitive DNA sequences of more than 80% (Stein et al., 2000). The three genomes of wheat are designated 'A', 'B' and 'D' and that of barley is designated 'H'(Gill and Friebe, 2009). Comparative mapping of the genomes of both species has shown homology of barley chromosomes 1H to 7H with the wheat chromosomes 7, 2, 3, 4, 1, 6 and 5, respectively (Kleinhofs and Han, 2002).

1.8.2 Genetic Disease Resistance

Disease resistance of plants was found to have high heritability in many cases (Young, 1996). This resistance expressed in plants may be divided into two main categories: qualitative and quantitative resistance. Qualitative resistance is controlled by one or a few genes, and individuals expressing this can be classified into distinct groups, e.g. fully resistant or fully susceptible (Poehlman, 1987). This kind of resistance, although complete, can easily and quickly be overcome by a pathogen population if there is enough selective pressure (Sakr et al., 2011). For example, the North Dakotan two-row barley cultivar Bowman, which was shown to have a single resistance gene effective against *B. sorokiniana* (Valjavec-Gratian and Steffenson, 1997a), was released as a moderately resistant cultivar to SB in 1984. However by 1990, Bowman had become highly susceptible (Valjavec-Gratian and Steffenson, 1997b). Thus it is more desirable for a plant to possess resistance controlled by multiple, independently acting genes. This kind of resistance is quantitative, and cannot be categorised into distinct groups. That is, there is a continuous distribution of phenotypes (from susceptible to resistant) expressed in a population with the trait (Collard et al., 2005). Genomic regions associated with quantitative traits are known as quantitative trait loci (QTL). Locating QTL associated with disease resistance is useful for the incorporation of genes from various sources into single varieties, to obtain more durable resistance.

Some novel mechanisms of resistance to *B. sorokiniana* have been identified in barley and wheat. Persson *et al.* (2009) investigated these mechanisms in barley by inoculating the *B. sorokiniana*-tolerant barley mutant *bst1* with an isolate of the pathogen as well as wild-type barley. The tolerance gene mutation is localised on chromosome 5HL. The group found results suggesting defense against the pathogen relies on pathogenesis related genes as well as some reactive oxygen species. A microscopy study of wheat infection by *B. sorokiniana* by Ibeagha *et al.* (2004) revealed that penetration of the wheat epidermis was prevented by cell wall apposition or by a hypersensitive reaction (involving reactive oxygen species) of single cells. Programmed cell death acted as a post-penetration defense mechanism.

1.9 Mapping QTL

The process of locating QTL associated with a particular trait is known as QTL mapping, and this involves four steps: (a.) production of an appropriate mapping population (b.) construction of a linkage map by use of molecular markers, (c.) phenotyping of the population for the trait of interest, and (d.) linkage analysis.

1.9.1 Mapping Populations

There are a number of populations useful in linkage analysis and QTL mapping of self-pollinating plants (such as wheat and barley). Primary methods used in plant mapping are F2, backcross (BC), recombinant inbred lines (RILs) and doubled haploid (DH) populations (Collard et al., 2005). F2 populations are produced by first making a cross of two homozygous parents that differ in particular traits of interest. The first generation of offspring is the F1, and these individuals should have highly similar phenotypes for the traits of interest if the parent lines are truly homozygous (Meksem and Kahl, 2005). To produce a segregating population (a population with a range of phenotypes) an F2 population is produced by selfing individual F1 plants. BC populations (although not usually used for mapping purposes in wheat) are produced by crossing the F1, or subsequent generations, back to one of the parent lines. A minimum of 200 individual lines is required for efficient QTL mapping (Meksem and Kahl, 2005). Neither F2 nor BC populations are homozygous, but have the advantage of being produced relatively quickly.

Examples of completely homozygous populations are RILs and DH lines. RILs can be produced by firstly selfing F2 individuals and then individuals of the subsequently produced generations usually to at least F6, which produces individuals homozygous at almost all loci with genomes made up of a mosaic of the parental genomes (Broman, 2005). DH lines in barley are most commonly produced by first generating haploid plants through tissue culture of microspore cells, usually anthers (Luckett and Smithard, 1992), while in the case of wheat, haploid embryos from wheat × maize crosses are used (Kammholz et al., 1996). Diploid plants are produced by treatment with colchicine, which prevents cell spindle formation during mitosis and lead to a doubling of the chromosome number in derived cells. DH lines are fully homozygous and enable more efficient breeding, and mapping. Association mapping makes use of natural diversity present over multiple generations by analysing populations made up of more distantly related individuals, or accessions (Collins, 2007; Deschamps *et al.*, 2012; Hall *et al.*, 2010). These populations can provide a higher resolution map than linkage mapping populations for mapping traits

of interest. Nested association mapping (NAM) combines the benefits of linkage analysis and association mapping by first selecting diverse parents and developing a large collection of related mapping progenies, which may be RILs, DH lines or other inbred lines (Deschamps et al. 2012, Yu et al. 2008). Once an appropriate mapping population has been produced a linkage map must be constructed, which is completed using molecular markers.

1.9.2 Molecular Markers

A number of molecular marker methods are available for generating linkage maps. These can be grouped into three classes: hybridisation-based, PCR-based or DNA-sequence-based (Collard et al., 2005). The most reliable polymorphic marker is restriction fragment length polymorphic (RFLP) DNA, which is hybridisation-based (Mohan et al., 1997). This method involves digestion of DNA with restriction endonucleases, which cleave at specific sequences. Specifically designed probes are used to narrow down the number of visualised bands. Although this is a reliable method, it requires a great deal of labour and high concentrations of DNA. The PCR-based marker type overcomes these issues.

Three examples of PCR-based markers are random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs) also known as microsatellites, and amplified fragment length polymorphisms (AFLPs). The microsatellite marker is based around simple repetitive DNA sequences. Repeat units consist of 1 to 5 bases, and microsatellites range in their number of repeats, hence the length in different individuals also varies (Saghai-Maroof et al., 1994). These regions can occur due to replication slippage or uneven crossing over during meiosis (Jarne and Lagoda, 1996; Morgante et al., 2002). Flanking primers specific to individual microsatellites are designed for amplification of the microsatellite. This marker type is codominant, and is relatively reliable among different plant species (McCouch et al., 1997).

The AFLP marker type, developed by Vos *et al.* (1995), is a combination of RFLP and PCR-based markers. The process begins like RFLPs, with digestion of DNA with restriction endonucleases, producing a large number of small fragments (Figure 1.7). The number of visualised fragments is reduced by ligating DNA adapters (of known sequence) to each end of the fragments. Primers complementing the adapters, with one selective base to complement the end base of some original fragments are used in PCR. A second round of selective amplification is performed with two selective bases at each end. These primers are fluorescently labelled, enabling only visualisation of selected fragments during polyacrylamide electrophoresis. This method enables a larger number of fragments to be analysed than the RFLP method, and is highly reproducible (Leisova-Svobodova et al., 2011).

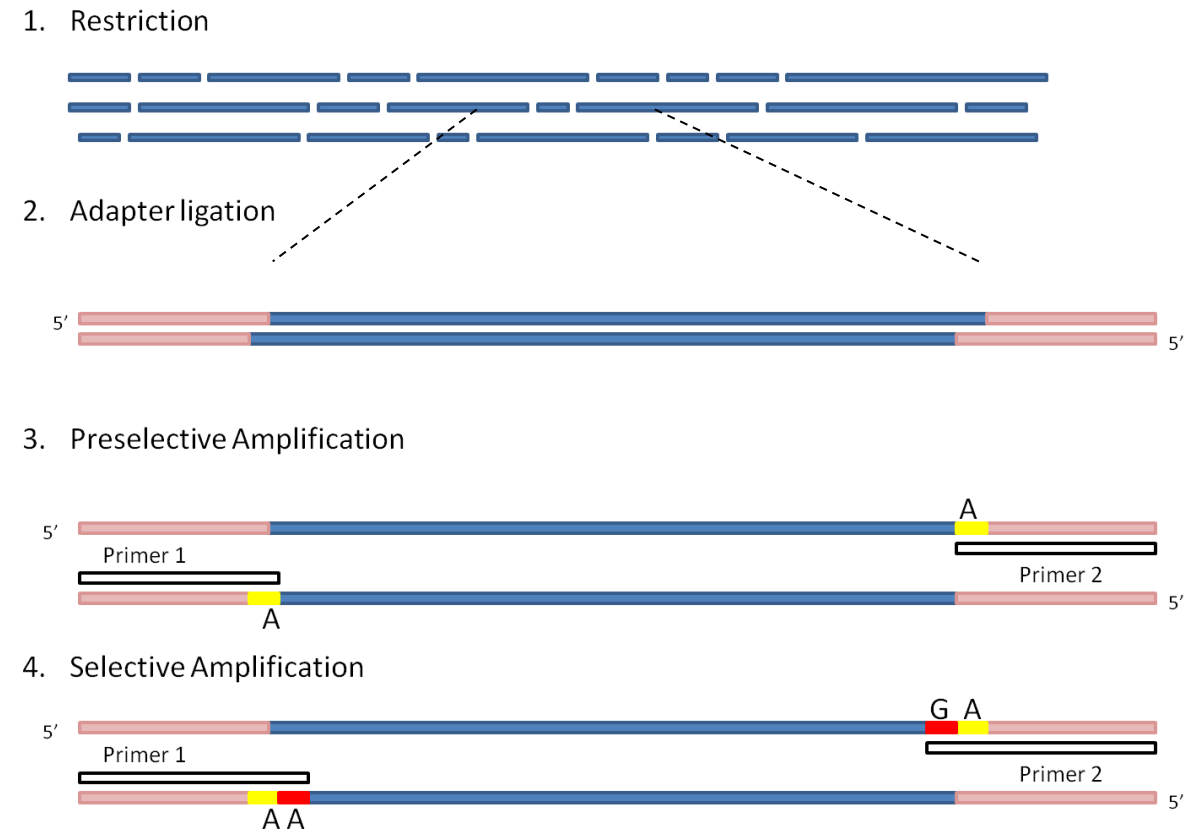


Figure 1.7 The four steps of amplified fragment length polymorphism (AFLP) molecular marker method (picture by Maree Horne).

RAPD markers are based on primers of short random sequence, which amplify multiple regions in the genome (Hadrys et al., 1992). The primary advantages of this method are that no prior knowledge of the genomic sequence is necessary, and only nanogram amounts of total genomic DNA are required for its use. However, reproducible results are not always obtainable between laboratories when using this method (Penner et al., 1993).

Some examples of DNA sequence-based markers are single nucleotide polymorphisms (SNPs) and expressed sequence tags (ESTs). SNPs are based on single base changes in a sequence and can be linked to traits of interest, particularly if found in coding regions (Liao and Lee, 2010). Expressed sequence tags are based on DNA transcripts which have been randomly isolated from tissue, converted to cDNA (complementary DNA) and the 3' and/or 5' end translated (Alba *et al.* 2004). This method targets only expressed regions.

In the genotyping of a population, markers are run across all individuals in the population as well as the parents, and polymorphic markers (that is, markers which differ between the parents) are scored. Individuals are allocated to groups depending on which parental genotype is matched (e.g. 'a' for Parent A and 'b' for Parent B genotypes). Scores are used to produce a linkage map. The theory behind map construction is that two markers in close proximity to each other on a chromosome are less likely to be involved in a chromosomal recombination (or crossover) event (Collard et al., 2005). Markers that have a high number of crossovers between them

are mapped further apart, and those with rare crossover events are mapped closer together. These markers act as an ordered map for locating positions of genes along the chromosomes associated with specific traits of interest.

1.9.3 Phenotypic testing

The method commonly used for SB testing is that described by Fetch and Steffenson (1999), explained in the 'Spot Blotch' section, and the method currently used for assessing CRR reaction is the QDAFF method described likewise in section 4.2. Other far less frequently used methods for phenotyping in SB trials, include estimation of lesion density and area under disease progress curve (AUDPC). Estimation of lesion density, described by Duveiller and Garcia Altamirano (2000) involves counting the number of lesions on a 7cm length of leaf tissue (third leaf), starting 3cm from the ligule, and an average leaf area is determined for the third leaf to calculate density. AUDPC is a more involved approach, where the disease severity is scored on a scale three times at 8 day intervals, and the progress of the disease over time is calculated using a formula (Bashyal *et al.*, 2010). Requirements for phenotypic data collection include at least partial replication for multiple isolates in multiple environments and across different growth stages. Once disease reaction has been assessed, the collected phenotypic data is used in the locating of QTL, after the construction of a linkage map.

1.9.3 Linkage Mapping

Linkage maps show the position of markers along a chromosome, and their genetic distances; (that is, the likelihood of crossover events occurring between any two markers). Linkage maps are also useful for finding positions of QTL, and markers closely linked to them (Collard et al., 2005). For small linkage maps including only a few markers, linkage mapping may be completed manually by performing chi-square tests; however, this is impossible with large numbers of markers (Young, 1996). Appropriate computer programmes are used for this purpose, including Mapmaker (Lander et al., 1987) and Mapmanager QTX (Young, 1996). Constructed linkage maps are then used in QTL mapping, which makes use of the phenotypic and genotypic data. The association between marker and trait is calculated by odds ratios (association vs no association), and this is expressed as a logarithm, known as the logarithm of odds (LOD) score. A LOD value of three indicates linkage is 1000 (10^3) times more likely than no linkage (Collard et al., 2005). In the analysis of marker-trait associations, a LOD score of three is commonly used as the threshold value above which the connection between markers and traits is accepted.

1.10 Known QTL Associated with Resistance

Limited research has been conducted in identifying QTL associated with resistance to CRR and black point. To date, three studies have identified QTL associated with black point resistance (Lehmensiek et al., 2004; March, 2008; Tah et al., 2010), and one has identified QTL associated with CRR resistance in barley (Lehmensiek et al., 2010). Two early studies identified CRR resistance in the wheat variety Apex, but only identified the chromosomes on which the resistance was located (Larson and Atkinson, 1970; Larson and Atkinson, 1981).

More comprehensive studies have investigated SB resistance QTL of barley. Two studies have each investigated QTL of 4 individual populations (Bilgic et al., 2005; Bovill et al., 2010). Bilgic *et al.* (2005) identified a significant seedling resistance (SLR) QTL located on the short arm of chromosome 7H, which was detected in all four of the populations investigated. A significant QTL associated with adult plant resistance (APR) was identified in the same region, on the short arm of chromosome 7H. SLR and APR QTL were also identified on the long arm of chromosome 3H, and were both detected in two of the four populations. Another APR QTL was also detected in two of the four populations on the short arm of chromosome 3H. Similar results were found in the study by Bovill *et al.* (2010). A significant QTL for APR was detected in two of the four populations on the short arm of chromosome 7H, and a significant SLR QTL was detected in all four populations in the same region on 7H. A second significant QTL for APR was detected on the short arm of chromosome 3H in all four populations examined. The APR and SLR resistances on chromosome 3HL detected in the Bilgic *et al.* (2005) study were not detected in this study.

1.11 Mapping Methods

A number of methods for QTL mapping have been developed, varying in their complexity and accuracy. The simplest method is called single marker analysis, and uses analysis of variance (ANOVA) to calculate significant differences between phenotypes among the genotypic classes of single markers (Coffman et al., 2003). No linkage map is required for this method. Two more complex methods make use of linkage maps and analyse marker intervals. These methods are simple interval mapping (SIM, Figure 1.8) (Lander and Botstein, 1989) and composite interval mapping (CIM) (Jansen, 1993). The less complex of these is SIM, which compensates for recombination between the marker and QTL by including two markers in the analysis (Collard et al., 2005). QTL locations are estimated by calculating LOD scores (one by one) for a number of intervals to determine the most likely position. LOD scores are often converted to likelihood ratio statistics (LRS), which is the ratio of two probabilities occurring in the same event under different hypotheses. It can take any value between zero and infinity, and may be calculated as the LOD score multiplied by 4.6. Results are presented as a graph, and the LRS (or LOD score) must exceed a predetermined threshold in order to be considered significant. The method of CIM combines both SIM and single marker analysis to consider the epistatic effects of QTL located elsewhere. Results are presented in a similar manner to SIM, and LOD scores must exceed a threshold to be considered significant (Jansen, 1993)

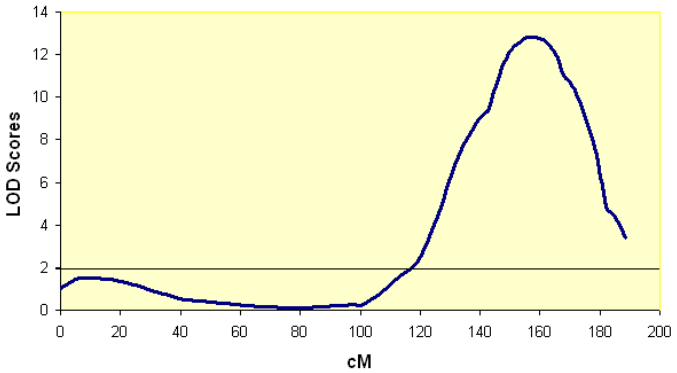


Figure 1.8 Example of simple interval mapping LOD curve with a LOD threshold of 2. The x-axis represents the distance or position along a the section of chromosome analysed, and in this case is given in centimorgans. The position at which the peak of the graph occurs is the most likely position of the QTL (Plant and Soil Sciences eLibrary, http://plantandsoil.unl.edu/croptechnology2005/pages/index.jsp?what=topicsD&information_ModuleId =1067442598&topicOrder=7&max=11&min=0&).

1.12 Fine Mapping

Fine mapping is the identification and placement of molecular markers within closer proximity to a gene of interest (Bennetzen, 1999), once the initial round of marker analysis is completed to identify chromosome regions occupied by a gene of interest. In ordinary biparental mapping populations, this can be problematic due to a lack of crossover events within the population, and therefore linkage disequilibrium (LD). LD, also referred to as gametic phase disequilibrium and allelic association, can be described as the non-random association between two or more alleles. This process occurs where certain allele combinations on a single chromosome are more likely than other allele combinations (Collins, 2007). Autogamous (inbreeding) populations have a higher level of LD than allogamous (outcrossing) populations, therefore decay of LD (eventually leading to linkage equilibrium) can be achieved by making wide crosses with distant relatives or related species. Numerous mapping populations, collections of cultivars, lines or landraces are used for identifying marker-trait associations for fine mapping.

Some examples of these populations are large F2 populations (described earlier), recombinant inbred lines (RILs), near isogenic lines (NILs) and multi-parent advanced generation intercross populations (MAGIC). Recombinant inbred lines are derived from F_2 populations, where single seeds are selected and self-pollinated over several generations, producing genetically stable lines. Near isogenic lines are identical at all loci, except in specific regions of interest. They are produced by first crossing a line or variety possessing the phenotype of interest with a standard line. The F1 is then selfed to produce the F2 population, and individuals with the trait of interest are selected and crossed repeatedly with the standard line (recurrent parent) over several generations to produce the RILs. MAGIC lines incorporate a number of genetically diverse accessions by randomly intercrossing them (Kover et al., 2009). This produces a population with greater linkage equilibrium, and the method takes advantage of the effects of multiple past recombination events over many generations.

Molecular marker methods based on effects with greater frequency in the genome are also utilized in fine mapping. These methods include high resolution melting (HRM), single nucleotide polymorphisms (SNPs), Amplified Fragment Length

Polymorphisms (AFLPs) and markers derived from syntenic relatives (that is, a close or distant relative with sections of markers within the genome that have a conserved order). Each of these methods enables denser placement of molecular markers by demonstrating a greater degree of polymorphism among individuals of a population. HRM detects small differences in the melting curves of PCR products due to single base differences (Lehmensiek et al., 2009), while syntenic relatives can be used to identify potentially polymorphic markers for regions of interest (Bennetzen, 1999; Mammadov et al., 2005). NILs use recombinant backcrosses to introduce traits of interest from a wild relative to a commercial cultivar. The lines are almost identical to the recombinant parent in all genetic regions except in the introduced region, which is often highly polymorphic (Young, 1996).

1.12.1 Next-Generation Sequencing

A more recent approach being utilised in fine mapping and identification of candidate genes is next-generation sequencing. This is a broad term used to describe a number of methods developed for the rapid sequencing of whole genomes. The earlier method of dideoxy sequencing developed by Sanger and Coulson (1975), also referred to as Sanger sequencing, has been largely replaced by next-generation sequencing methods, as they are cheaper and more rapid (Morozova and Marra, 2008; Schuster, 2008; Solieri and Dakal, 2012). The Sanger sequencing method is based upon synthesis of a complementary DNA strand in a reaction supplied with the natural deoxynucleotides (dNTPs), and with dideoxynucleotides (ddNTPs) that act as terminators. Each of the four ddNTPs in the synthesis reaction are labelled with different coloured fluorescent dyes, and the reaction produces different fragment lengths of the same original sequence with a fluorescently labelled terminator base (Schuster, 2008). Fragments can be resolved in capillary electrophoresis and the DNA sequence is identified due to the fluorescent labelling. Three widely-used platforms for next-generation sequencing are the Roche/454 FLX system, the Illumina/Solexa Genome Analyser and the Applied Biosystems SOLiD System (Liu et al., 2012; Mardis, 2008; Morozova and Marra, 2008).

The most useful method in fine mapping is the genotyping-by-sequencing (GBS) approach, which enables marker discovery and genotyping in a single step. This method is relatively straightforward in organisms with small genomes, but requires reduction in complexity in larger genomes such as barley (Elshire *et al.*, 2011). GBS usually utilises restriction endonucleases coupled with barcoded adapters to target a small and specific region of the genome, and multiplex libraries are produced for next-generation sequencing (Poland and Rife, 2012). The sequence data is then used in SNP detection, and software such as MAQ, SAMtools, GATK,SNIP-Seq and MapNext are used for this purpose to identify polymorphisms (Deschamps *et al.*, 2012).

1.12.1.1 Roche/454 FLX

In this process, DNA is randomly fragmented, 454-specific adapters are ligated to the ends, fragments are denatured into single strands and attached to agarose amplification beads. These beads have oligonucleotides attached which are complimentary in sequence to the adapters, allowing each bead to be associated with

a single fragment. Beads are separated into individual micelles and individual fragments are amplified in PCR to approximately 1 million copies (Mardis, 2008). After the library is produced, fragments are sequenced by pyrosequencing, which involves the sequential addition of each individual nucleotide (dATP, dGTP, dTTP, dCTP) followed by a PCR step. Each time a dNTP is incorporated, this leads to a series of reactions which lead to light emission by the enzyme luciferase. The amount of light emitted is proportional to the number of bases added, however this is less accurate if longer chains of a single base are added (Mardis, 2008), which means the procedure can be less accurate in regions with long single base repeats.

1.12.1.2 Applied Biosystems SOLiD

The Applied Biosystems Sequencing by Oligonucleotide Ligation and Detection (SOLiD) system sequences two bases at a time. Library production begins with random fragmentation of DNA, ligation of adapters to fragments, and attachment of individual fragments to amplification beads (Ansorge, 2009). The individual fragments are amplified and transferred to a glass surface, with clones clustered together, and different sequences spatially separated. Fluorescently labelled dinucleotides (a different label for each dNTP) are added to the reaction for sequencing, and PCR is performed with two complementary nucleotides added per cycle. Each position is probed twice, and the two nucleotides are identified by laser excitation of the DNA clusters, which emits one of 16 possible colours, depending on which two nucleotides have been ligated (Morozova and Marra, 2008). The advantage of this procedure is its accuracy, as it is possible to distinguish between a sequencing error and a sequence polymorphism (an error is detected in only one ligation reaction, a polymorphism is detected in both) (Liu et al., 2012).

1.12.1.3 Illumina/Solexa Genome Analyser/Hiseq

Like the previous two methods, libraries are fixed to a solid surface and amplified. This process uses a sequencing-by-synthesis approach, with all four nucleotides added to the sequencing PCR at once. Each nucleotide is fluorescently labelled (a different label for each of the four nucleotides), and has a removable blocker, which enables incorporation of a single base at a time. (Liu et al., 2012). At each cycle, one base is incorporated, followed by a fluorescent detection step, and the removal of the blocker(Mardis, 2008). The process is repeated until the fragment is sequenced.

1.13 Determination of Association between Pathogen and Disease

Before a pathogen can be named as the causal agent of a specific disease, there are a number of requirements that must be met. Koch's postulates, developed in 1890, are the accepted method for determining whether a pathogen (fungal, microbial or viral) is the cause of a specific disease (Evans, 1976). These postulates are: (1) the microorganism is present in the lesions of the disease, (2) the organism can be isolated from the host in pure culture and grown for several generations, and (3) the pure culture of the organism is able to produce the symptoms of the disease in question when inoculated onto the host species and (4) the organism can be reisolated from the diseased host (Evans, 1976). However, since new developments

in disease epidemiology have been made, proposals for the expansion of these postulates have been made. These expanded points include factors such as statistical significance of disease-pathogen association, consistency of results among researchers, and specificity of the pathogen to the disease factor (i.e. is the disease caused specifically by the one pathogen, or are other pathogens also associated with the disease?)(Hunter, 2003). The postulates can be tested using a number of methods, including surveys of microbial agents isolated from tissue displaying the disease, and inoculating the species susceptible to the disease and observing symptoms. However, since these tests are not always possible or indicative of a disease-pathogen relationship, other techniques including molecular marker methods can be used to measure association.

1.14 Quantitative PCR

Quantitative PCR (qPCR) is a method which enables the amount of DNA amplified in PCR to be measured. It has been used to estimate the amount of fungal tissue of specific pathogens present in plant tissues (Bates et al., 2001; Hogg et al., 2007; Knight et al., 2012; Li et al., 2006; Moya-Elizondo et al., 2011b; Zhao et al., 2007). Fluorescent dyes are used for quantification, as the amount of fluorescence is directly related to the amount of PCR product in each reaction (Schena et al., 2004). The advantage of qPCR over traditional methods such as microscopy, is that fungal DNA can be quantified, and this information can be more readily used to investigate relationships between symptoms and fungal colonisation. Microscopy can only be scored as presence or absence of a particular fungal species.

There are three main phases of qPCR: the background, exponential, and plateau phases. The start of the reaction is the background phase, where no change in fluorescence can be detected due to an excess of background fluorescence. The cycle at which the fluorescence intensity of the product ascends above the background is called the threshold cycle (C_t) (Bustin, 2005; Heid et al., 1996). This is where the exponential phase commences and the amount of product increases rapidly. In an optimal reaction, each cycle will result in the product doubling each cycle ("Quantitative Real-Time PCR-The Essentials" Roche Diagnostics, 2009). However, due to a number of factors including a reduction in reagent concentration, build-up of by-products, and reannealing of the amplification products, this does not occur in reality. Efficiency of a reaction is notated as the average number of times the template is multiplied. For example, if 80% of the DNA in a reaction is replicated per cycle, this results in 1.8 times as much DNA as the starting amount, thus the efficiency is 1.8 (Brankatschk et al., 2012). The efficiency decreases over the cycles until the plateau phase, when replication ceases abruptly. The only measurement in the reaction which can be used for making calculations is the early exponential phase, as this is the only point at which the reaction follows predictable kinetics ("'Quantitative Real-Time PCR- The Essentials"Roche Diagnostics, 2009).

In order to calculate the amount of DNA present in unknown samples, a series of known DNA dilutions is prepared to produce a standard curve (Figure 1.9A) where the Cp is plotted against the log concentration of DNA, and read off the result graph for the PCR run (Figure 1.9B).

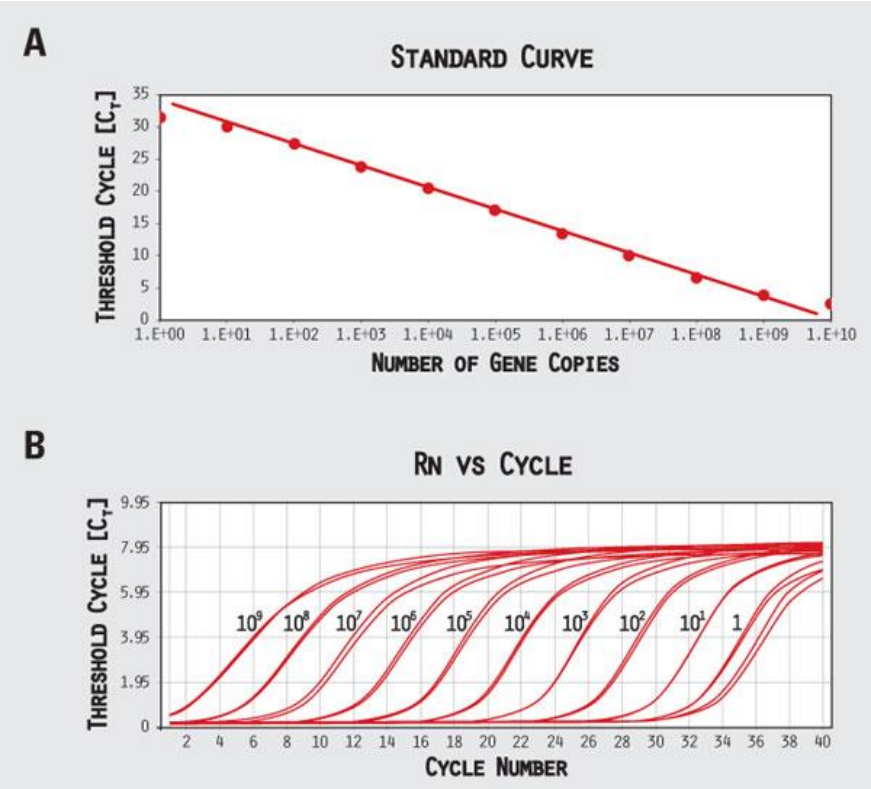


Figure 1.9 (A) Standard curve generated in quantitative PCR, (B) where the crossing point (Cp) is plotted against the log concentration of DNA (**http://www.sabiosciences.com/pathway7.php**).

In a number of previous studies, qPCR has been utilised in assessing the level of specific fungal pathogens in tissues displaying disease symptoms. The level of *B. sorokiniana* has been assessed both in roots displaying CRR symptoms (Moya-Elizondo et al., 2011b) and in tillers of plants inoculated in-furrow (Moya-Elizondo et al., 2011a). If the level of pathogen measured in the tissue is directly related to the level of disease, this provides evidence of an association between symptom and pathogen.

1.15 Project Goals

1. Firstly, to determine whether genetic differences exist between *B. sorokiniana* isolates from different hosts (wheat and barley) and different tissues (primarily root and leaf tissue) in Australia. Secondly, to determine whether there are genetic differences among isolates originating from different geographic regions (addressed in Chapter 2). Little is known about the population structure of Australian isolates of *B. sorokiniana* and the pathogen's main modes of genetic diversification. The questions raised by the previous preliminary study of an Australian *B. sorokiniana* population conducted by Knight *et al.* (2010), which revealed significant genetic differences between leaf and root isolates, will be addressed in this project by examining an expanded collection of isolates, including SB isolates taken from wheat plants and other isolates not examined in the previous work. This section of the project will aim to determine whether there are significant genetic differences among isolates from different hosts and tissues within the Australian population of *B. sorokiniana*.

Hypothesis 1: The genetic relationships among *B. sorokiniana* isolates are explained by host species (wheat and barley) and tissue (leaf root and other) combinations, as well as geographic regions from which the isolates were originally sampled.

2. To investigate the extent of genetic variation of isolates of *B. sorokiniana* sampled from different leaf sections or individual lesions of a single plant in the field, and to compare the genetic fingerprint stability of single source isolates sequentially inoculated and sampled from different host genotypes (addressed in Chapter 2).

The sexual stage of the pathogen, *C. sativus*, has only once been reported in nature, and it is thought that other modes of recombination, such as mutation and parasexual recombination, play a major role in the genetic diversity of *B. sorokiniana*. A recent study of the genetic diversity of individual isolates from the same lesion or tissue piece of barley (Leisova-Svobodova et al., 2011) claimed that the level of diversity within single lesions was too high to be accounted for by infection of single lesions with multiple spores. An earlier study which examined single spores isolated from different wheat genotypes inoculated with the same original isolate, claimed that significant genetic variation could emerge within a single generation, depending on the genotype of the host plant (de Oliveira et al., 2002). These theories will be investigated further by analysing isolates from single lesions within naturally infected field plots, and isolates taken from different host genotypes initially inoculated with the same isolate. These isolates will be subjected to AFLP analysis and compared for genetic similarity.

Hypothesis 2: There is a higher level of genetic variation among isolates from the same lesion from plants in the field than is expected in an asexual organism.

Hypothesis 3: Genetic variation among clonally propagated isolates is influenced by host genotype.

3. To determine whether there is any level of host or tissue specificity for *B. sorokiniana* isolates when inoculated onto leaf tissue by assessing the seedling infection response of barley and wheat leaf tissue to infection (addressed in Chapter 3).

Previous studies have investigated virulence patterns of SB isolates on leaf tissue of different barley and wheat varieties, but no previous work has investigated host or tissue specificity of CRR isolates.

Hypothesis 4: Host specificity does exist for isolates originating from wheat and barley plants from CRR and SB infections when inoculated onto leaf tissue of both hosts.

Hypothesis 5: Tissue specificity exists for isolates originating from wheat and barley plants from CRR and SB infections when inoculated onto leaf tissue of both hosts.

4a. To design an appropriate inoculation protocol of barley and wheat root to assess virulence of *B. sorokiniana*.
4b. To investigate the degree of host or tissue specificity in CRR and SB isolates for both hosts when inoculated onto root tissue (addressed in Chapter 4).

To investigate the degree of tissue and host specificity of isolates within the population of *B. sorokiniana*, a selection of isolates from CRR and SB infections of wheat and barley will be assessed for virulence on roots of a selection of wheat and barley genotypes.

Hypothesis 6: Host specificity exists for isolates originating from CRR and SB infections of both hosts when inoculated onto root tissue.

Hypothesis 7: Tissue specificity exists for isolates originating from CRR and SB infections of both hosts when inoculated onto root tissue of both hosts.

5. To fine map the barley 3H region associated with SB resistance to enable future identification of candidate genes (addressed in Chapter 5).
There are two major known field resistance QTL for SB resistance of barley, on chromosomes 7HS and 3HS. Since fine mapping of the 7HS region has recently been the subject of a significant study based in the USA (Drader and Kleinhofs, 2010), the 3H region will be fine mapped in this study. This region is known for its low level of polymorphism, and has not been fine mapped previously. The work will enable easier incorporation of the resistance region into agronomically adapted varieties, as well as enabling sequencing of the region to be undertaken in the future, and the identification of candidate genes.

Hypothesis 8: The barley 3H region associated with spot blotch resistance can be fine mapped using markers from other mapping studies.

6. To investigate whether there is a relationship between the abundance of *B. sorokiniana* mycelia as measured by the fungal DNA content in barley grain, and level of BP symptoms (from null to conspicuous symptoms, addressed in Chapter 6).

This final section of the study will employ qPCR to assess the level of fungal DNA present in a number of seeds with black point symptoms of varying levels, and seed with no black point symptoms. Results will be compared to other studies investigating various aspects of black point, and seeds have been sampled from a

number of barley genotypes from either fungicide treated or untreated plots, with or without symptoms of black point

Hypothesis 9: Evidence does exist for a relationship between *B. sorokiniana* and the grain defect black point.

Hypothesis 10: *B. sorokiniana* isolates recovered from symptomless kernels are genetically unrelated to those recovered from BP kernels.

Chapter 2: Genetic diversity and population structure of Australian *Bipolaris sorokiniana* isolates

2.1 General Introduction

Bipolaris sorokiniana is an economically important pathogen of wheat and barley in Australia, and causes foliar spot blotch (SB) and common root rot (CRR) in both crops. With regard to crown and root diseases, CRR causes damages of $30 million to wheat and $13 million to barley per year (Murray and Brennan, 2009; Murray and Brennan, 2010). Spot blotch (SB) is not a significant disease of wheat in Australia, and is a relatively minor disease of barley in the Northern grain region encompassing northern NSW and southern and central Queensland (Murray and Brennan, 2009; Murray and Brennan, 2010). In contrast, the disease is one of the most economically important diseases of wheat in warmer and more humid regions of the world, such as South Asia, Latin America and Africa (Acharya et al., 2011; Bhandari et al., 2010). For example, grain yield loss in South Asia due to spot blotch ranged from 4% to 38% and 25% to 43% in 2004 and 2005, respectively (Acharya et al., 2011).

There are a number of available control methods for both common root rot and spot blotch in wheat and barley, including stubble removal, crop rotation, seed treatment, and the use of resistant varieties. Additionally, spot blotch may be controlled by use of foliar fungicides (Acharya et al., 2011; Bovill et al., 2010; Couture and Sutton, 1978; Duveiller et al., 2005; Fetch and Steffenson, 1999). The most efficient and environmentally sustainable means of control is by use of resistant cultivars, and a number of studies have identified loci conferring resistance to spot blotch in barley (Bilgic et al., 2005; Bilgic et al., 2006; Bovill et al., 2010; Roy et al., 2010), while resistance to common root rot in barley has also been identified (Lehmensiek et al., 2010). However, disease assessment of common root rot is time-consuming and difficult; hence production of common root rot resistant cultivars has been slow.

In order to carry out more effective resistance studies for both diseases, investigation of pathogen population structure is imperative. Population studies have been undertaken in various countries, including India, Syria, Poland, Brazil, Pakistan, Australia, Czech Republic and USA (Aggarwal *et al.*, 2010; Arabi and Jawhar, 2007b; Bashyal *et al.*, 2010; Baturo, 2005; de Oliveira *et al.*, 2002; Gyawali *et al.*, 2012; Iram and Ahmad, 2004; Jaiswal *et al.*, 2007; Knight *et al.*, 2010; Leisova-Svobodova *et al.*, 2011; Zhong and Steffenson, 2001b). A distinct clustering of isolates according to geographic origin was observed by Aggarwal *et al.* (2010), and Leisova-Svobodova *et al.* (2011), whereas two earlier studies detected a lack of genetic diversity across geographic regions (Arabi and Jawhar, 2007; Zhong and Steffenson, 2001b). Most studies have solely investigated isolates from leaves of either wheat or barley and inoculated only one of these hosts. Recently Knight *et al.* (2010) investigated the issue of host specificity by including isolates from both hosts in a study which investigated both genetic relatedness and pathotype. They found a correlation between genetic relatedness and host specificity of the isolates, although only a very few wheat-derived leaf isolates were examined. Gyawali *et al.* (2012) also included isolates from both wheat and barley and observed genetic diversity according to the host of origin.

Research comparing isolates from roots and leaves has also been limited. Knight *et al.* (2010) included Australian isolates from both leaves and roots, which grouped

into distinctly separate clusters in a dendrogram of genetic similarity. In an earlier study of Syrian *B. sorokiniana* isolates, which investigated genetic variation in relation to pathogenicity, Arabi and Jawhar (2007b) included a relatively small number of root isolates (five out of 22), but made no analysis of the relationship between genetic diversity and tissue of origin. Baturo (2005) compared isolates from grain, stem base and root tissues, and found no correlation between the tissue of origin and the genetic diversity. Cluster analysis in the Australian study (Knight et al., 2010) indicated a genetic divergence between 19 isolates from foliar spot blotch infections of barley and 26 isolates taken from infected roots of wheat and barley. However since 22 of the 26 common root rot isolates were from wheat, it was not clear whether this genetic diversity was a response to host tissue or host species.

In this current study we have addressed whether a genetic relationship among *B. sorokiniana* isolates from Australia is affected by host species and/or tissue from which they were sampled, by analysing AFLP fingerprints of 126 isolates. These included a selection of 27 isolates from the previous study (Knight et al., 2010). Isolates were sampled from both wheat and barley and from a range of locations on the plants (from CRR and SB infections), with the goal of determining the degree to which different subpopulations of the fungus had diversified in response to the different host species and/or the different positions of the infection site on the host. All isolates were from naturally occurring infections in the field.

In an additional preliminary study, we examined multiple isolates that were taken from a single leaf of a barley plant in the field. The field had been inoculated with the commonly used *B. sorokiniana* isolate SB61. The aim of this study was to test the results obtained in a Czech study (Leisova-Svobodova et al., 2011) in which the genetic variability observed among multiple isolates from a single lesion using AFLP analysis was too high to be explained by multiple infection sites alone. Since the previous study looked at isolates from naturally infested fields, this section aimed to investigate whether the same level of variability is observed when the field is inoculated with a single isolate.

A third study examined isolates that were inoculated onto different wheat and barley genotypes in the glasshouse, and reisolated from leaf tissue. The aim of this section was to examine previous results obtained in a study by de Oliveira *et al.* (2002) in which isolates were observed to have different RFLP marker patterns when reisolated from different plant genotypes. The current study used the genotyping method based on AFLP markers, which requires much lower quantities of target DNA than RFLP analysis, to test if these variations could be observed among Australian isolates.

2.2 Methods

2.2.1 Collection of Single Spore Isolates

2.2.1.1. Population Study

One hundred and twenty six (126) Australian single spore isolates were collected primarily from wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) tissues, with some additional samples from barley grass, prairie grass, triticale and durum. Samples were collected within Queensland and New South Wales (with the exception of two from Victoria and one from Western Australia) between 1995 and 2011. Tissue sources included leaves, seeds, stubble, crowns, subcrown internodes and roots. Samples collected prior to 2007 were the subject of an earlier analysis by Knight *et al.* (2010). Isolates from NSW were provided by Steven Simpfendorfer of the Tamworth Agricultural Institute, NSW Department of Primary Industries, and the majority of the QLD isolates were from the collections of the QDAFF Hermitage Research Station or the University of Southern Queensland. Infected tissue was surface-sterilized (5 seconds in 70% ethanol followed by 2 minutes in bleach and rinsed three times in demineralised water) and placed onto moistened filter paper in petri dishes. These were incubated at room temperature under normal night/day light conditions for three to five days for production of conidia. Single spores were picked with a glass needle and transferred to potato dextrose agar (PDA), incubated for 48 hours at 24^{o}C, then subcultured onto fresh PDA plates.

2.2.1.2. Multiple Isolates from a Single Leaf of Barley

This section aimed to investigate whether a high level of variation among *B. sorokiniana* isolates can occur within a single leaf and/or a single lesion of a barley plant in the field, as observed by Leisova-Svobodova *et al.* (2011). One barley plant was collected from a field inoculated with the spot blotch isolate SB61 at the Department of Agriculture, Fisheries and Forestry Redland Bay Research Facility in 2011. One section of leaf was sampled, and cut into four ~2cm sections, surface-sterilised (as described above) and placed in a petri dish on moistened filter paper. These plates were again incubated at room temperature for three to five days, and B. sorokiniana isolates were collected from individual lesions from each section of leaf, while others were sampled from the same lesion as described in Table 2.1... A total of 29 single spore isolates were placed on PDA, as described in Section 2.2.1.1.

Table 2.1 Isolates of *B. sorokiniana* sampled from a barley leaf from the field. The leaf was cut into four sections (numbered 1 to 4), isolates were collected from different lesions within the same section of leaf (labelled lesions a,b,c, etc) and two of the lesions had four isolates sampled from them (labelled in Roman numerals I to IV).

Sample Number	Lesions	Isolates	Monosporic Isolate Accession
USQ11125-1	1		
USQ11125-2a	2	a	
USQ11125-2b		b	
USQ11125-2c		c	
USQ11125-2d		d	
USQ11125-2e		e	
USQ11125-3a	3	a	
USQ11125-3b		b	
USQ11125-3c		c	
USQ11125-3d		d	
USQ11125-4a	4	a	
USQ11125-4b		b	
USQ11125-4c		c	
USQ11125-4d		d	
USQ11125-4e		e	
USQ11125-4f		f	
USQ11125-4g		g	
USQ11125-4h (I)		h	I
USQ11125-4h (II)			II
USQ11125-4h (III)			III
USQ11125-4i (I)		i	I
USQ11125-4i (II)			II
USQ11125-4i (III)			III
USQ11125-4i (IV)			IV
USQ11125-4j		j	
USQ11125-4k		k	
USQ11125-4l		l	
USQ11125-4m		m	

2.2.1.3. Reisolated *B. sorokiniana* from Inoculated Wheat and Barley Plants

This study aimed to investigate whether variation among isolates could be observed when isolates had been inoculated onto different host genotypes, as reported by de Oliveira *et al.* (2002). A selection of *B. sorokiniana* isolates from a variety of sources (one isolate each from wheat root, crown and stubble tissue; five isolates from barley leaf tissue and one isolate from barley root tissue) examined in the population study was used to individually inoculate leaves of a set of wheat and barley differential plants in a separate study (described in full in Chapter 3). Isolates were sampled from the wheat differentials Wyalkatchem, Magenta and Calingiri, and the barley differentials Delta, Stirling, Lindwall, Gilbert, CI 1227, NRB 091084, Bowman and Conlon. Briefly, isolates were placed on starch nitrate agar for 10 days at 24^{o}C, after

which each plate was flooded with 5mL of a solution made up of six drops of Tween20 in 100mL water, and spores were dislodged from the plate with a paint brush treated with 70% ethanol. The spore mixture was made up to 50mL in a volumetric flask, transferred to a beaker and agitated constantly by placing on a magnetic stirrer. Concentration of spores was estimated using a haemocytometer, and a dilution of 10 000 spores/uL was made for each isolate (24mL per set of 18 plants). Seeds were planted in small pots in a randomised order (three seeds per differential, three differentials per pot) and grown in a glasshouse for 14 days. After this time, plants were inoculated with the spore suspension by spraying evenly using a Preval Power Unit sprayer system. Plants were transferred to a growth cabinet at 24ºC and 100% humidity for 24 hours to enable infection, and then transferred to the glasshouse. After 10 days, leaf material of three to five of the differentials per isolate displaying spot blotch symptoms were randomly selected and single spores were isolated as described in Section 2.2.1.1. Isolates were collected from nine of the barley differentials and three of the wheat differentials.

2.2.2. Species-specific primers

Isolates from the population study were identified as *B. sorokiniana* (*Bs*) by verification with species-specific PCR markers for *B. sorokiniana* (Matusinsky et al., 2010) and *Pyrenophora teres* f. *maculata* (Lu et al., 2010), a fungal pathogen with similar symptoms on leaf tissue to *B. sorokiniana*. Four additional primers were designed in the same region as the *B. sorokiniana*-specific marker designed by Matusinsky (2010), due to the unreliability of the previously designed primers (discussed further in the 'Results' section). The new primers were designed using Primer3 software and the *B. sorokiniana* Brn1 sequence (accession number AB011653.1). PCR was run using a total of five combinations of the primers. A pair of primers designed by Moya-Elizondo (2011b) in the glyceraldehyde-3-phosphate dehydrogenase region for isolate ND93-1 (Accession number:EF513209.1), which amplify in many fungal species were employed as a positive control for the PCR reaction. These primers are referred to as the 'gly' primers. The sequences of all the primers are given in Table 2.2, and the relative positions and length of fragments are shown in Figure 2.1.

Table 2.2 Cos primer sequences, designed for *Brn1* sequence and glyceraldehyde-3-phosphate dehydrogenase region.

Primer	Sequence	Reference
CosA_F_519	5'-TCAAGCTGACCAAATCACCTTC	Matusinsky 2010
CosA_R	5'-CTTCTCACCAGCATCTGAATATATGA	Matusinsky 2010
CosA_R_248	5'-AATGTCGAGCTTGCCAAAGT	current study
CosA_F_248	5'-CACCATCAACACTCGTGGTC	current study
Cos_F_839	5'-CACCGGCTCAGGTAAGACAT	current study
Cos_R_839	5'-CCTTTCCGTTAACCCAGTCA	current study
gly_F	5'-GAAGGACCCCGCCAACA	Moya-Elizondo et al 2011
gly_R	5'-CCGCTACACTCGACGACGTAGT	Moya-Elizondo et al 2011

Each PCR reaction using the Cos (conserved orthologous set) primers contained: 1μL template DNA, 0.5 U GoTaq® Flexi DNA Polymerase, 4μL 5x reaction buffer, 1.5 mM $MgCl_2$, 200μM dNTPs, 2.5μM of each Cos primer (F and R) and 2.5μM of each Gly primer, with a total volume of 20μL. PCR conditions were: 7mins at 95ºC, followed by 35 cycles of 94ºC for 30secs, 55ºC for 30 secs, and 72ºC for 30 secs, with a final extension at 72ºC for 10 mins. These primers were screened across all 126 *Bipolaris* isolates, and also several fungal isolates of other species (*Fusarium pseudograminearum*, *F. graminearum*, *F. culmorum*, *F. scirpi*, *F. semitectum*, *F. proliferatum*, *F. compactum*, *F. poae*, *F. equisetum*, *F. crookwellense* supplied by P. Davies, University of Sydney, and *Alternaria alternata* and *Epicoccum nigrum*, which were identified with the assistance of A. Martin (USQ) and R. Fowler (QDAFF)).

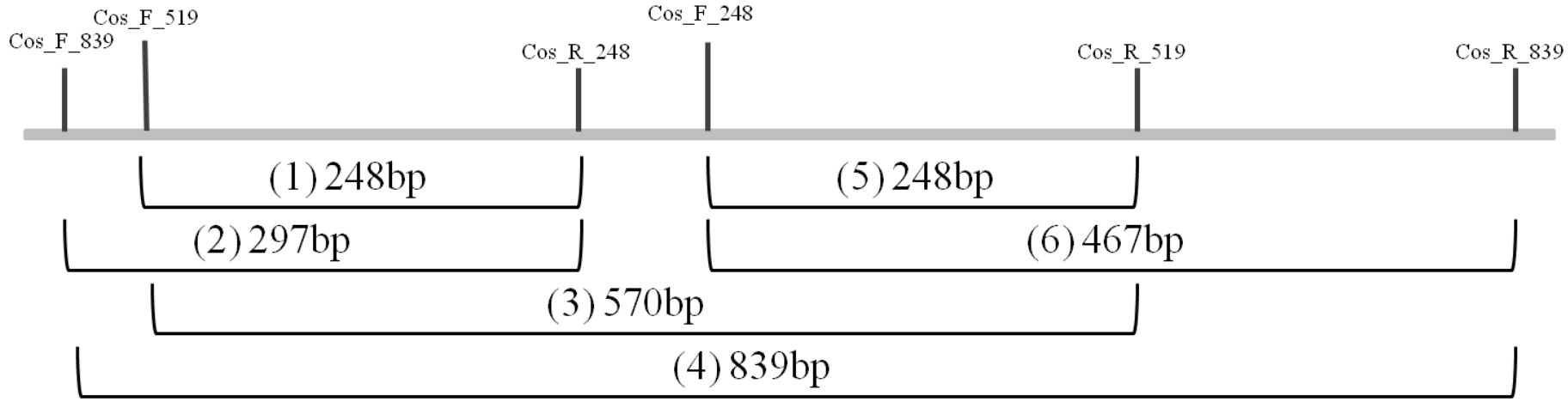


Figure 2.1 Position of *B. sorokiniana* species' specific primers and expected PCR amplification fragment sizes of *Brn1* locus. Primers description in Table 2.1..

2.2.3. DNA extractions

Mycelia were harvested from single-spore cultures of all isolates grown on solid PDA incubated at 24ºC for 1 week. DNA was extracted from mycelia using the cetyl trimethyl ammonium bromide (CTAB) based method of Saghai-Maroof *et al.* (1984). Extracted DNA was quantified using an Implen Nanophotometer (Integrated Sciences) and diluted to a concentration of 25ng/µL.

2.2.4. AFLP analysis

AFLP analysis was performed for all isolates using an Invitrogen AFLP core reagent kit and eight selective EcoRI/MseI primer combinations. Approximately 150ng of DNA was restricted with the EcoRI and MseI restriction enzymes. A 1:10 dilution was prepared for each sample using TE buffer after adapter ligation. Pre-selective amplification with EcoRI (E-G or E-A) and MseI (M-A or M-G) primers, each having one selective nucleotide, was carried out with the diluted restriction-ligation mix. Each reaction contained 5µL restricted-ligated DNA, 0.5U GoTaq® Flexi DNA Polymerase (Promega Corporation), 4µL 5x reaction buffer, 1.5mM $MgCl_2$, 200µM dNTPs and 0.25µM EcoRI and MseI primers, with a total volume of 20µL. PCR conditions were 20 cycles of 94ºC for 30 secs, 56ºC for 1 min and 72ºC for 1 min. Selective amplification was completed with EcoRI and MseI primers, each having two selective nucleotides, which were fluorchrome-labelled for visualisation (E-AA with M-AG or M-AA; E-AG with M-AT or M-AC; E-GC with M-GC, M-GT, M-GG or M-GA). Each reaction contained 2µL preselective amplified DNA, 0.5 U GoTaq® Flexi DNA Polymerase, 3µL 5x reaction buffer, 1.5 mM $MgCl_2$, 200µM dNTPs, 0.25µM EcoRI and MseI primers, with a total volume of 15µL. PCR conditions for selective amplification were 12 cycles of 94ºC for 30 s, 65ºC for 30 s, 72ºC for 1 min, followed by 23 cycles of 94ºC for 30 s, 56ºC for 30 s, and 72ºC for 1 min. Three microlitres of formamide loading buffer was added to each well, DNA was denatured at 94ºC for 4 min, and markers were visualised on 6% denaturing polyacrylamide gel using a Gelscan 2000^{TM} fragment analyser (Corbett Life Sciences) at 2300 V. Polymorphic and monomorphic markers were scored as binary data. Controls were included in the analysis to test consistency between DNA extractions and restriction ligation reactions.

2.2.4.1. Distance-based clustering analysis

A similarity matrix was constructed for each of the three groups of isolates (population, reisolated and whole plant isolates) using the Dice coefficient (Dice, 1945) in the Qualitative Data programme within the NTSYS-pc version 2.20f software package. Cluster analysis of the matrix data was performed with NTSYS-pc using the unweighted pair-group method with arithmetic mean (UPGMA, Sneath and Sokal, 1973) provided in the SAHN programme. A dendrogram was constructed using TREE PLOT.

2.2.4.2. AMOVA

The AFLP data of the *B. sorokiniana* population was converted from Microsoft Office Excel format to the ARLEQUIN file format using the programme TRANSFORMER-3 (Caujape-Castells and Baccarani-Rosas, 2005). Analysis of molecular variance (AMOVA) was performed using the software package ARLEQUIN version 2.0 (Excoffier et al., 1992; Schneider et al., 2000) with 1000 permutations. Genotypic data was grouped to assess major genetic differences between hosts (wheat or barley), tissues, locations (regions of QLD and NSW) and years of collection (early, collected before 2007; late, after 2007). An additional grouping classified isolates into isolate type: basal and aerial. Leaf and seed isolates were classified as aerial, and isolates from root, stubble, crown and subcrown internode were classified as basal. Default settings were used but at a 0.01 significance level.

2.2.4.3. Model-based clustering analysis

The genetic structure implies number of groups or populations (K), which were inferred for the *B. sorokiniana* population using STRUCTURE version 2.2 (Pritchard et al., 2000). Ten independent runs were completed for each value of K, with K values ranging from 1 to 10. The default settings of the programme were used (Falush et al., 2007; Pritchard and Wen, 2003), and each run was set to a burn-in period of 10 000 iterations, followed by 100 000 Monte Carlo Markov Chain (MCMC) iterations. The likelihood of each individual belonging to each of the two populations calculated in the model-based analysis was then calculated using the programme CLUMPP 1.1.1.

2.3 Results

2.3.1 Species-Specific Primers

DNA of all 126 individuals from the *B. sorokiniana* population were analysed with five combinations of the Cos primers, however no one pair of primers amplified in all individuals. It was particularly difficult to obtain positive results with the Matusinsky (2010) primer pair, and in most of the PCR runs with this pair no markers were amplified in any individuals. Only 45 out of 126 individuals were positive for the Matusinsky (2010) marker, which was the lowest proportion of positives out of all six primer pairs. On the other hand, when these two primers were individually paired with the one of the other Cos primers, amplified markers were clearly visible in several (although not all) individuals. The DNA of only 12 (out of a total of 126) *B. sorokiniana* isolates amplified with all primer combinations, while a further 28 individuals amplified with all four remaining combinations, when the Matusinsky (2010) primers were excluded. Three individuals were negative for all five primer combinations, and when the Matusinsky (2010) primer results were omitted, a further eleven isolates were negative for the remaining four combinations. The marker with the highest proportion of positives in the population was Cos_F_248 + Cos_R_519, which was positive in 99 out of the 126 isolates. The cultures and spore morphology of these isolates were rechecked and each confirmed as *B. sorokiniana*. These individuals were randomly distributed in the dendrogram, and were closely associated with other *B. sorokiniana* isolates that amplified with the markers. There also appears to be no consistent pattern in the tissues or hosts of origin when comparing the patterns of the Cos primers.

The alternate fungal species were also tested with all combinations of Cos primers. The majority of isolates did not amplify with most of the Cos primers; however a few isolates did produce a fragment of the same size as the *B. sorokiniana* isolates. None of the isolates of other species amplified with more than one primer combination. Cos primer combinations 3 and 5 (see Figure 2.1) failed to amplify a product in any isolates; combinations 1 and 4 amplified multiple markers, with combination 4 also amplifying the same marker size as *B. sorokiniana* in two individuals (*F. semitectum* and *F. proliferatum*). Combination 2 amplified the *B. sorokiniana* marker in both isolates of *F. equisetum*, and combination 6 amplified the *B. sorokiniana* marker in four of the *A. alternata* isolates and three *E. nigrum* isolates.

2.3.2 AFLP analysis of the *B. sorokiniana* Population

Fourteen duplicate isolates were included in the analysis as controls, with 11 being from independent DNA extractions and three using the same DNA to produce new restriction ligation reactions. All duplicated samples gave identical results. Only markers that could be scored consistently were included in the analysis. A total of 245 markers were scored, 83 monomorphic and 162 polymorphic.

2.3.3 Distance-based clustering analysis

Collectively in the distance-based cluster analysis (Figure 2.2, with details of each isolate listed in Table 2.3), all isolates shared a similarity index of more than 76%. Three main clusters of isolates were observed: primarily barley aerial isolates (cluster I), primarily wheat basal isolates (cluster II), and isolates from a mixture of sources (cluster III). The majority of the seed isolates segregated into cluster I, while the stubble isolates segregated into the other two clusters. A total of 56 isolates grouped into cluster I, none of which were stubble, root, or subcrown internode isolates. Only one crown isolate (from wheat) grouped with cluster I. Only three of the isolates were from a species other than barley; two from wheat (once crown and one leaf isolate), the other from barley grass (leaf). Cluster II consisted of 51 isolates, primarily from crown, subcrown internode and root tissue. Three of the isolates originated from leaf tissue (of barley, wheat and barley grass) and one was an isolate from wheat seed. Nineteen individuals grouped into cluster III, four of which were leaf isolates (two from wheat, one of triticale and one of prairie grass), and two seed isolates (wheat and barley). The remaining isolates were from basal tissues from both wheat (6) and barley (3). A separate cluster analysis (not shown) was also performed using only the current data for isolates previously analysed by Knight *et al.* (2010). A comparison of the clustering results indicated a high level of consistency between the two studies, with the wheat CRR, the barley SB and the mixed cluster all grouping together in the same way as the original study, and the outlier 20004 remaining as an outlier.

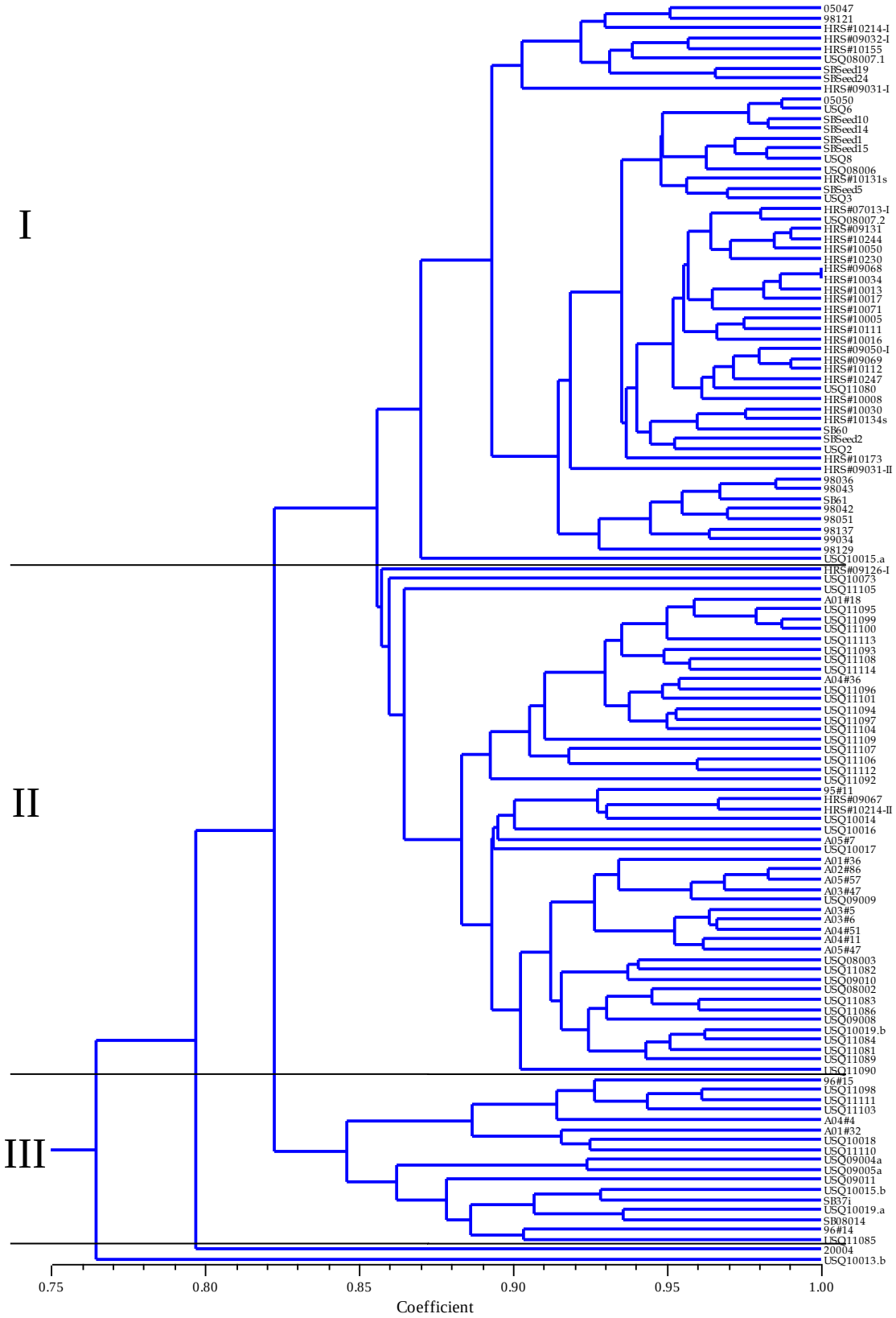


Figure 2.2 Dendrogram of Australian *B. sorokiniana* isolates using AFLP data. The relationship among isolates was determined using the DICE similarity coefficient and UPGMA (Unweighted Pair Group Method with Arithmetic Means).The three main clusters, I, II and III are indicated at the left of the figure, and partitioned with horizontal black lines.

Table 2.3 All *B. sorokiniana* isolates included in the AFLP population analysis, presented in the same order as the dendrogram (Figure 2.2). The 'Region' column indicates the designation given to isolates clustered together along the central region of the east coast. The 'North' region includes isolates located between Gladstone and Dalby, QLD; the 'Mid' region is allocated to isolates located between Dalby, Queensland and Yamba, New South Wales; the 'South' region is designated to isolates located between Yamba and Sydney, New South Wales; isolates with no region allocation were located elsewhere in Australia and were excluded from the regional analysis.

No	ID	Host	Tissue	State	Town	Region	Year
1	05047	Barley	Leaf	Qld	Gatton RS	Mid	2005
2	98121	Barley	Leaf	NSW	Tamworth	South	1999
3	HRS#10214-I	Barley	leaf	Qld	Mt Stuart		2010
4	HRS#09032-I	Barley	leaf	Qld	Sladevale	Mid	2009
5	HRS#10155	Barley Grass	leaf	Qld	Inglewood	Mid	2010
6	USQ08007.1	Barley	leaf	Qld	Bundaberg	North	2008
7	USQSeed19	Barley	Seed	Qld	Bundaberg	North	2006
8	USQSeed24	Barley	Seed	Qld	Bundaberg	North	2006
9	HRS#09031-I	Barley	leaf	Qld	Mt Whitestone	Mid	2009
10	05050	Barley	Leaf	Qld	Pilton	Mid	2005
11	USQ10006	Barley	leaf	Qld	Wellcamp	Mid	2010
12	USQSeed10	Barley	Seed	Qld	Bundaberg	North	2009
13	USQSeed14	Barley	Seed	Qld	Bundaberg	North	2009
14	USQSeed1	Barley	Seed	Qld	Bundaberg	North	2009
15	USQSeed15	Barley	Seed	Qld	Bundaberg	North	2009
16	USQ10008	Barley	leaf	Qld	Wellcamp	Mid	2010
17	USQ08006	Barley	leaf	NSW	Acacia Plateau	Mid	2008
18	HRS#10131s	Barley	leaf	NSW	North Star	Mid	2010
19	USQSeed5	Barley	Seed	Qld	Bundaberg	North	2009
20	USQ10003	Barley	leaf	Qld	Wellcamp	Mid	2010
21	HRS#07013-I	Barley	leaf	NSW	Grafton	South	2010
22	USQ08007.2	Barley	leaf	Qld	Bundaberg	North	2008
23	HRS#09131	Barley	leaf	Vic	Minyip		2010
24	HRS#10244	Barley	leaf	Qld	Massie	Mid	2010
25	HRS#10050	Barley	leaf	Qld	Murgon	North	2010
26	HRS#10230	Barley	leaf	Qld	Unknown		2010
27	HRS#09068	Barley	leaf	Qld	Atherton		2009
28	HRS#10034	Barley	leaf	Qld	Pittsworth	Mid	2010
29	HRS#10013	Barley	leaf	Qld	Bundaberg	North	2010
30	HRS#10017	Barley	leaf	Qld	Hermitage RS	Mid	2010
31	HRS#10071	Barley	leaf	Qld	Gatton RS	Mid	2010
32	HRS#10005	Barley	leaf	Qld	Hermitage RS	Mid	2010
33	HRS#10111	Barley	leaf	Qld	Kalbar	Mid	2010
34	HRS#10016	Barley	leaf	Qld	Redlands RS	Mid	2010
35	HRS#09050-I	Barley	leaf	Qld	Wellcamp	Mid	2009
36	HRS#09069	Barley	leaf	Qld	Atherton		2009
37	HRS#10112	Barley	leaf	Qld	Gatton RS	Mid	2010
38	HRS#10247	Barley	leaf	Qld	NB85 Nursery HRS	Mid	2010
39	USQ11080	Wheat	leaf	Qld	Wellcamp	Mid	2011
40	HRS#10008	Barley	leaf	Qld	Applethorpe RS	Mid	2010
41	HRS#10030	Barley	leaf	Qld	Bundaberg	North	2010
42	HRS#10134s	Barley	leaf	NSW	Yallaroi	Mid	2010
43	SB60	Barley	Leaf	Qld	Hermitage RS	Mid	1999
44	USQSeed2	Barley	Seed	Qld	Bundaberg	North	2009
45	USQ10002	Barley	leaf	Qld	Wellcamp	Mid	2010
46	HRS#10173	Barley	leaf	Qld	Gatton RS	Mid	2010
47	HRS#09031-II	Barley	leaf	Qld	Mt Whitestone	Mid	2009
48	98036	Barley	Leaf	NSW	Grafton	South	1999
49	98043	Barley	Leaf	Qld	Biloela RS	North	2000
50	SB61	Barley	Leaf	Qld	Monto	North	2001
51	98042	Barley	Leaf	Qld	Monto	North	1999
52	98051	Barley	Leaf	Qld	Logan Point	Mid	2000
53	98137	Barley	Leaf	NSW	Cobbitty	South	1999
54	99034	Barley	Leaf	Qld	Jandowae	North	1999
55	98129	Barley	Leaf	NSW	Moree	Mid	1999
56	USQ10015a	Wheat	crown	NSW	Coonamble	South	2010
57	HRS#09126-I	Barley Grass	leaf	WA	Wooree		2010
58	USQ10073	Barley	roots	Qld	Wellcamp	Mid	2010
59	USQ11105	Wheat	stubble	NSW	Narrabri	South	2011
60	A01#18	Wheat	root	Qld	Wellcamp	Mid	2001
61	USQ11095	Wheat	stubble	NSW	Walgett	South	2011
62	USQ11099	Wheat	stubble	NSW	Rowena	South	2011
63	USQ11100	Wheat	stubble	NSW	Narrabri	South	2011

Key	
Abbreviation	**State**
Qld	Queensland
NSW	New South Wales
Vic	Victoria
WA	Western Australia

Table 2.3 (continued) Information about each of the isolates, in the same order of the dendrogram.

No	ID	Host	Tissue	State	Town	Region	Year
64	USQ11113	Wheat	stubble	NSW	Bombala		2011
65	USQ11093	Wheat	stubble	NSW	Burren Junction	South	2011
66	USQ11108	Wheat	stubble	NSW	Rowena	South	2011
67	USQ11114	Wheat	stubble	NSW	Bombala		2011
68	A04#36	Barley	root	Qld	Tummaville	Mid	2004
69	USQ11096	Wheat	stubble	NSW	Burren Junction	South	2011
70	USQ11101	Wheat	stubble	NSW	Narrabri	South	2011
71	USQ11094	Wheat	stubble	NSW	Burren Junction	South	2011
72	USQ11097	Wheat	stubble	NSW	Burren Junction	South	2011
73	USQ11104	Wheat	stubble	NSW	Walgett	South	2011
74	USQ11109	Wheat	stubble	NSW	Burren Junction	South	2011
75	USQ11107	Wheat	stubble	NSW	Walgett	South	2011
76	USQ11106	Wheat	stubble	NSW	Bombala		2011
77	USQ11112	Wheat	stubble	NSW	Bombala		2011
78	USQ11092	Wheat	stubble	NSW	Walgett	South	2011
79	95#11	Barley	root	Qld	Millmerran	Mid	1995
80	HRS#09067	Barley	leaf	Qld	Atherton		2009
81	HRS#10214-II	Barley	root	Qld	Mt Stuart		2010
82	USQ10014	Wheat	crown	NSW	Wellington	South	2010
83	USQ10016	Wheat	stubble	NSW	Tamworth	South	2010
84	A05#7	Barley	root	Qld	Billa Billa	Mid	2005
85	USQ10017	Wheat	seed	NSW	Gunnedah	South	2010
86	A01#36	Wheat	root	NSW	Moree	Mid	2003
87	A02#86	Wheat	root	Qld	Dulacca	North	2002
88	A05#57	Wheat	root	Qld	Nindigully	Mid	2005
89	A03#47	Wheat	root	NSW	Spring Ridge	South	2003
90	USQ09009	Wheat	crown	NSW	Cryon	Mid	2009
91	A03#5	Wheat	root	Qld	Goondiwindi	Mid	2003
92	A03#6	Wheat	root	Qld	Goondiwindi	Mid	2003
93	A04#51	Wheat	root	Qld	Tara	North	2004
94	A04#11	Wheat	root	Qld	Wallumbilla	North	2004
95	A05#47	Wheat	root	Qld	Wandoan	North	2005
96	USQ08003	Durum	husk	NSW	Quirindi	South	2008
97	USQ11082	Wheat	stubble	NSW	Moree	Mid	2011
98	USQ09010	Wheat	crown	NSW	Cryon	Mid	2009
99	USQ08002	Durum	SCI	NSW	Gunnedah	South	2008
100	USQ11083	Wheat	stubble	NSW	Moree	Mid	2011
101	USQ11086	Wheat	stubble	NSW	Walgett	South	2011
102	USQ09008	Wheat	leaf	Qld	Applethorpe	Mid	2009
103	USQ10019b	Wheat	stubble	NSW	Tamworth	South	2010
104	USQ11084	Wheat	stubble	NSW	Burren Junction	South	2011
105	USQ11081	Wheat	stubble	NSW	Rowena	South	2011
106	USQ11089	Wheat	stubble	NSW	Narrabri	South	2011
107	USQ11090	Wheat	stubble	NSW	Narrabri	South	2011
108	96#15	Wheat	root	Qld	Nindigully	Mid	1996
109	USQ11098	Wheat	stubble	NSW	Narrabri	South	2011
110	USQ11111	Wheat	stubble	NSW	Bombala		2011
111	USQ11103	Wheat	stubble	NSW	Walgett	South	2011
112	A04#4	Barley	root	Qld	Wellcamp	Mid	2004
113	A01#32	Barley	root	NSW	Blackville	South	2001
114	USQ10018	Wheat	seed	NSW	Gunnedah	South	2010
115	USQ11110	Wheat	stubble	NSW	Bombala		2011
116	USQ09004a	Wheat	leaf	Qld	Applethorpe	Mid	2009
117	USQ09005a	Wheat	leaf	Qld	Applethorpe	Mid	2009
118	USQ09011	Wheat	crown	NSW	Edgeroi	South	2009
119	USQ10015b	Wheat	crown	NSW	Coonamble	South	2010
120	SB37i	Barley	seed	Vic	Woomelang		1999
121	USQ10019a	Wheat	stubble	NSW	Tamworth	South	2010
122	08014	Triticale	leaf	NSW	Acacia Plateau	Mid	2008
123	96#14	Barley	root	Qld	Nindigully	Mid	1996
124	USQ11085	Wheat	stubble	NSW	Rowena	South	2011
125	20004	Prairie Grass	Leaf	NSW	Casino	Mid	2000
126	USQ10013b	Wheat	crown	NSW	Tamworth	South	2010

Key	
Abbreviation	**State**
Qld	Queensland
NSW	New South Wales
Vic	Victoria
WA	Western Australia

2.3.4 Model-based clustering analysis

For the model-based cluster analysis the ad hoc statistic method was chosen (Figure 2.3) to determine the number of clusters (Evanno et al., 2005). In this method, K is the number of populations and ΔK is the rate of change in the log probability of data between successive K values. The log-likelihood values divided the collection into two populations.

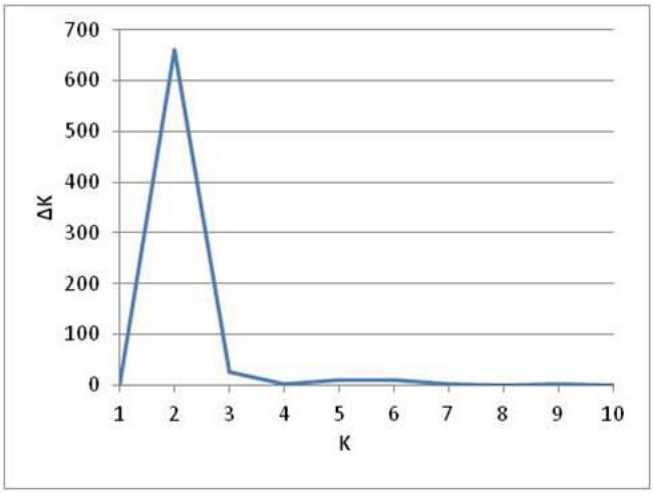


Figure 2.3 Model-based clustering analysis to infer the number of clusters (where K is the number of populations and ΔK is the rate of change in the log probability of data between successive K values).

For the graphed CLUMPP analysis (Figure 2.4), the 56 leaf and seed isolates from cluster I in Figure 2.2 showed a high probability of belonging to population 1 while the second and third clusters from Figure 2.2 and the two outliers, showed a high probability of deriving from population 2. Cluster 3 generally showed an even higher likelihood in this regard than Cluster 2. Three individuals in Cluster I indicated a likelihood of belonging to Population 2 of 45% or higher. One of these was a wheat basal (crown) isolate and was the isolate with the lowest similarity coefficient in the cluster. The other two isolates belonged to the same sub-cluster as each other within Cluster I and were both barley leaf isolates.

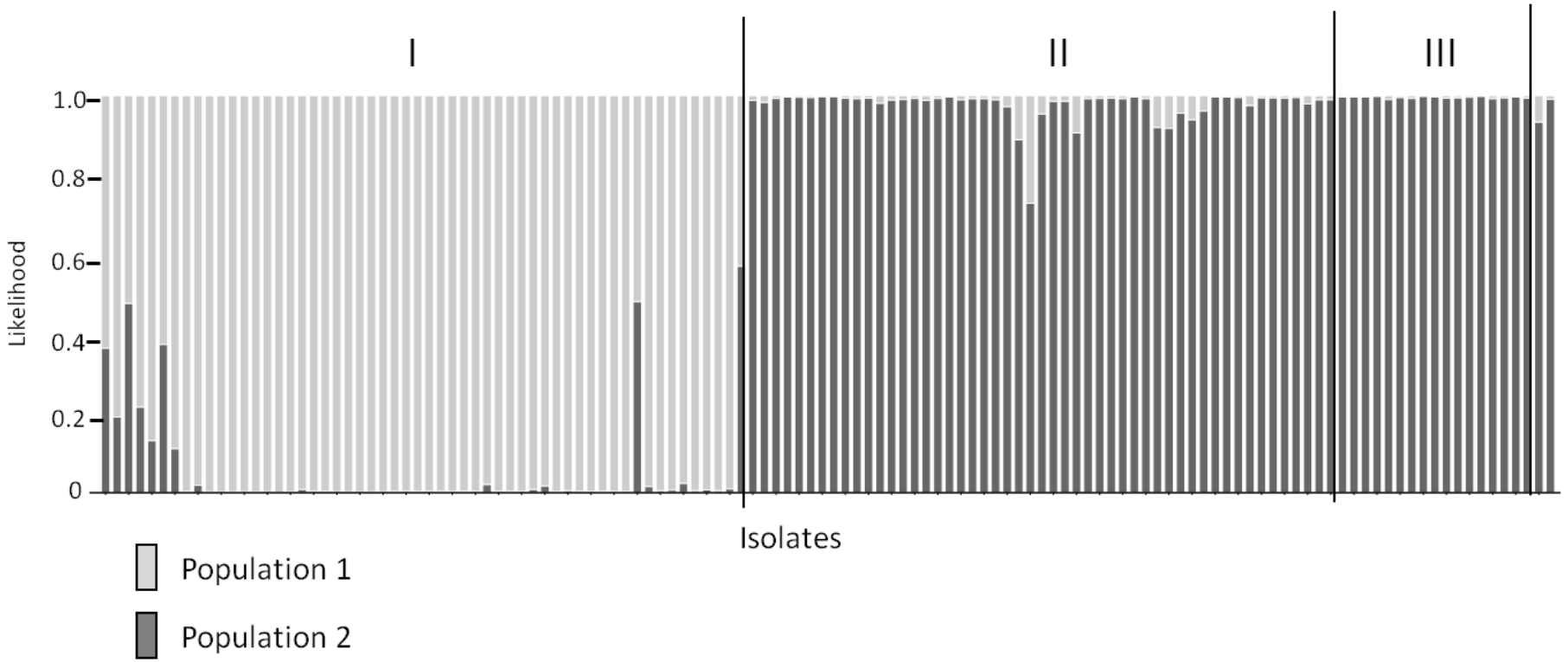


Figure 2.4 Bar graph comparing the likelihood that each individual belongs to each of the two populations. Each bar along the x-axis represents a separate individual, in the same order as the dendrogram (Figure 2.2) vertical black lines at the top of the figure indicate the three main clusters, as described in Figure 2.2. The y-axis shows the likelihood that each individual belongs to each of the two populations (Population 1 likelihood represented by light grey, and population 2 likelihood represented by dark grey), estimated to exist in the model-based cluster analysis.

2.3.5 AMOVA

Analysis of molecular variance (AMOVA) is a statistical test similar to analysis of variance (ANOVA). It compares the similarity of genotypes (in this case the AFLP patterns) of groups of isolates. Isolates were grouped according to the host and tissue of origin, and geographic location. The method was used in conjunction with the previous methods in order to determine the significance of the differences among the groups, which cannot be determined from the dendrogram and model-based analyses alone. Isolates were grouped according to host (wheat or barley) and further grouped into plant position (aerial and basal). Since isolates from stubble and below-ground plant parts clustered along with the root isolates, these were classified as basal. The leaf and seed isolates tended to cluster together, and so were classified as aerial.

Table 2.4 Summary of AMOVA for groups of Australian *B. sorokiniana* isolates.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups (wheat and barley)	1	97.192	0.77495	15.33ns
Among populations (aerial and basal isolates) within groups (wheat and barley)	2	32.289	0.97613	19.31**
Within populations	118	389.986	3.30496	65.37**

** 0.00001 level of significance

ns- Not significant

For the analysis of molecular variance (AMOVA, Table 2.4), significant differences were observed among tissue types (aerial and basal) when separated into host groups (wheat and barley). That is, significant differences were observed among aerial and basal isolates from wheat (at the 0.00001 level), and among aerial and basal isolates from barley (at the 0.00001 level). Isolates did not differ significantly from each other when separated into groups based on host. When pairwise comparisons of only wheat and barley aerial isolates were made (Table 2.5a), highly significant differences were observed (0.00001 level), and when only wheat and barley basal isolates were compared, significant differences at the 0.05 level were observed, indicating significant differences among hosts when grouped into tissue sources. Highly significant differences (at the 0.00001 level) were also observed among barley aerial and basal isolates, whereas significant differences at 0.04 level were observed among aerial and basal groups of wheat isolates.

Table 2.5 P-values obtained from pairwise Fst analysis. (a) Comparisons of wheat and barley isolates originating from aerial and basal tissues and (b) Pairwise comparisons of aerial and basal tissue isolates between northern (Nth) middle (Mid) and southern (Sth) regions of Queensland and New South Wales. Number of isolates are indicated in brackets.

(a)	**Barley Basal**	**Barley Aerial**	**Wheat Basal**	**WheatAerial**
Barley Basal (8)				
Barley Aerial (55)	0.00000			
Wheat Basal(52)	0.05237	0.00000		
WheatAerial (7)	0.24429	0.00000	0.03462	

(b)	**MidAerial**	**MidBasal**	**NthAerial**	**NthBasal**	**SthAerial**	**SthBasal**
MidAerial (34)						
MidBasal (14)	0.00000					
NthAerial (21)	0.28322	0.00000				
NthBasal (5)	-0.000090	0.29483	0.00000			
SthAerial (6)	0.08382	0.02985	0.00592	0.31146		
SthBasal (41)	0.00000	0.26091	0.00000	0.01313	-0.000090	

Isolates were divided into regions (Figure 2.5) to compare any differences between those separated by greater distances. Isolates located from Gladstone to Dalby (north to south) were grouped in the northern region, those located from Oakey to Moree were classified as mid-region, and individuals between Grafton and Newcastle were grouped in the southern region. Isolates outside of these boundaries were excluded from the analysis, as they were separated from the bulk by greater distances and the clusters were too small to make any assumptions about significant differences from the rest of the isolates. Pairwise comparison of aerial and basal isolates from the three regions (Table 2.5b) revealed mostly significant differences among aerial and basal isolate groups. However, the north and south aerial isolates differed significantly from each other (0.006 level) and the north and south basal isolates also differed significantly from each other (0.01 level), indicating that there may be some geographic differences among isolates.

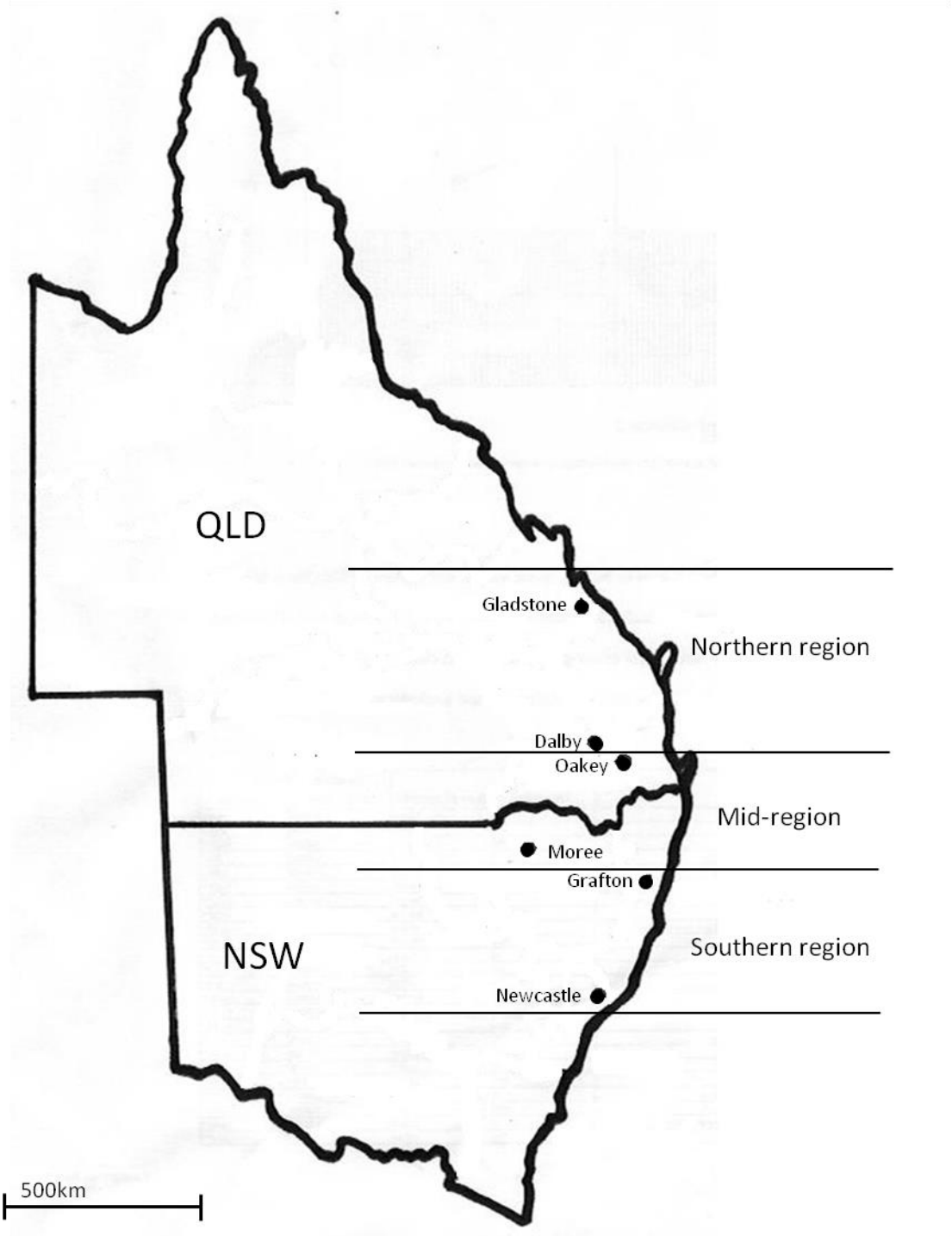


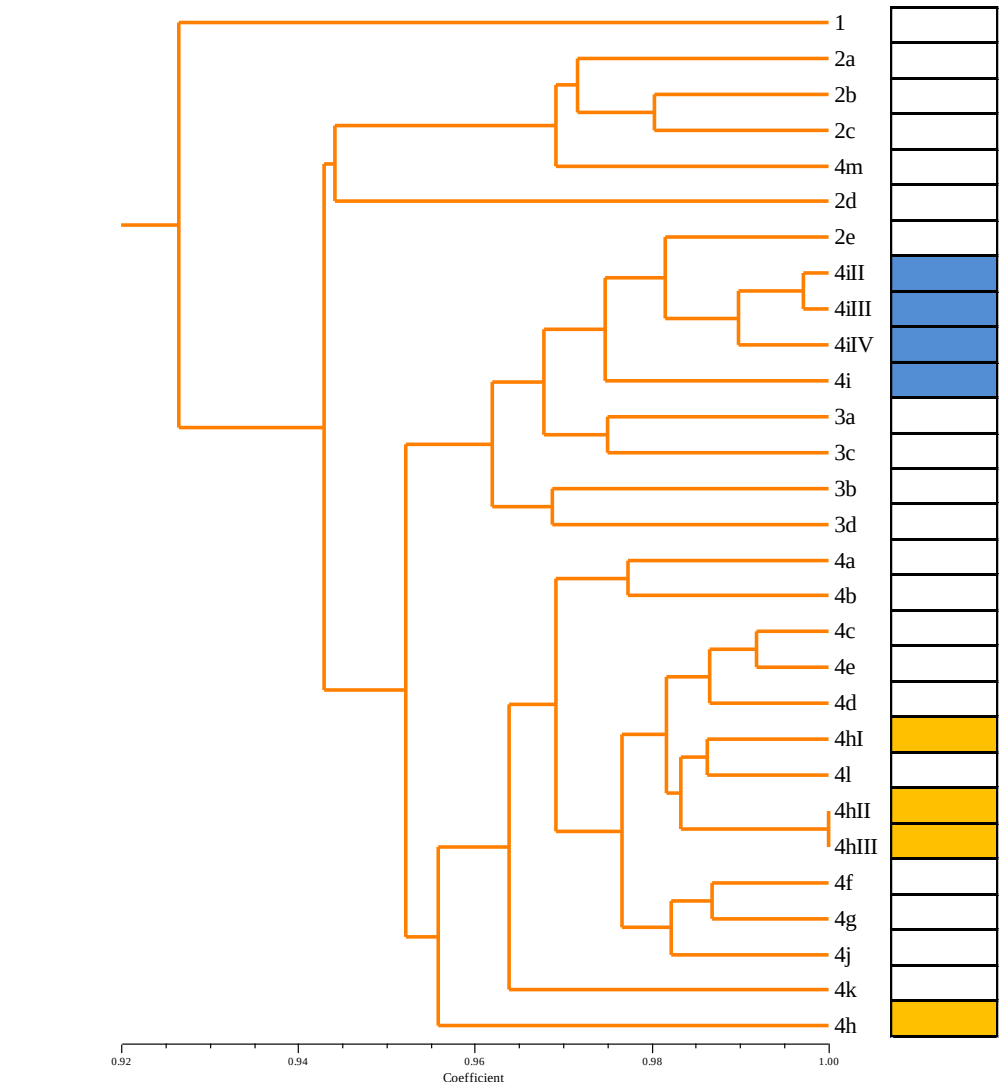
Figure 2.5 Northern, middle and southern regions within Queensland (QLD) and New South Wales (NSW).

2.3.6 AFLP Analysis of the Isolates Collected from Single Lesions from an Individual Plant in the Field

When AFLP patterns of the isolates were subjected to distance-based clustering analysis (Figure 2.6), isolates had a minimum similarity coefficient of 93 percent. Some clustering of isolates according to leaf piece was evident, but this was not always consistent. Isolates I to IV from lesion 'i' of tissue piece '4' clustered together, but none of these isolates were completely identical to each other. Isolates I to IV of lesion 'h' of the same tissue piece did not cluster as closely to each other as the lesion 'i' isolates, but instead clustered amongst the other isolates from that section of leaf. Only isolates II and III of this lesion had identical AFLP patterns.

2.3.7 AFLP Analysis of the Reisolated *B. sorokiniana* Isolates

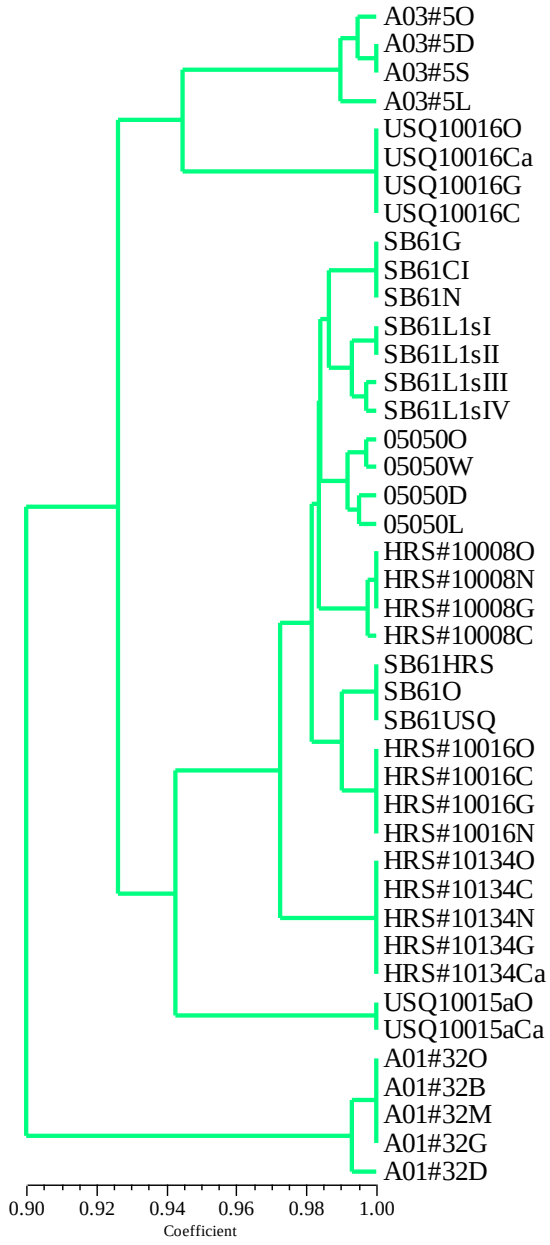
de Oliveria *et al*. (2002) reported genetic variation among isolates of *B. sorokiniana* inoculated onto leaves of different plant genotypes. The question being addressed in this section is whether this variation can occur among Australian isolates when inoculated onto different plant genotypes in controlled glasshouse conditions. When subjected to distance-based clustering analysis (Figure 2.7), the reisolated *B. sorokiniana* isolates clustered in a similar manner to the original population cluster analysis, with the root isolates clustering separately to the leaf isolates. Isolates of the same original source tended to cluster together, however were not all completely identical, and had a maximum difference in AFLP patterns of 1%. In particular, the SB61 isolates did not all cluster together and showed a much higher level of variability.



Isolates from lesion ‘i’ of leaf piece 4

Isolates from lesion ‘h’ of leaf piece 4

Figure 2.6 Dendrogram (from AFLP analysis) of multiple isolates collected from a single barley leaf from a plant inoculated in the field with the spot blotch isolate SB61. Each leaf piece (2cm segments of the same leaf, adjacent to each other) is numbered (1 to 4), each isolate sampled from a separate lesion is allocated a letter, and each isolate sampled from the same lesion is additionally assigned a roman numeral (I to IV).



Original Isolate	Original Host Species	Original Host Tissue
A03#5	Wheat	Root
USQ10016	Wheat	Stubble
SB61	Barley	Leaf
O5050	Barley	Leaf
HRS#10008	Barley	Leaf
HRS#10016	Barley	Leaf
HRS#10134	Barley	Leaf
USQ10015	Wheat	Crown
A01#32	Barley	Root

Abbreviation	Differential Line	Differential Species
O	Original	Barley
D	Delta	Barley
S	Stirling	Barley
L	Lindwall	Barley
G	Gilbert	Barley
CI	CI 1227	Barley
N	NRB091084	Barley
W	Wyalkatchem	Wheat
B	Bowman	Barley
M	Magenta	Wheat
C	Conlon	Barley
Ca	Calingiri	Wheat

Figure 2.7 Dendrogram of the reisolated *B. sorokiniana* isolates inoculated onto wheat and barley plants, recovered after infection and analysed with AFLPs. The letter after the isolate name represents the wheat or barley line from which the isolate was taken. Abbreviations and their lines are given in the table to the right of the dendrogram.

2.4 Discussion

The primers designed by Matusinsky *et al.* (2010) were not useful as species-specific primers for identifying Australian *B. sorokiniana* isolates. In fact, the *Brn1* locus by which these primers were designed showed a high level of variation among isolates within the species, even with the new markers. No marker was present in every individual, and the marker with the highest proportion of positives within the population of 126 isolates, Cos_F_248 + Cos_R_519, was still negative in 37 of the isolates. Therefore none of the markers was found to be reliable for the purpose of a species-specific marker, and other loci which are more conserved within the species should be investigated for this purpose.

The 126 isolates in the current study were from basal and aerial tissues of both wheat and barley. The genetic similarity among the 126 Australian isolates in this study was comparable to studies of *B. sorokiniana* in other countries (Ghazvini and Tekauz, 2012; Gyawali *et al.*, 2012; Zhong and Steffenson, 2001b), all of which included isolates from wheat and barley hosts. All isolates in the current study had a higher similarity coefficient (>76%) compared with the study by Knight *et al.* (2010) (>52%), which may have been due to a larger number of markers scored in the current study. The variability was hierarchically partitioned, as 65% was found among isolates derived from the same tissue-host combination, 19% from isolates derived from different tissue within a host, while host species did not appear to be as important. This is consistent with the lack of difference observed between isolates from barley roots and wheat leaves according to the AMOVA test. The most significant genetic divergence in the examined population of isolates consistently observed in all tests, was between the common root rot group and spot blotch group of isolates. The two groups clearly segregated in both the model- and distance-based cluster analyses, and significant differences were also apparent in the AMOVA at the 0.00001 level for both wheat and barley. These results indicate that tissue specificity may be relevant for Australian *B. sorokiniana* isolates, while host specificity, if any, may be weak.

Isolates originating from root, crown, SCI and stubble tissue tended to cluster separately from those originating from leaf and seed tissue. Isolates from seed and upper stem tissue clustered with leaf isolates, which indicates these isolates are able to infect all these tissues. This was also observed for isolates from stubble, SCI and crown tissue, which clustered with root tissue isolates. Limited research has been conducted to investigate differences between isolates originating from basal and aerial tissues. Gyawali *et al.* (2012) completed a population study using AFLP markers, examining 208 *B. sorokiniana* isolates from North Dakota: 76 isolates from barley leaves, 97 from barley roots and 35 from wheat roots. This study found comparable results to ours, as populations differentiated according to tissues, with barley leaf and barley root isolates forming two distinct subpopulations. Arabi and Jawhar (2007b) included five barley root isolates and 17 barley leaf isolates in an RFLP study on the genetic diversity of *B. sorokiniana* from Syria, and observed a total of 476 markers, 189 of which were polymorphic. They did not observe clustering of isolates according to tissue of origin. Knight *et al.* (2010) undertook an extensive study that compared isolates from different tissues, which included mostly spot blotch isolates from barley plants and common root rot isolates from wheat plants. A total of 134 polymorphic amplicons were observed, and they observed significant genetic divergence between the common root rot and spot blotch isolates,

but could not separate the effect of host origin from isolate tissue source. A factor which may have had an effect on the degree of variation among isolates in this study is the year in which the isolates were collected. The earlier isolates were collected in 1999 and the later isolates were collected in 2009 to 2011. The earlier isolates do not necessarily reflect what is currently present in the field.

In the current study, considerable efforts were made to include a higher proportion of *B. sorokiniana* isolates from barley roots and wheat shoots than included in earlier studies. Difficulty in obtaining isolates from barley common root rot infections was encountered, possibly due to minimal awareness of the symptoms in association with this crop. Wheat spot blotch is a rare disease state in Australia, and was represented by five isolates collected from Queensland in summer increase plots near Stanthorpe or from winter plots at Wellcamp. By comparison, 46 barley leaf isolates were included in the analysis. Despite this imbalance, when only barley and wheat spot blotch isolates were compared using AMOVA, highly significant differences were observed (P= 0.00000). This suggests there may be a level of host specificity among the spot blotch isolates. When the 51 wheat and eight barley basal tissue isolates were compared, significant differences at the 0.05 level were observed. This also suggested a level of host specificity among isolates of common root rot. Earlier studies also found evidence of host specificity of *B. sorokiniana*, based on disease expression of inoculated plants in the field and glasshouse. An investigation of wheat and barley basal isolates was conducted by Conner and Atkinson (1989) in Canada. Wheat and barley seeds were plotted in soil that had been cropped continuously with either wheat or barley naturally infested with *B. sorokiniana* spores. Wheat grown in soil that had been cropped to barley only, did not display symptoms of common root rot. The same was true for barley plants grown in soil cropped continuously to wheat. Ghazvini and Tekauz (2007) tested the virulence of 16 Canadian leaf isolates taken from three hosts (10 from wheat, 5 from oat and 1 from rye) on a differential set of barley leaves, and found that seven of the wheat isolates were assigned to the avirulent pathotype 0.0.0.0. This indicated none of the differential lines were susceptible. The single rye isolate also belonged to pathotype 0.0.0.0, as did three of the isolates collected from oat leaves. In contrast, most of the barley isolates tested caused a susceptible infection response in at least two barley genotypes from the differential set of 12 barley lines (a total of 87 isolates out of 105). These results suggest that the genetic clusters according to tissue and host origin observed in this current study may be reflected in the disease phenotypes produced when a range of wheat and barley lines are inoculated with isolates from each tissue and or host. Chapters 4 and 5 address this question.

Evidence of a relationship between geographic location and genetic diversity of *B. sorokiniana* was detected in this study, although the majority of the SB isolates were collected from Qld, and most basal isolates from NSW. Nonetheless, when aerial and basal tissue isolates were separately compared between the three regions (north, mid and south), significant differences were observed. The aerial isolates had highly significant differences between the northern and southern regions, as did the basal isolates, although less than displayed by the aerial isolates. This is unexpected, since aerial isolates would be subject to wind movements between regions, as well as transport on seeds and straw, thus encouraging gene flow (Couture and Sutton, 1978). Movement of the common root rot isolates on the other hand, is intuitively less likely due to the location of most spore bearing tissues at or below soil level and

the much more limited movement of material from this zone. This result might be attributed to low statistical power due to the lower numbers of isolates in some groups used in the comparison. Further studies of this issue are required. In the previous preliminary study, Knight *et al.* (2010) found a geographical trend in the location of CRR and SB isolates, with the CRR isolates being from more inland areas, and spot blotch isolates being sampled from more coastal locations. This group did not observe genetic differences among isolates from different geographical locations.

The genetic fingerprints of *B. sorokiniana* may have an affinity with location (Aggarwal et al., 2010; Leisova-Svobodova et al., 2011), however this research is weakly supported. Zhong and Steffenson (2001a) used AFLP marker analysis, and found isolates from a number of distinct geographic regions worldwide, including USA, China, and Australia, did not differ significantly from each other. UPGMA analysis revealed that these isolates did not cluster according to the region of origin. Similarity coefficients ranged from 0.45 to 1.00. Leisova-Svobodova *et. al.* (2011), on the other hand, found a relationship between genetic diversity of isolates with geographic origin from regions within the Czech Republic. The study determined that a significant amount of genetic variation occurred among isolates from different regions within areas of up to 80-100km, but that variation did not increase further at larger distance scales. Previous studies of other pathogens have also found significant genetic differences among various geographic regions. Bayon *et al.* (2009) investigated isolates of the heteroecious willow rust *Melampsora larici-epitea* originating from 3 sites in the UK. Meybodi *et al.* (2011) investigated isolates of the causal agent of Fusarium head blight of cereal grain, *Fusarium graminearum*, originating from four regions within the northern part of Iran. Both of these studies partitioned isolates from a single small region into even smaller regions and detected significant differences.

In general, previous population studies of *B. sorokiniana* have found an unexpectedly high level of genetic variation, considering the pathogen is thought to reproduce only by asexual means. Similarly we found that DNA fingerprints from reisolated *B. sorokiniana* strains were similar but not identical to that of the original isolate sources (Figure 2.6). Leisova-Svobodova *et. al.* (2011) also tested multiple *B. sorokiniana* isolates from the same lesion in the field and found "genetic diversity of *C. sativus* populations…was much higher than expected for an asexual organism". A study examining genetic diversity of *B. sorokiniana* reisolated from different host genotypes also claimed to find high diversification of isolates within a single generation according to the host genotype (de Oliveira et al., 2002). This study used RAPD markers, which are less reliable than AFLP markers, so the theory was tested by our group using AFLP markers. Similar results were obtained, with isolates which were infected with the same original isolate source and from the same lesion being highly similar, but not identical. This suggests that some form of recombination, or mutation, may be occurring within the single generation timescale of these experiments. A high level of variability was observed among the multiple SB61 isolates, some of which clustered separately. Other possibilities for the cause of this variability could be the effect of artificial medium on the isolate after multiple subcultures, or spore contamination during any stage in inoculum preparation. Further study should be conducted in future into the effect of artificial growth media on genetic variability of *B. sorokiniana* isolates by comparing DNA fingerprints

from an isolate recultured several times in artificial media with that of the original sampling event.

The sexual stage of *B. sorokiniana* has been reported to occur in the wild only once (Kumar et al., 2002), although it can be induced *in vitro* (Tinline, 1960). This stage is not believed to play a significant role in the lifecycle. This raises the question, how is the high level of genetic variation in this fungal pathogen generated? It has been suggested that parasexual recombination is involved in providing new sources of genetic variability. Parasexual recombination is a process by which hyphae of two genetically similar isolates can fuse after physical contact to form a heterokaryon. The heterokaryon can be defined as a hyphal cell containing two or more nuclei from genetically distinct individuals. Recombination can occur between chromosomes from the different nuclei and the haploid state is subsequently restored by abortion of extra chromosomes, producing genetically different offspring to the parents via asexual means (Burdon and Silk, 1997). This process is believed to be rare in nature due to vegetative incompatibility genes, also known as *het* or *vic* genes, which act to prevent heterokaryon formation between isolates that differ at one or more *het* loci (Clutterbuck, 1995). However, evidence for the parasexual cycle occurring in *B. sorokiniana* has been observed in laboratory conditions (Poloni et al., 2009; Tinline, 1962), but the number or location of *het* loci in *B. sorokiniana* has not been studied. The process has been well documented in *Neurospora crassa*, which is known to possess at least ten such genes and mating type genes that are also involved (Mir-Rashed et al., 2000).

Another fungal pathogen believed to reproduce only asexually, but which has unexpectedly high genetic variation is *Colletotrichum cereale*, a pathogen of turfgrasses. An investigation of transposons of this pathogen's genome revealed high levels of a directed mutation process known as repeat-induced point mutation, or RIP (Crouch et al., 2008). This process acts by mutating multicopy DNA within the genome by substituting GCs with ATs in both copies. These segments of multicopy DNA were observed to be susceptible to RIP through six generations. However this process was previously thought only to occur during the sexual cycle (Crouch et al., 2008). *B. sorokiniana* was found to have high variation in chromosome lengths between isolates (Zhong and Steffenson, 2007), which indicates the presence of extra copies of genes in some individuals. If *B. sorokiniana* completes sexual reproduction, even rarely, it may produce the general level of genetic variation observed in this and previous studies. This variation may then be maintained by parasexual recombination at individual infection sites. The testing of these hypotheses requires a focussed study of the genetic systems which operate in this fungus.

Chapter 3: Phenotypic Assessment of Spot Blotch on Leaf Tissue of Wheat and Barley

3.1 General Introduction

Results from the studies in the previous chapter indicate that isolates causing SB are genetically distinct from isolates that cause CRR. This raises the question as to whether isolates from roots are able to cause infection on wheat or barley leaves, and whether isolates from leaf infections of wheat and barley are virulent on barley and wheat roots, respectively.

The most efficient and effective method of control is the use of varieties with genetic disease resistance, and this is complemented by knowledge of the disease cycle, and also the disease epidemiology. Assessments of SB isolate pathogenicity have been made by various groups. Most of these studies tested isolates on differential sets of either wheat or barley lines, some only testing on a very limited number of differentials, yielding little information about pathotype diversity. A US study by Ghazvini and Tekauz (2007) tested the virulence diversity of 127 isolates on 12 barley genotypes and identified 8 pathotypes. A study conducted by Knight *et al.* (2010) examined virulence patterns of 31 isolates from common root rot (CRR) and SB infections on a set of 18 differentials and observed 11 pathotypes. Another study by Valjevec-Gratian and Steffenson (1997b) investigated the virulence of 36 isolates (only one of which was from a common root rot infection) on 3 barley differentials. However, due to the very minimal number of differentials tested, only three pathotypes were observed in this study. The aim of this chapter was to determine virulence levels on wheat and barley leaf tissue, of *B. sorokiniana* isolated from SB and CRR infections of wheat and barley.

3.2 Materials and Methods

3.2.1 Selection of Isolates

A selection of 25 isolates (Table 3.1) was made from the collection of 127 isolates analysed genotypically in Chapter 2. These isolates were selected according to the tissue and host of isolation and their degree of genetic relatedness. This ensured that the isolates selected for this study were genetically diverse. Eight isolates originated from barley leaves, four from barley root material, four from wheat leaves and seven from wheat roots. Two additional isolates were from other tissues (one from barley seed and one from wheat stem tissue). Agar plugs of isolates stored in glycerol were recovered from storage in the -80 degree freezer, heat shocked at 45^{o}C for 3 mins and placed on potato dextrose agar (PDA). Isolates were grown for 5 days at 24^{o}C, after which they were transferred to starch nitrate agar (SNA) to optimise spore production. Two plates per isolate were prepared by distributing five plugs of the PDA culture on each SNA plate which was incubated for 8 days at 24^{o}C.

3.2.2 Selection of Differentials

A total of eighteen wheat and barley SB differentials were selected according to their varying susceptibility to isolates of the disease and differing genetic backgrounds. A total of eight wheat varieties were selected; six current Australian wheat varieties

were recommended for testing by the breeding companies LongReach ('Wyalkatchem', 'Scout' and 'EGA Gregory') and Intergrain ('King Rock', 'Calingiri', 'Magenta' and again 'Wyalkatchem'). The two wheat varieties 'Chirya3', developed by CIMMYT and 'Ning8201' , a Chinese cultivar, were found to have SB resistance in a quantitative trait loci mapping study by Kumar *et al.* (2010), and so were also included in the differential set. Ten barley varieties (Table 3.2) were selected from a set of differentials used in testing at the Queensland Department of Agriculture, Forestry and Fisheries' (QDAFF) Hermitage Research Facility, which included the varieties ND B112, Stirling, Conlon, Delta, Gilbert, Lindwall and Tolar, and the lines NRB091084, CI1227 and CI6311, all with known differential reactions to various Australian isolates.

Table 3.1 Original infection types of *B. sorokiniana* isolates (12 from spot blotch (SB) infections, 11 from common root rot (CRR) infections, one isolated from seed tissue and one from lower stem tissue) that were phenotypically assessed on 18 differential genotypes of wheat and barley. The host species and infection type (SB or CRR) from collected isolates, the origin, and the dendrogram cluster in which the isolate appeared in Figure 2.2 are described.

USQ ID	Host	Tissue	Town	State	Cluster
05050	Barley	SB	Pilton	QLD	I
HRS#09069	Barley	SB	Atherton	QLD	I
HRS#10214-1	Barley	SB	Mt Stuart	QLD	I
USQ08007.1	Barley	SB	Bundaberg	QLD	I
SB61	Barley	SB	Monto	QLD	I
USQSeed14	Barley	Seed	Bundaberg	QLD	I
HRS#09131	Barley	SB	Minyip	Vic	I
HRS#10134s	Barley	SB	Yallaroi	NSW	I
USQ08006	Barley	SB	Acacia Plateau	NSW	I
USQ09004a	Wheat	SB	Applethorpe	Qld	III
USQ09005a	Wheat	SB	Applethorpe	Qld	III
USQ09008	Wheat	SB	Applethorpe	Qld	II
USQ11080	Wheat	SB	Wellcamp	Qld	I
A05#7	Barley	CRR	Billa Billa	QLD	-
HRS#10214-II	Barley	CRR	Mt Stuart	QLD	II
USQ10073	Barley	CRR	Wellcamp	QLD	II
A01#32	Barley	CRR	Blackville	NSW	III
A03#5	Wheat	CRR	Goondiwindi	QLD	II
A05#16	Wheat	CRR	Moonie	QLD	-
A06#1	Wheat	CRR	Wallumbilla	QLD	-
USQ10016	Wheat	Stem	Wellcamp	Qld	I
USQ09011	Wheat	CRR	Edgeroi	NSW	III
USQ10013b	Wheat	CRR	Tamworth	NSW	Outlier
USQ10015a	Wheat	CRR	Coonamble	NSW	I
A01#36	Wheat	CRR	Moree	NSW	II

Table 3.2 Barley differentials selected from the set recommended and used by the Department of Agriculture, Fisheries and Forestry. The attributes with regards to reaction to spot blotch in previous tests is listed along with the reference for the studies in which these reactions were found.

No.	Differential	Species	Attribute	Reference
1	CI 1227	Barley	Differentiates in Aust	Meldrum et al. 2004
2	CI 6311	Barley	Differentiates in Aust	Meldrum et al. 2004
3	Conlon	Barley	Differentiates in Canada	Ghazvini & Tekauz 2007
4	Delta	Barley	Differentiates in Aust	Knight et al. 2010
5	Gilbert	Barley	Differentiates in Aust	Meldrum et al. 2004
6	Lindwall	Barley	Differentiates in Aust	Meldrum et al 2004
7	ND B112	Barley	Source of resistance	Valjavec-Gratian & Steffenson 1997
8	NRB091084	Barley	Superior resistance in Aust	Franckowiak (pers. comm.)
9	Stirling	Barley	Differentiates in Aust	Meldrum et al. 2004
10	Tolar	Barley	Different resistance from ND B112	German (pers. comm.)
11	Scout	Wheat	Suggested as possible differential by LongReach	N/A
12	EGA Gregory	Wheat	Suggested as possible differential by LongReach	N/A
13	Wyalkatchem	Wheat	Suggested as possible differential by LongReach and Intergrain	N/A
14	King Rock	Wheat	Suggested as possible differential by InterGrain	N/A
15	Calingiri	Wheat	Suggested as possible differential by InterGrain	N/A
16	Magenta	Wheat	Suggested as possible differential by InterGrain	N/A
17	Ning8201	Wheat	Resistance identified in a QTL mapping study	Kumar et al. 2010
18	Chirya3	Wheat	Resistance identified in a QTL mapping study	Kumar et al. 2011

3.2.3 Preparation of Inoculum

Inoculum was prepared using isolates that had been grown on SNA. To remove conidia, six drops of Tween 20 were added to 100mL distilled water, and each plate was flooded with 5mL of this mixture. A paintbrush sterilised with 70% ethanol was used to dislodge conidia from the plate, which were then rinsed with distilled water and strained through a fine sieve. This suspension was transferred to a volumetric flask and made up to 50mL with distilled water. The conidial suspension was placed on a magnetic stirrer to prevent conidia from settling, and after an even distribution of the conidia occurred, a small amount was pipetted onto a haemocytometer to determine spore concentration. The suspensions were each diluted to an optimum 10 000 conidia per mL (Meldrum et al., 1999).

3.2.4 Preparation of Differentials

Three differentials were planted per pot, three seeds per differential, and two replicates of each differential set were planted per isolate (as shown in Figure 3.1). Each of the two replicates was arranged in a different randomised order to avoid positional effects and pots were assigned a number, such that rating of the pots could be conducted “blind” with no knowledge of which cultivar was being rated until the codes in Figure 3.1 were subsequently translated into cultivar names. Plants were fertilised with Aquasol (1 gram per litre of water) twice before inoculation, which occurred fourteen days after planting, at approximately Zadok’s growth stage 13.

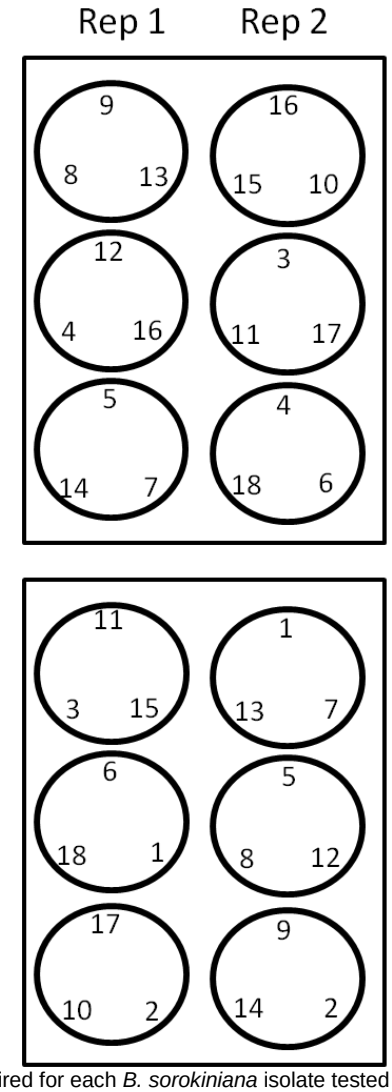


Figure 3.1 Diagram of the pots required for each *B. sorokiniana* isolate tested on each set of differentials. Each test was carried out in duplicate (with replicate 1 and replicate 2). Three differentials (three seeds each) were planted per pot, as represented by the numbers in the figure, and the order of differentials was randomised for each of the two replicates.

3.2.5 Inoculation

The differential sets were evenly sprayed with 24 mL of the diluted spore suspension, using a Preval Power Unit sprayer system. The bottle containing the spore suspension was swirled between sprays to ensure spores did not settle during application. Plants were transferred to a growth cabinet set at 100% humidity in the dark for 24 hours at 24^{o}C to establish infection. Plants were moved to the glasshouse after this incubation period and were allowed to grow and develop infection for 12 days. A total of 25 isolates were tested on the differential set, with seven of these isolates being tested in two separate experiments on the 18 differentials. A single reference isolate was included in each inoculation experiment, to allow comparisons across experiments inoculated on different days. These reference isolates were inoculated onto barley differentials only. SB61 was most frequently used as a reference, however when the SB61 isolate did not have enough growth on the PDA plate to produce sufficient inoculum, SB080 and SB015a were used as alternative reference isolates in some tests. Due to a lack of space in the growth cabinet, only four isolates including the reference could be inoculated onto a differential set in a single 24 hour period. Timing of inoculation and disease ratings also limited the

number of isolates tested to only 12 per week. As a result, only a selection of isolates were tested more than once.

3.2.6 Assessment of Disease Severity

After 12 days growth, the second leaf of all three plants in each replicate of either wheat or barley was rated for SB severity using the method of Fetch and Steffenson (1999) on a 9 point scale, described in section 1.2.1.3. Individual disease scores are also referred to as infection responses (IRs). Once data was collected for all isolates, the results were converted to coded triplet nomenclature, as described by Limpert and Müller (1994). This method summarises the virulence of each isolate on all of the differentials tested by producing a code of four digits. The differentials are kept in the same order for each isolate and grouped into threes, and each individual in the triplicate is designated a number according to whether it is resistant (SB disease score of 4.5 or less) or susceptible (SB disease score above 4.5). Resistant individuals are always designated zero, but the first individual in a triplet will be designated '1' if susceptible, the second is designated '2' if susceptible, and the third designated '4'. The total of these is a unique number which indicates the susceptible response (see example Table 3.3).

Table 3.3 Example of Triplet Coded Nomenclature described by Limpert and Muller (1994). Each susceptible genotype in a triplet is designated a unique number (1, 2, or 4, respectively). The pathotype number is the sum of the individual scores, i.e. 1 + 2 + 0 = 3. In the example below, the final pathotype would be presented as 3.7.2.0.

	Genotype A	Genotype B	Genotype C	Pathotype
Score →	**1**	**2**	**4**	
e.g.	S	S	R	3
	S	S	S	7
	R	S	R	2
	R	R	R	0

3.2.7 ANOVA

The infection responses induced by the SB and CRR isolates on the differential set were compared with ANOVA using the programme SPSS. Infection responses were compared among the following four groups: SB isolates sampled from wheat or barley, and CRR isolates sampled from either wheat or barley.

3.3 Results

3.3.1 Infection Responses of Differential Wheat and Barley Lines to Fungal Isolates

Reactions of the wheat and barley differentials to the isolates originating from different host and tissue sources were initially collected and recorded as infection responses, as per the method described by Fetch and Steffenson (1999) outlined in Section 1.2.1.3. In the following sections, these infection responses are compared between experiments (for the SB61 and additional reference isolates), between replicates within experiments, and among groups of differentials and isolates.

3.3.1.1 Comparison of Infection Responses among Replicates and Experiments

Results for the reference isolates are presented in Appendix 3.0. The number of times each isolate was tested, and the experiment(s) in which the isolates were applied are presented in Appendix 3.1. Infection responses (IRs) of the SB61 isolate were tested on all differentials for one experimental run, and on nine of the barley differentials for seven experimental runs, to test for consistency between experiments. The wheat lines were excluded from repeated testing since their response to SB61 was uniformly at the avirulent end of the scale. Two additional isolates, SB080 and USQ10015, were used as references and were tested on all differentials in one experiment each, and on nine barley differentials in one and two experiments, respectively. Five further isolates (USQ09008, HRS#10214-I, A03#5, USQ10073 and A01#32) were tested twice each on all differentials. Results were compared statistically among tests for each isolate using paired sample t-tests, with all results presented in Appendix 3.2. For the purposes of comparison, since some isolates in some experiments were tested on only a selection of the barley differentials, only results from these nine barley differentials were compared.

Of the eight SB61 reference runs (Appendix 3.2), four out of 28 pairwise FST comparisons were significantly correlated at the 0.05 significance level: set 1 and set 10; set 2 and set 7; set 5 and set 7(2); set 7 and set 7(2). However, of the remaining eight reference isolates, only one out of nine paired comparisons among repeated isolates did not correlate significantly: isolate SB015a tested in Set 4 was not significantly correlated with the same isolate tested in Set 3 and Set 3(2).

The raw SB data for isolate runs tested on all 18 of the differentials, presented in Tables 3.4 to 3.7, was used to make comparisons among groups of isolates. The infection responses (IRs) induced by each of the isolates on the wheat and barley differentials are presented for all replicates and repeats in Appendix 3.0. Un-inoculated plants in parallel experiments displayed no SB symptoms. One of the isolates (USQ10013b) was excluded due to failed infections in some replicates and visible levels of infection on the same differential in other replicates. A second isolate (USQ08006) was excluded from the final analysis due to large inconsistencies between replicates and experiments. Comparison of the individual IR scores between replicates of an isolate x host combination (complete differential sets only) shows that these scores do not differ by more than one for 540 interactions, with only 25 pairs of replicate scores differing by more than one across all experiments, and four of these scores differing by more than two across experiments. Paired sample t-tests comparing the two replicates of each isolate in each test run (Appendix 3.2) revealed significant correlations between 26 of the 30 pairs of isolate replicates at a 0.05 significance level.

When examining individual infection responses induced by isolates on each of the wheat differentials (Tables 3.4 to 3.7), the barley SB isolates caused few IRs above 3, the wheat SB isolates caused few IRs above 4 and the barley CRR isolates induced IRs no higher than 5. The wheat CRR isolates caused the highest individual infection responses in the wheat differentials, with a maximum IR of 7. On the barley differentials, higher individual IRs were observed, with maximum IRs of 9, 8, 7 and 8 for the barley SB, wheat SB, barley CRR and wheat CRR isolates, respectively. Highly susceptible scores (7 to 9) were observed on all of the barley

differentials, except ND B112 (a breeding line developed in North Dakota with known SB resistance). The breeding line NRB 091084, a recent barley line displaying high levels of spot blotch resistance, displayed highly susceptible scores when inoculated with the barley SB isolate HRS#10134s and the wheat SB isolate SB005a. Two of the barley differentials, Stirling and Gilbert, displayed the highly susceptible reactions in one replicate each to the wheat root isolate A06#1.

Table 3.4 Isolates collected from barley spot blotch infections, tested on the eight wheat and ten barley differentials with replication. Data presented includes only the experimental runs tested on the complete differential set.

Fungal Isolate Name	Wheat								Average
	Magenta	Wyalkatchem	King Rock	Calingiri	EGA Gregory	Scout	Ning8201	Chirya 3	
	1	1	2	1	1	1	1	1	**1.13**
USQ08007.1	2	2	1	2	2	1	2	1	**1.63**
	0	0	0	0	0	0	0	1	**0.13**
HRS#10214-1	0	0	0	0	0	0	0	0	**0.00**
	0	0	0	0	0	0	0	0	**0.00**
HRS#10214-1	0	0	0	0	1	0	0	0	**0.13**
	2	1	2	1	1	3	1	1	**1.50**
HRS#09069	2	2	1	1	2	1	2	2	**1.63**
	2	1	2	1	2	2	1	2	**1.63**
HRS#09131	2	2	2	1	2	1	1	2	**1.63**
	4	3	3	3	3	4	3	4	**3.38**
HRS#10134s	3	3	4	3	3	3	4	3	**3.25**
	1	1	1	1	1	1	1	1	**1.00**
USQSeed 14	2	1	1	1	1	1	1	1	**1.13**
	0	0	1	0	1	1	2	1	**0.75**
O5050	0	0	1	0	0	0	3	1	**0.63**
	3	3	3	2	2	2	2	3	**2.50**
SB61	3	2	4	2	3	3	3	4	**3.00**
Average	**1.50**	**1.22**	**1.56**	**1.06**	**1.39**	**1.33**	**1.50**	**1.56**	

Fungal Isolate Name	Barley										Average
	ND B112	NRB091084	Stirling	Conlon	Delta	Gilbert	Lindwall	CI 1227	Tolar	CI 6311	
	2	3	3	3	5	3	6	5	3	4	**3.70**
USQ08007.1	3	3	3	3	5	5	7	6	3	5	**4.30**
	0	1	1	1	2	1	2	2	1	2	**1.30**
HRS#10214-1	0	0	1	1	2	2	3	3	2	3	**1.70**
	1	0	1	3	4	2	3	3	2	2	**2.10**
HRS#10214-1	0	0	2	3	4	3	4	4	3	3	**2.60**
	4	4	4	4	6	6	8	5	4	8	**5.30**
HRS#09069	3	4	4	6	6	7	7	6	3	6	**5.20**
	3	3	5	4	5	5	4		4	4	**4.11**
HRS#09131	3	5	5	3	4	4	5	7	5	4	**4.50**
	5	7	6	5	6	6	6	7	5	6	**5.90**
HRS#10134s	4	6	6	4	7	7	-	7	6	7	**6.00**
	3	3	5	3	4	5	4	4	5	4	**4.00**
USQSeed 14	3	4	4	4	5	5	6	5	3	4	**4.30**
	3	2	5	4	6	0	5	5	5	5	**4.00**
O5050	2	3	5	3	6	4	4	4	4	6	**4.10**
	4	6	9	7	9	9	9	7	8	8	**7.60**
SB61	4	5	9	7	-	8	9	6	9	8	**7.22**
Average	**2.61**	**3.28**	**4.33**	**3.78**	**5.06**	**4.56**	**5.41**	**5.06**	**4.17**	**4.94**	

Table 3.5 Isolates collected from wheat spot blotch infections, tested on the eight wheat and ten barley differentials with replication. Data presented includes only the experimental runs tested on the complete differential set.

Fungal Isolate Name	Wheat								Average
	Magenta	Wyalkatchem	King Rock	Calingiri	EGA Gregory	Scout	Ning8201	Chirya 3	
USQ09004a	3	3	4	3	3	4	4	4	**3.50**
	4	4	4	4	4	4	4	4	**4.00**
USQ09005a	3	4	5	5	3	3	2	4	**3.63**
	4	4	5	4	4	4	3	5	**4.13**
USQ09008	0	1	1	1	2	1	0	1	**0.88**
	0	1	2	2	2	1	1	1	**1.25**
USQ09008	0	1	1	3	3	2	1	1	**1.50**
	0	1	3	4	4	3	2	1	**2.25**
USQ11080	3	3	3	3	2	2	2	3	**2.63**
	3	2	4	4	3	3	2	2	**2.88**
Average	**2.00**	**2.40**	**3.20**	**3.30**	**3.00**	**2.70**	**2.10**	**2.60**	

Fungal Isolate Name	Barley										Average
	ND B112	NRB091084	Stirling	Conlon	Delta	Gilbert	Lindwall	CI 1227	Tolar	CI 6311	
USQ09004a	5	6	3	4	4	4	3	4	3	5	**4.10**
	5	5	3	3	4	3	3	4	4	4	**3.80**
USQ09005a	5	7	4	-	6	6	7	4	5	7	**5.67**
	4	8	5	6	6	5	8	4	4	7	**5.70**
USQ09008	1	1	1	1	1	1	1	2	1	1	**1.10**
	1	1	2	1	1	1	1	2	1	1	**1.20**
USQ09008	1	1	1	1	1	1	1	3	1	2	**1.30**
	2	1	3	1	1	1	1	2	2	1	**1.50**
USQ11080	4	2	4	4	5	4	4	5	4	5	**4.10**
	4	4	-	3	5	3	3	4	4	5	**3.89**
Average	**3.20**	**3.60**	**2.89**	**2.67**	**3.40**	**2.90**	**3.20**	**3.40**	**2.90**	**3.80**	

Table 3.6 Isolates collected from barley common root rot infections, tested on the eight wheat and ten barley differentials with replication. Data presented includes only the experimental runs tested on the complete differential set.

	Wheat								
Fungal Isolate Name	**Magenta**	**Wyalkatchem**	**King Rock**	**Calingiri**	**EGA Gregory**	**Scout**	**Ning8201**	**Chirya 3**	**Average**
	2	2	4	3	3	3	3	4	**3.00**
HRS#10214-II	3	3	3	2	3	2	2	3	**2.63**
	2	3	3	3	4	2	3	2	**2.75**
USQ10073	1	2	3	2	3	3	2	2	**2.25**
	3	3	4	4	4	3	3	3	**3.38**
USQ10073	2	4	4	3	4	4	2	3	**3.25**
	3	3	4	3	2	3	3	3	**3.00**
A01#32	3	4	4	3	2	2	4	3	**3.13**
	3	4	4	3	3	3	3	4	**3.38**
A01#32	2	4	3	2	3	3	3	5	**3.13**
	2	2	2	3	4	3	4	3	**2.88**
A05#7	2	1	3	4	3	3	2	4	**2.75**
	2	2	4	2	2	4	3	2	**2.63**
A05#16	2	3	5	2	3	4	3	1	**2.88**
Average	**2.29**	**2.86**	**3.57**	**2.79**	**3.07**	**3.00**	**2.86**	**3.00**	

	Barley										
Fungal Isolate Name	**ND B112**	**NRB091084**	**Stirling**	**Conlon**	**Delta**	**Gilbert**	**Lindwall**	**CI 1227**	**Tolar**	**CI 6311**	**Average**
	3	3	4	2	3	4	3	3	4	3	**3.20**
HRS#10214-II	3	4	3	3	4	4	3	3	3	4	**3.40**
	1	1	1	1	2	1	1	2	1	1	**1.20**
USQ10073	1	2	1	1	1	1	1	2	1	2	**1.30**
	1	2	1	1	2	2	1	2	2	2	**1.60**
USQ10073	2	3	1	1	1	2	2	2	1	3	**1.80**
	4	5	3	3	2	2	3	3	4	3	**3.20**
A01#32	4	4	4	4	2	3	3	2	4	2	**3.20**
	5	6	7	5	-	5	6	4	4	4	**5.11**
A01#32	4	5	7	5	3	5	6	4	5	5	**4.90**
	3	1	4	3	2	3	3	4	2	2	**2.70**
A05#7	2	2	3	4	3	4	2	3	3	2	**2.80**
	2	2	3	1	2	3	3	4	1	2	**2.30**
A05#16	2	1	2	2	1	3	3	3	2	1	**2.00**
Average	**2.64**	**2.93**	**3.14**	**2.57**	**2.15**	**3.00**	**2.86**	**2.93**	**2.64**	**2.57**	

Table 3.7 Isolates collected from wheat common root rot infections, tested on the eight wheat and ten barley differentials with replication. Data presented includes only the experimental runs tested on the complete differential set.

Fungal Isolate Name	Wheat								
	Magenta	Wyalkatchem	King Rock	Calingiri	EGA Gregory	Scout	Ning8201	Chirya 3	Average
USQ10016	4	2	4	4	3	4	4	5	**3.75**
	4	3	4	4	4	4	4	1	**3.50**
USQ09011	1	0	0	3	0	1	2	1	**1.00**
	2	0	0	3	1	2	2	2	**1.50**
A03#5	4	-	5	4	4	3	5	5	**4.29**
	4	5	5	4	5	4	4	5	**4.50**
A03#5	6	6	7	5	5	5	6	5	**5.63**
	7	5	6	6	4	5	5	6	**5.50**
A01#36	4	3	3	2	3	5	4	4	**3.50**
	5	4	4	1	3	4	3	3	**3.38**
A06#1	7	6	6	4	3	4	5	4	**4.88**
	3	6	5	3	4	4	4	5	**4.25**
USQ10015a	5	4	4	4	4	4	4	4	**4.13**
	4	4	5	5	4	4	4	4	**4.25**
Average	**4.29**	**3.69**	**4.14**	**3.71**	**3.36**	**3.79**	**4.00**	**3.86**	

Fungal Isolate Name	Barley									
	ND B112	NRB091084	Stirling	Conlon	Delta	Gilbert	Lindwall	CI 1227	Tolar	CI 6311	Average
USQ10016	1	3	1	2	2	1	1	2	1	2	**1.60**
	1	2	1	2	2	1	-	2	1	2	**1.56**
USQ09011	3	1	0	1	0	1	2	0	2	2	**1.20**
	3	2	1	2	3	1	2	2	2	1	**1.90**
A03#5	2	5	4	4	4	3	6	4	2	4	**3.80**
	3	4	3	5	-	4	5	3	2	4	**3.67**
A03#5	2	4	4	5	5	5	7	4	3	5	**4.40**
	2	5	5	5	4	5	6	3	3	4	**4.20**
A01#36	1	4	3	4	3	5	3	3	2	3	**3.10**
	1	3	3	4	3	6	3	2	2	2	**2.90**
A06#1	3	5	8	5	3	7	6	5	3	4	**4.90**
	3	6	7	6	-	8	6	4	3	5	**5.33**
USQ10015a	5	5	4	5	5	6	4	5	6	4	**4.90**
	6	5	5	5	5	5	5	4	6	5	**5.10**
Average	**2.57**	**3.86**	**3.50**	**3.93**	**3.25**	**4.14**	**4.31**	**3.07**	**2.71**	**3.36**	

3.3.1.2 Average Infection Response Induced By Isolates on Barley Differentials

Average infection response of each barley differential to the barley and wheat SB and CRR isolates is presented in Figure 3.2. Only data from experiments tested on the complete differential set are used for the comparisons, to prevent biasing of data due to the use of only barley differentials in some tests. The isolate group inducing the most consistently high average IRs on the barley differentials were the barley SB isolates, with Delta, Lindwall and CI6311 having the highest IRs, and ND B112 having the lowest IR. The variety Delta is known to have genetic resistance to CRR, and ND B112 has known SB resistance. The isolates with the most consistently low IRs on the barley differentials were the barley CRR isolates, with Stirling having the highest IR and Delta the lowest.

3.3.1.3 Average Infection Response Induced By Isolates on Wheat Differentials

The average IRs induced by each isolate source on the wheat differentials is presented in Figure 3.3. The isolates with the most consistently high IRs induced on the wheat differentials were the wheat CRR isolates, with Magenta and King Rock having the highest IRs and Wyalkatchem and EGA Gregory the lowest. The isolates with the most consistently low IRs induced on the wheat differentials were the barley leaf isolates, with Chirya 3 and Wyalkatchem having the highest IRs and Calingiri the lowest. Chirya 3 is a synthetic wheat developed at CIMMYT with known SB resistance (Kumar et al., 2010).

3.3.1.4 Comparison of Infection Response Induced on Differentials by Isolates Sampled from Different Tissues and Hosts

When comparing the average IRs induced by each host/tissue group of isolates on the barley differentials (Figure 3.4a) it is apparent that the barley SB isolates induced a significantly higher average IR than the other three groups. Statistical t-tests comparing pairs of groups (Appendix 3.3) showed this difference to be highly significant, and there was also a significant difference (at the 0.05 level) between the wheat and barley CRR reactions induced on the barley differentials. Comparisons of the average IRs induced on the wheat differentials by each isolate group (Figure 3.4b) showed the lowest response of the differentials to the barley SB isolates and the highest response to the wheat CRR isolates. T-tests comparing pairs of isolate sources (Appendix 3.3) showed the barley SB isolates to induce significantly lower reactions on the wheat differentials than all other categories (at the 0.001 level), and the wheat CRR isolates to induce significantly higher levels of infection on wheat than all other categories (at the 0.001 significance level).

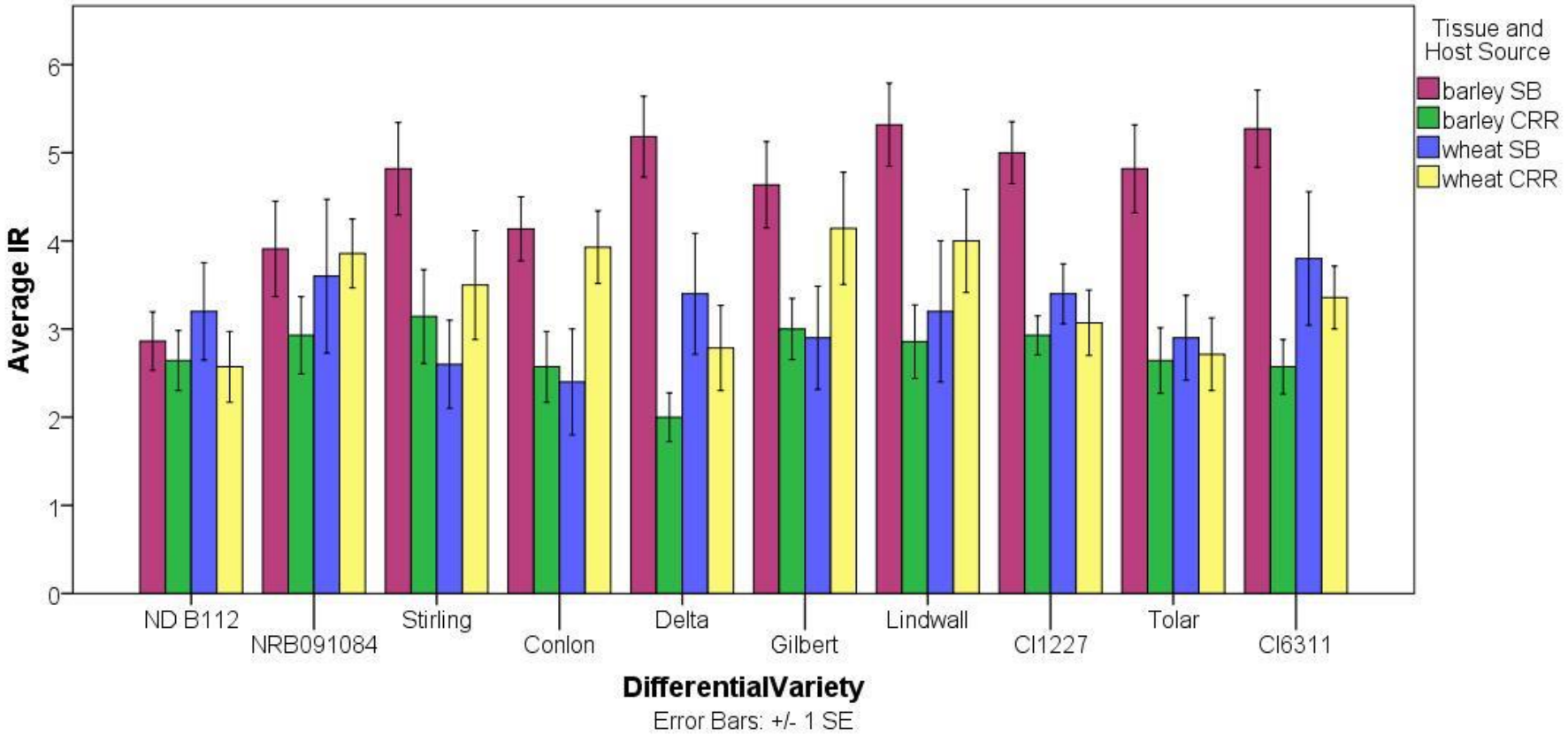


Figure 3.2 Average infection response (IR) induced on each barley differential by each isolate source (barley SB, wheat SB, barley CRR, wheat CRR).

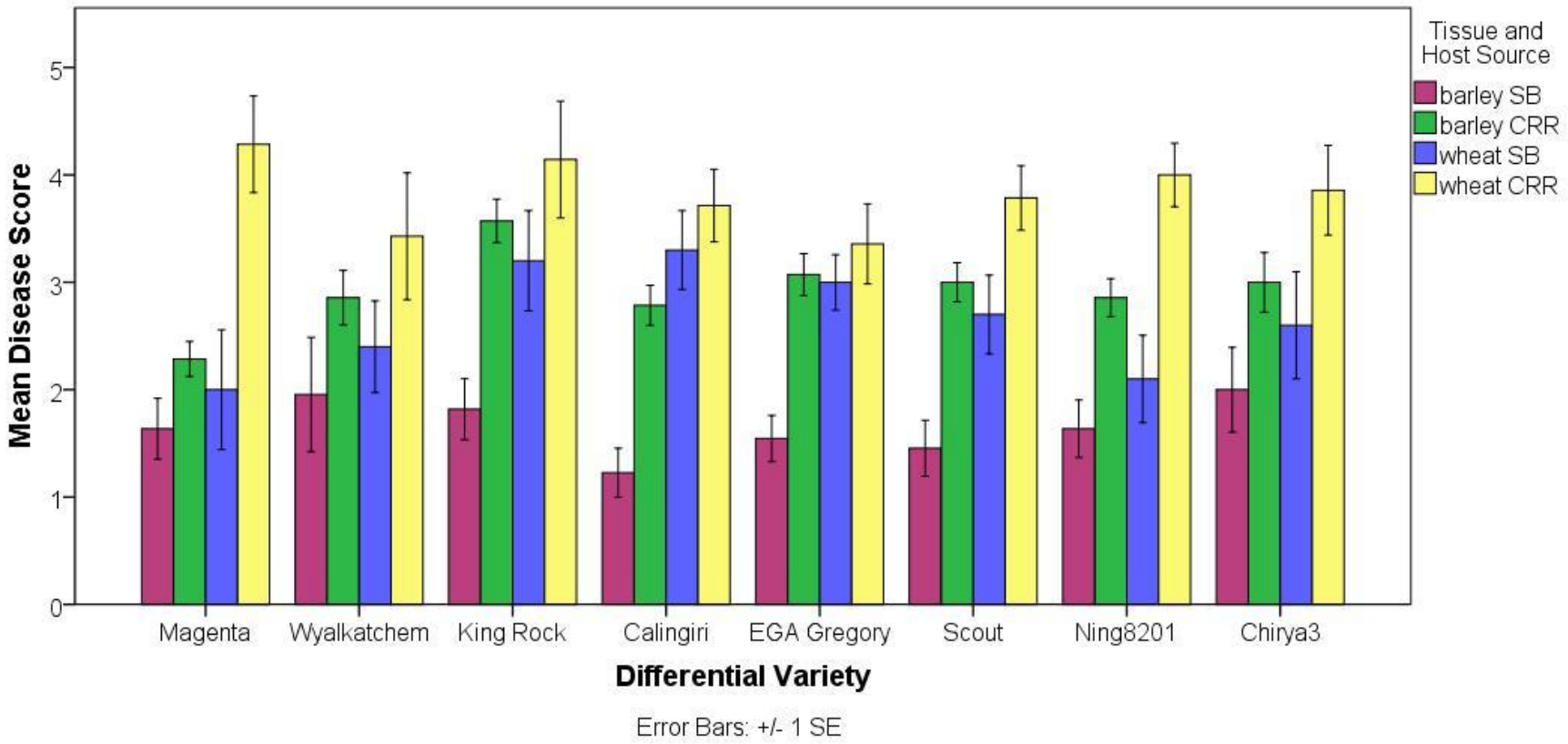


Figure 3.3 Average infection response (IR) induced on each wheat differential by each isolate source (barley SB, wheat SB, barley CRR, wheat CRR).

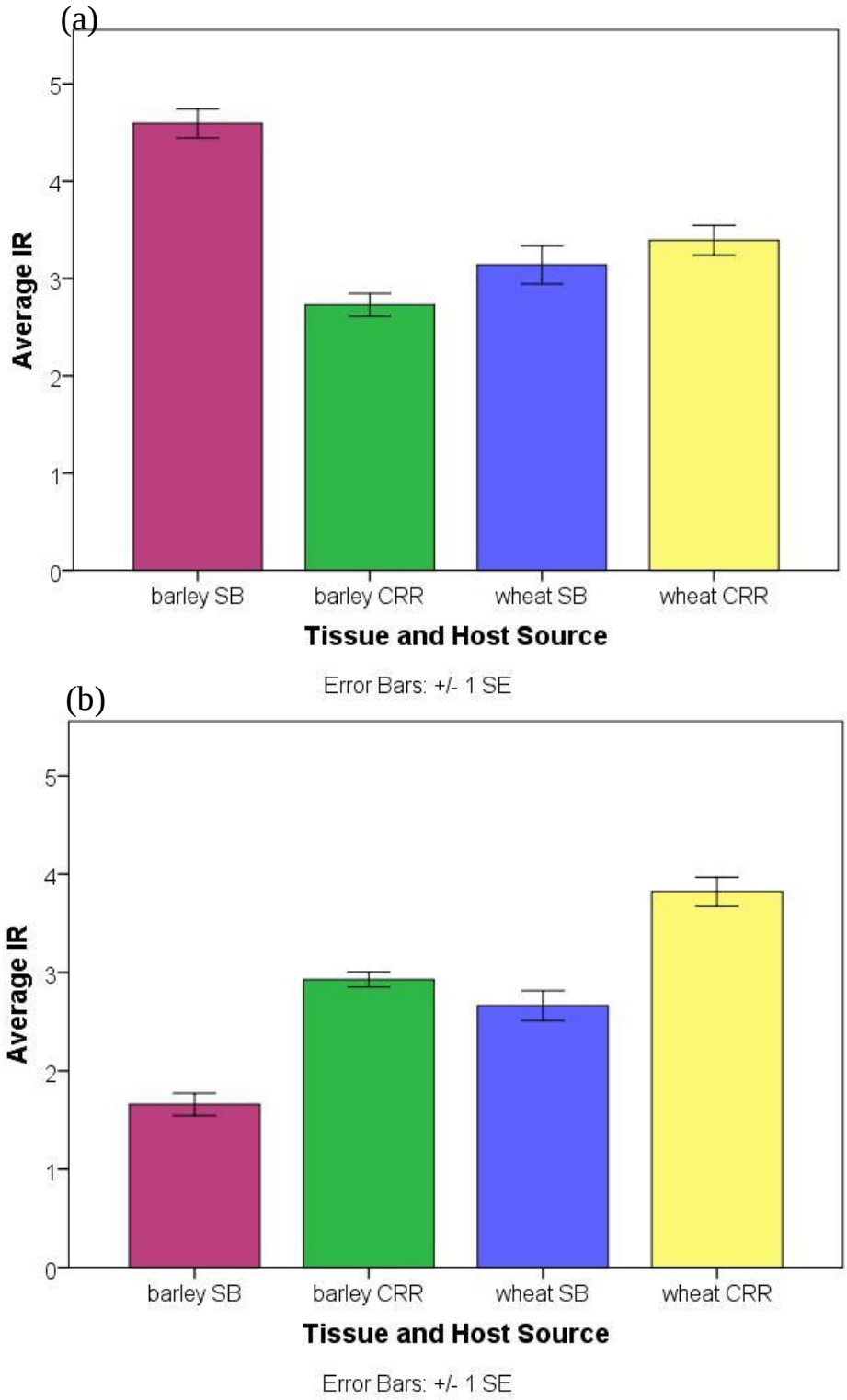


Figure 3.4 Average infection response (IR) induced by each isolate source (barley SB, wheat SB, barley CRR, wheat CRR) on (a) the barley differentials and (b) the wheat differentials.

3.3.2 Barley and wheat spot blotch pathotypes determined by triplet coded nomenclature

The IRs of isolates were averaged over replicates for each differential (using only data tested on the complete differential set) and then converted to either resistant or susceptible responses in order to assign a pathotype to each isolate. For the purposes of this exercise, a resistant IR was defined as a score of 0 to 4.5, while a susceptible IR was any score above 4.5. Since the wheat differentials EGA Gregory and Scout were classified as resistant to all isolates, and hence would not affect the pathotype scoring, these were not included in the final results. To make the number of differentials multiple of three for the purposes of generating pathotypes, wheat variety Calingiri was removed, as it had the same pattern as Chirya3 and Ning8201. A total of 16 pathotypes were observed among the 23 isolates analysed. The resistant and susceptible patterns for each of these pathotypes is given in Table 3.8. The two barley varieties susceptible to the least number of isolates were ND B112 and Tolar, which are known to possess different genetic resistance genes from each other. ND B112 was susceptible to only one isolate (USQ09004a), to which Tolar showed a reasonable level of resistance. Tolar was susceptible to three isolates (HRS#10134s, SB61 and USQ10015a) to which ND B112 displayed resistance.

Table 3.8 Summary of the virulence patterns for each pathotype of the spot blotch and common root rot isolates on the wheat and barley differential set. For all varieties susceptible to a given isolate, the first number in each triplicate is scored as 1, the second as 2, and the third as 4. These numbers are added together for each set of three and given as a five digit pathotype score. The number of isolates belonging to each pathotype is given in the final column.

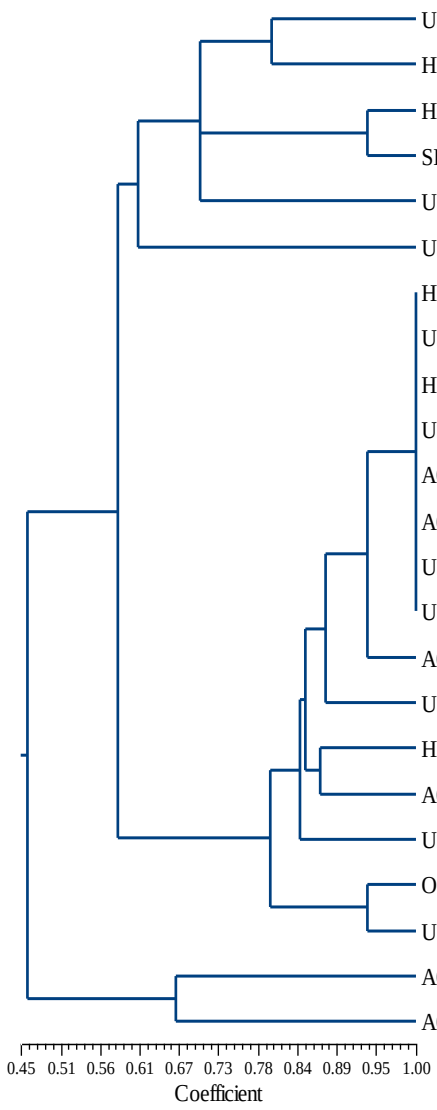
Number	Magenta	Wyalkatchem	King Rock		Ning8201	Chirya 3	ND B112		NRB091084	Stirling	Conlon		Delta	Gilbert	Lindwall		CI 1227	Tolar	CI 6311		Pathotype	No. Isolates
Code →	**1**	**2**	**4**		**1**	**2**	**4**		**1**	**2**	**4**		**1**	**2**	**4**		**1**	**2**	**4**			
1	R	R	R		R	R	R		R	R	R		R	R	R		R	R	R		**0.0.0.0**	**8**
2	R	R	R		R	R	R		R	R	R		S	R	R		R	R	S		**0.0.0.1.4**	**1**
3	R	R	R		R	R	R		R	R	R		R	S	R		R	R	R		**0.0.0.2.0**	**1**
4	R	R	R		R	R	R		R	R	R		S	R	S		S	R	R		**0.0.0.2.6**	**1**
5	R	R	R		R	R	R		R	R	R		R	S	S		R	R	R		**0.0.0.6.0**	**1**
6	R	R	R		R	R	R		R	S	R		R	R	R		S	R	R		**0.0.2.0.1**	**1**
7	R	R	R		R	R	R		R	S	R		S	R	R		R	R	S		**0.0.2.1.4**	**1**
8	R	R	R		R	R	R		S	S	R		R	R	R		R	R	R		**0.0.3.0.0**	**1**
9	R	R	R		R	R	R		S	S	R		S	S	S		S	S	S		**0.0.3.7.7.**	**1**
10	R	R	R		R	R	R		R	R	S		S	S	S		S	R	S		**0.0.4.7.5**	**1**
11	R	R	R		R	R	R		S	S	S		S	S	S		S	S	S		**0.0.7.7.7**	**1**
12	R	R	R		R	R	S		S	R	R		R	R	R		R	R	R		**0.4.1.0.0**	**1**
13	R	R	R		R	R	S		S	R	S		S	S	R		R	S	R		**0.4.5.3.2**	**1**
14	R	R	S		R	R	R		S	R	S		S	S	S		R	R	S		**4.0.5.7.4**	**1**
15	S	S	S		R	R	R		S	S	S		R	S	S		R	R	R		**7.0.7.6.0**	**1**
16	S	S	S		S	S	R		R	R	S		R	R	S		R	R	R		**7.3.4.4.0**	**1**

Pathotypes of individual isolates and their sources of origin are presented in Table 3.9. Six out of the eight isolates showing low virulence on all of the differentials (pathotype 0.0.0.0.0) originated from common root rot infections, and two isolates of this pathotype originated from SB infections, one from wheat and one from barley. Each of the remaining isolates, which caused susceptible reactions in at least one of the differentials, had its own individual pathotype, and caused a range of reactions on the differential set. These isolates caused susceptible reactions in 1 to 9

differentials (2 to 9 for the SB isolates, and 1 to 8 for the common root rot isolates). The dendrogram of the pathotypes (Figure 3.5) shows that isolates did group to an extent with regards to host or tissue of origin, with isolates mainly clustering according to the infection type (CRR or SB). The three clusters differed in the average infection response, with Cluster 1 displaying a lower IR on wheat and a higher IR on barley, isolates in Cluster 2 isolates the least aggressive on both wheat and barley differentials, and Cluster 3 isolates displaying moderate IRs on both the wheat and barley differentials. The majority of isolates in Cluster 1 were isolated from barley SB infections, explaining the higher IRs on barley differentials

Table 3.9 Summary of the pathotype designated to each isolate tested on leaves of wheat and barley. The type of infection (common root rot, CRR; spot blotch, SB) and host species from which the isolates were collected are indicated.

Host	Infection Type	Fungal Isolate Name	Pathotype
Barley	SB	USQ08007.1	0.0.0.2.6
Barley	SB	HRS#10214-I	**0.0.0.0.0**
Barley	SB	HRS#09069	0.0.4.7.5
Barley	SB	HRS#09131	0.0.2.0.1
Barley	SB	HRS#10134s	0.0.3.7.7.
Barley	SB	USQSeed 14	0.0.0.6.0
Barley	SB	O5050	0.0.2.1.4
Barley	SB	SB61	0.0.7.7.7
Wheat	SB	USQ09004a	0.4.1.0.0
Wheat	SB	USQ09005a	4.0.5.7.4
Wheat	SB	USQ09008	**0.0.0.0.0**
Wheat	SB	USQ11080	0.0.0.1.4
Barley	CRR	HRS#10214-II	**0.0.0.0.0**
Barley	CRR	USQ10073	**0.0.0.0.0**
Barley	CRR	A01#32	0.0.3.0.0
Barley	CRR	A05#7	**0.0.0.0.0**
Barley	CRR	A05#16	**0.0.0.0.0**
Wheat	CRR	USQ10016	**0.0.0.0.0**
Wheat	CRR	USQ09011	**0.0.0.0.0**
Wheat	CRR	A03#5	7.3.4.4.0
Wheat	CRR	A01#36	0.0.0.2.0
Wheat	CRR	A06#1	7.0.7.6.0
Wheat	CRR	USQ10015a	0.4.5.3.2



Cluster	Host	Tissue	Pathotype	Average IR on Wheat Diffs	Average IR on Barley Diffs
1	Barley	SB	0.0.0.2.6	2.85	5.54
	Barley	SB	0.0.4.7.5		
	Barley	SB	0.0.3.7.7.		
	Barley	SB	0.0.7.7.7		
	Wheat	SB	4.0.5.7.4		
	Wheat	CRR	0.4.5.3.2		
2	Barley	SB	**0.0.0.0.0**	2.34	2.85
	Wheat	SB	**0.0.0.0.0**		
	Barley	CRR	**0.0.0.0.0**		
	Barley	CRR	**0.0.0.0.0**		
	Barley	CRR	**0.0.0.0.0**		
	Barley	CRR	**0.0.0.0.0**		
	Wheat	CRR	**0.0.0.0.0**		
	Wheat	CRR	**0.0.0.0.0**		
	Wheat	CRR	0.0.0.2.0		
	Barley	SB	0.0.0.6.0		
	Barley	SB	0.0.2.0.1		
	Barley	CRR	0.0.3.0.0		
	Wheat	SB	0.4.1.0.0		
	Barley	SB	0.0.2.1.4		
	Wheat	SB	0.0.0.1.4		
3	Wheat	CRR	7.3.4.4.0	4.84	4.34
	Wheat	CRR	7.0.7.6.0		

Figure 3.5 Dendrogram of the pathotypes determined using Triplet Coded Nomenclature. The relationship among isolates was determined using the Simple Matching (SM) similarity coefficient and UPGMA (Unweighted Pair Group Method with Arithmetic Means). The dendrogram was divided into three clusters, as indicated in the first column of the table to the right. Average infection responses (IRs) demonstrated by the isolates in each cluster, on the wheat and barley differentials are presented in the final two columns.

3.4 Discussion

The results from the examined isolates of *B. sorokiniana* indicate a certain level of host and tissue specificity. When examining the reactions of the barley differentials to each isolate group, the barley SB isolates induced significantly higher average IRs than all other isolate groups (Figure 3.4a). The wheat CRR isolates also produced significantly higher average IRs on the barley differentials than did the barley CRR isolates, based on t-tests (Appendix 3.3). These results indicate host specificity on barley, as the wheat isolates were far less aggressive on the barley differentials. They also indicate tissue specificity, as the barley CRR isolates also were far less virulent on leaves of barley plants than barley SB isolates. These results confirm the findings suggested by the preliminary study of Knight *et al.* (2010), and the genetic diversity study by Gyawali *et al.* (2012), in which the wheat and barley CRR isolates were found to be genetically distinct.

Inoculations of the wheat differentials, an aspect which has not previously been investigated on multiple wheat genotypes using Australian isolates from different host and tissue sources, had contrasting results to those of the barley differentials. The most virulent isolates were those from the wheat CRR infections, with the wheat SB isolates producing significantly lower IRs on the wheat differentials. Since SB is known to be insignificant to wheat in Australia (Murray and Brennan, 2009), it could be suggested that these wheat SB isolates are genetically more adapted to infection of barley. However, the average virulence of the wheat SB isolates was the same on barley and wheat. So although spot blotch of wheat is insignificant in Australia, there appear to be isolates that possess a significant level of host specificity. The high level of wheat CRR virulence on wheat leaves indicates that virulent spot blotch infection of wheat does have the potential to occur with isolates originating from CRR infections, and while historically Australian environmental conditions do not tend to be conducive to development of the disease (Acharya et al., 2011), any change in climate towards warmer, humid springs, especially in the northern grains region, may lead to an increase in this disease in Australian wheat crops.

There is a distinct lack of literature investigating virulence of common root rot isolates on leaf tissue. A virulence study conducted in the USA by Valjavec-Gratian and Steffenson (1997b) examined virulence of only a single *B. sorokiniana* root isolate from wheat (out of 36 total isolates) on a minimal differential set of three barley varieties (ND 5883, Bowman, and ND B112). Using the SB rating method described by Fetch and Steffenson (1999), this group found the root isolate to have low virulence. A second US study by Zhong and Steffenson (2001b) tested only one root isolate, which was the same isolate tested by Valjavec-Gratian and Steffenson (1997b), and was again tested on the same three barley differentials using the same rating scale, with comparable results. A third study conducted in Mexico (Duveiller and Altamirano, 2000) tested virulence of a total of 27 wheat *B. sorokiniana* isolates (9 each from root, leaf and seed tissue) on leaves of a single wheat variety (Ciano T-79). This group assessed severity of SB by counting the number of lesions per leaf after inoculation, and did not estimate lesion size. However, this group did find wheat root isolates to be more aggressive than wheat leaf and seed isolates on the leaf tissue of this single cultivar.

It was observed in the genetic diversity study in Chapter 2, and also in previous work by Knight *et al.* (2010) and Gyawali *et al.* (Gyawali *et al.*, 2012)(2012) that there is

some evidence for host specificity of the pathogen. Previous investigations tested a small selection of isolates from alternate hosts on barley differentials. For example, Ghazvini and Tekauz (2007) tested virulence of 105 barley isolates, 10 wheat, three oat and one rye isolate on leaves of 12 barley differentials. Out of the barley isolates, 18 were found to have the low virulence pathotype of 0.0.0.0. Of the isolates from the alternate hosts, three out of ten wheat isolates and two out of five oat isolates induced a response classified as 'susceptible' on one or more of the barley differentials. The single rye isolate was not virulent on any differentials (i.e. 0.0.0.0). The earlier mentioned study by Zhong and Steffenson (2001b) included 48 barley isolates (11 from seeds and 37 from leaf tissue) and seven wheat isolates (six from seeds and one from root tissue), which were tested for virulence on the three barley differentials. This group found four of the wheat seed isolates caused reactions classified as 'susceptible' (according to the coded triplet nomenclature method of Limpert and Muller (1994)) on one of the differentials, while the other three isolates (including the single wheat root isolate) were avirulent on all of the differentials. Of the 11 barley kernel isolates, three caused susceptible reactions in at least one differential, and of the 37 barley leaf isolates, 19 caused susceptible reactions on at least on differential. This experiment tells us very little about the overall host specificity of isolates since it includes a very small set of differentials, all developed in North Dakota. The study by Knight *et al.* (2010) has been the most comprehensive on the question of host specificity to date, as it assessed the SB virulence of 22 wheat and 21 barley isolates on a differential set of 15 barley genotypes and one triticale, rye and wheat differential each. Results of the study indicated that the isolates from barley were significantly more virulent than those from wheat. However, since all of the 22 wheat isolates were sampled from common root rot infections, and 19 of the 21 barley isolates were from SB infections, and the differential set included only a single wheat genotype, it was not possible to separate the effect of the sources of the isolate from the effect of the host onto which it was inoculated. The current study has addressed these issues by including an increased number of wheat differentials and relatively more isolates from barley roots and wheat leaves.

Individuals from each group of isolates (host/tissue combinations) displayed varying degrees of pathogenicity on the differentials, so although barley SB isolates caused the highest average infection responses on the barley differentials, some isolates were unable to cause virulent infections in several or all of the lines. This variability in the host genotype/fungal isolate interaction suggests the existence of pathotypes (Ghazvini and Tekauz, 2007; Knight *et al.*, 2010; Limpert and Muller, 1994; Meldrum *et al.*, 2004; Valjavec-Gratian and Steffenson, 1997b; Zhong and Steffenson, 2001b) in the fungal population and does not arise from simple differences in aggressiveness between isolates.

A total of 16 pathotypes were observed in the current study, and of the 23 isolates examined, 8 were found to have the avirulent pathotype 0.0.0.0.0. Clustering of pathotypes was dependent to an extent on tissue of origin. The findings in the current study are comparable with the previous work by Knight *et al.* (2010) in which 11 pathotypes were observed using 12 differentials in the pathotype designation. Ghazvini and Tekauz (2007), despite using the same number of differentials and a very large number of isolates (127), found only 8 pathotypes. This may be attributed to the source of all of the lines being from Canada and North America, whereas in

the current study, the differentials were sourced from a variety of continents and hence may have possessed a wider range of host genes contributing to resistance.

The method of Triplet Coded Nomenclature, although being useful in comparisons of the virulence of various isolates, is an arbitrary system that divides isolates into 'resistant' and 'susceptible' classifications when the isolates are not always so different in their reactions. For example, differentials with an infection response to an isolate of 4 are classified as 'resistant', and yet a differential with an infection response of 5 is classified as susceptible. An isolate that is designated as the pathotype 0.0.0.0., and therefore avirulent by this classification, could in fact induce infection responses of 4 on the Fetch and Stephenson (date) scale in many of the differentials, which while defined as a resistant response still produces small lesions capable of some spore production. Therefore caution must be exercised in applying this system to the definition of unique pathotypes.

The two barley varieties which displayed resistance to the most isolates were ND B112 and Tolar, which were also susceptible to different isolates. Lines derived from ND B112 such as Morex have been used in mapping of spot blotch resistance, and possess the major source of spot blotch resistance in commercial varieties (Gubis et al., 2010). These varieties contain quantitative trait loci associated with resistance on chromosomes 7H and 3H (Bovill et al., 2010). ND B112 and Tolar possess differing resistances to spot blotch, and could produce crosses with a greater level of resistance to a broader range of *B. sorokiniana* isolates. To date the resistance loci in Tolar have not been mapped.

Chapter 4: Virulence of Australian *Bipolaris sorokiniana* isolates of varying origin on Root tissue of Wheat and Barley

4.1 General Introduction

In furtherance of the work from the previous chapter, reactions of wheat and barley roots to isolates of *B. sorokiniana* were examined. A selection of isolates from wheat and barley aerial and basal tissue was tested on roots of wheat and barley genotypes. There were two primary aims for these experiments. First we aimed to determine whether an appropriate seedling screening technique could be used to rapidly and accurately assess virulence of *B. sorokiniana* isolates towards wheat and barley varieties, and secondly to determine whether host or tissue specificity exist for isolates with regard to infection of root tissue.

Common root rot (CRR) due to infection by *B. sorokiniana*, is a widespread disease, occurring wherever wheat and barley are grown. The disease symptoms include brown to black oblong necrotic lesions on roots, subcrown internodes (SCIs) and crowns of plants. In more severe cases CRR can cause the entire SCI to blacken and shrivel, which may result in eventual death of the plant if secondary roots are poorly developed. Above-ground symptoms include a decreased number of tillers and heads, as well as a decrease in grain size resulting in reduced yields (Duczek, 1984; Duczek, 1989; Ledingham et al., 1973; Piening, 1973; van Leur et al., 1997).

Occurrence of CRR is heavily influenced by environment. Conditions conducive to CRR development include environmental stresses, such as low water availability and lack of necessary nutrients (Hill and Blunt, 1994). Nutrients such as nitrogen and chloride have been found to improve root health and hence resistance of plants to the disease (Bailey and Lazarovits, 2003; Goos et al., 1987; Shefelbine et al., 1986), and have also been found to inhibit spore production and pathogenicity of *B. sorokiniana* (Arabi and Jawhar, 2009). In relation to this, some research also suggests that wheat lines which support the growth of N-fixing bacteria may also display greater levels of resistance to the disease (Deacon and Lewis, 1982).

Crop management practices that aid in the control of CRR include ley farming (where land is used for pasture or left fallow for a period between cropping), rotation with non-cereals (Burgess and Griffin, 1968; Conner and Atkinson, 1989; Wildermuth, 1986), reduced tillage (Matheison et al., 1990) and use of fungicides (Shefelbine et al., 1986; Verma, 1983). Other researchers have suggested the use of biocontrol agents, where non-pathogenic fungal isolates or bacteria are used as competitors to reduce populations of *B. sorokiniana* in the soil (Dal Bello et al., 2003; Shivanna et al., 1996).

The most efficient method of fungal disease control is incorporation of genetic resistance into commercial cultivars. However, most of the research investigating resistance to *B. sorokiniana* has been conducted on foliar spot blotch, and little research has investigated sources of genetic resistance to CRR in wheat and barley. This is partly due to the laborious field trial-based assessment of cultivar reactions to the disease. CRR resistance based on field trial data was identified in the wheat line Apex (Larson and Atkinson, 1970; Larson and Atkinson, 1981), while more recently Lehmensiek *et al.* (2010) identified markers for resistance on chromosome 4HL and 5HL in the barley cultivar Delta.

The current accepted method of CRR assessment is to grow plants in a field inoculated with *B. sorokiniana*, and assess plants for common root rot at, or near, maturity. In Australia, barley is usually planted in late April to June, and harvested in October to late November (http://www.daff.qld.gov.au/plants/field-crops-and-pastures/broadacre-field-crops/barley/planting-nutrition-harvesting). Time to maturity is broadly similar to wheat, but varies according to variety (Gomez-Macpherson and Richards, 1995). Natural inoculum levels in the soil also vary, and environmental conditions have a strong influence on the development of CRR. Due to these varying parameters and time required for field trials, development of a seedling assessment method would benefit research into CRR.
Some research groups have trialled potential methods for virulence assessment of *B. sorokiniana* on roots. Syrian researchers Arabi and Jawhar proposed multiple methods in two separate studies (Arabi and Jawhar, 1999; Arabi and Jawhar, 2001). In the earlier study, an *in vitro* trial assessed common root rot severity on two commercial cultivars and three breeding lines of barley. Seeds were inoculated with a mixture of isolates using a spore suspension mixed with sterilised peat moss, and were grown on moist filter paper in petri dishes. Seedlings were assessed for disease severity by visually rating SCI tissue discolouration two weeks after inoculation. The latter study by Arabi and Jawhar (2001) compared visual assessment of common root rot to an in vitro method. SCIs were surface-sterilised, cut into 1.5mm segments and placed on agar to allow growth of fungal colonisers. Disease severity was calculated as the percentage of SCI fragments colonised with *B. sorokiniana*, and this value was found to correlate strongly with the visual discolouration assessment. Almgren *et al.* (1999) proposed a similar *in vitro* assessment method in which seeds were grown in rolls of cellulose filter paper for ten days before inoculation with agar discs of fresh inoculum, placed directly on the roots. Severity of CRR was assessed three, five and seven days after inoculation by measuring the length of discoloured root. Preliminary trials of a seedling assessment method were also conducted by Sheedy and Reen (2010) in which wheat and barley seedlings were grown in pots with inoculum placed in a layer above the seed and visually assessed for disease severity after 5 weeks.

The first goal of this study was to develop a seedling assay for crown rot resistance that might prove to be predictive of field performance, thus providing a faster, simpler, more efficient and less environmentally dependent assay than currently available from field trials. The second aim of the study was to assess the ability of *B. sorokiniana* from different sources to cause infections on roots. Isolates from both hosts and both tissues need to be tested on root tissue of wheat and barley in order to determine whether the genetic differences among *B. sorokiniana* root and leaf isolates of wheat and barley also are applicable to virulence. Previous investigations examined reactions of wheat and/or barley leaf tissue to a small number of pathogen isolates from CRR infections and compared them to leaf tissue reactions to isolates from spot blotch lesions (Arabi and Jawhar, 2007b; Duveiller and Altamirano, 2000; Meldrum et al., 2004; Valjavec-Gratian and Steffenson, 1997b). However assessment of the ability of leaf isolates to infect roots was not previously reported. The aim of the following study was to investigate how effectively isolates from leaf tissue can infect root tissue, and to investigate host specificity of CRR isolates.

4.2 Methods

4.2.1 Selection of Host Differentials

Eight CRR differential lines (four barley and four wheat) with varying reactions to CRR disease were selected based on varying reactions to the disease in field trials and some pot trials at the Queensland Department of Agriculture, Forestry and Fisheries (QDAFF) Leslie Research Facility in Toowoomba, Queensland (Table 4.1). These included the barley varieties 'Franklin', 'Flagship', 'Tallon' and 'Delta' and the wheat varieties 'Sunlin', 'Timgalen', 'Leichardt' and 'Janz'. Seed was provided by the QDAFF Leslie Research Facility.

Table 4.1 Reaction of each wheat and barley differential to common root rot (VS = Very susceptible; S = Susceptible; MS = Moderately susceptible; MR = Moderately resistant; R = Resistant). Wheat differentials were previously assessed in field and seedling trials, but the barley reactions were assessed in field trials only.

Species	Differential	CRR Response
Barley	Franklin	S
	Flagship	S
	Tallon	MS
	Delta	R
Wheat	Sunlin	R-MR
	Leichardt	MR-MS
	Janz	S
	Timgalen	VS

4.2.2 Selection and Preparation of Isolates

Isolates were selected from the main collection, which was subjected to genotypic analysis in Chapter 5. Eight single spore isolates were selected according to their tissues and hosts of origin, and all were previously scored for spot blotch reactions. Isolates and their origins are listed in Table 4.2.

Table 4.2 *B. sorokiniana* isolates used to inoculate basal tissue of wheat and barley differentials in Experiments 1 to 3.

Isolate	Host	Tissue
O5050	Barley	Leaf
SB61	Barley	Leaf
HRS#10008	Barley	Leaf
A01#32	Barley	Root
A05#7 *	Barley	Root
USQ09005a	Wheat	Leaf
A06#1 *	Wheat	Root
A05#16 *	Wheat	Root

* Isolates used in the inoculum mixture in Experiment 3

Isolates were first grown on potato dextrose agar (PDA) for 5 days or until sufficient growth was established to transfer agar plugs to starch nitrate agar (SNA). Each isolate was grown for 8 days in the dark at 24^{o}C, after which the plate was flooded

with 10 mL sterile demineralised water and agitated with a paintbrush sterilised with 70 % ethanol. The spore suspension was collected and added at approximately 1 % concentration, or 2.5 mL to 250 mL of starch nitrate broth in a 500 mL Schott bottle. The bottle was placed on a shaker for 10 days at 20^{o}C, after which the broth culture was transferred to a beaker and blended with a sterilised handheld blender until smooth.

4.2.3 Preparation of Dried Grain Inoculum

For each isolate, 60 g of a mixture of two parts wheat (40 g) and one part barley (20 g) grain was prepared and poured into 250 mL Schott bottles. The grain was covered with sterilised demineralised water with extra to allow for grain expansion, and bottles were placed in the cold room overnight. The next day, grain was drained of excess water and placed in the autoclave with the lids loosely screwed on for 4 mins at 121^{o}C, and again bottles were transferred to the cold room overnight. Bottles were autoclaved again for 11 mins and then allowed to cool in the laminar flow cabinet. Each 60 g sample of grain was inoculated with 1.5 mL blended broth inoculum, and shaken well to distribute. The inoculated bottles of grain were placed in the incubator in the dark at 24^{o}C and after one week were shaken every second day. After another three weeks in the incubator, bottles were removed and grain transferred to trays lined with blotting paper. The grain was covered with another piece of blotting paper and allowed to dry for two weeks on the bench top. Grain was stirred every second day. After the drying period, inoculated grain was pulverised to a fine powder in a sterilised coffee blender. The powder was transferred to a sterilised 100 mL Schott bottle for storage. Spore viability was checked by sprinkling 1g of the ground inoculum on solid PDA growth media and incubated for 24 hours in the dark at 24^{o}C, then viewed under a light microscope.

4.2.4 Preparation of Host Differentials

4.2.4.1 Experiment 1

Each host cultivar, or differential, was tested in duplicate. That is, two pots containing eight to 12 seeds were prepared per differential per isolate. Differentials were planted in 90 mm square, 150 mm high pots in pasteurised soil collected from the field in Wellcamp. A base layer (410 g) of soil was added to the pot, followed by 10 to 15 seeds of the appropriate differential. The seeds were covered with 100 g soil, and ground grain-based inoculum of a single isolate (0.55 g, 25 to 27 week old inoculum) was sprinkled evenly on top to ensure all plants would contact inoculum on emergence. A final layer of 35 g of sterilised soil was added to the top. The pots were set up in a randomised order on a self-watering bench system and allowed to grow for five weeks.

4.2.4.2. Experiment 2

Differentials were tested in duplicate, and planted in 90 mm square pots. A base layer (310 g) of soil was added to the pot, followed by 10 to 15 seeds of the appropriate differential. The seeds were covered with 160 g soil, and ground wheat

inoculum of a single isolate (0.70 g, 28 to 30 week old inoculum) was sprinkled evenly on top to ensure all plants would contact inoculum on emergence. A final 75 g layer of sterilised soil was added to the top, giving a total seed depth of 4 cm to enable optimal emergence and SCI length. The pots were set up in a randomised order on a self-watering bench system and allowed to grow for five weeks.

4.2.4.3 Experiment 3

In a third experiment, the precise conditions developed by Sheedy and Reen (2010) were tested. This was conducted due to the low level of disease observed overall in Experiments 1 and 2, the high number of escapes (uninfected plants) which was likely due to the larger pot size and hence larger surface area. Briefly, three replicates of the four wheat and four barley cultivars were grown in 7 cm square, 15 cm high pots containing 210 g pasteurised soil. Ten to 15 seeds were covered with 90 g soil, then 0.66 g ground grain inoculum, produced during the trials for the initial study in 2010. This inoculum consisted of a three isolate mixture, one from barley roots (A05#7), and two from wheat roots (A05#16 and A06#1), as used in the previous preliminary assessment (Sheedy and Reen, 2010). Inoculum was sprinkled evenly on top, and the pots were capped with another 30 g soil. The pots were set up in a randomised order on a self-watering bench system and allowed to grow for five weeks.

4.2.5 Assessment of Common Root Rot severity

After five weeks, plants were harvested and roots rinsed and assessed for CRR severity. Eight plants were rated per pot, but when less than eight seeds germinated, fewer plants were rated. Two replicates per treatment were rated in Experiment 1 and 2, and three replicates per treatment in Experiment 3. All replicates were pooled together for the statistical analysis. The accepted method for rating CRR is to visually rate the percent discolouration of the SCI surface, which is directly associated with yield losses and decrease in biomass (Kokko et al., 1993; Verma et al., 1976). This was completed by first washing the SCI and removing any dead leaf sheath tissue, then estimating discolouration of the entire surface of the SCI, which was rated as a percentage (Sheedy and Reen, 2010). SCIs of eight individual plants were rated per treatment in each replicate and all data presented was based on analysis of these individual scores.

4.2.6 Statistical Analysis

The percentage of SCI discolouration of individual isolates and differentials were compared using parametric (ANOVA) and non-parametric (Independent samples Kruskall-Wallis) tests in the statistical programme SPSS. Both methods were used, as parametric tests assume normal distribution of data, but all data was severely skewed towards lower percentages. The escapes were excluded from the final analysis.

4.3 Results

4.3.1 Comparison of Common Root Rot ratings Experiments 1, 2 and 3

Experiments 1 and 2 contained the same differentials and isolates, thus results for these two experiments can be compared. Experiment 3 tested a single mixture of three isolates (A05#7, A05#16 and A06#1) to establish a more consistent and reproducible test that reliably differentiates between lines based on their field screening results. The seed sample of the differential variety Janz showed a very low germination rate of 1.25 % when tested in independent germination tests in the laboratory, and as a result this variety was excluded completely from further analysis. For both Experiments 1 and 2, the escapes were evenly distributed across differentials for the isolates A06#1, A05#7, USQ09005a and HRS#10008, except when Timgalen was inoculated with A06#1, no escapes were observed. The remaining four isolates had a minimal number of escapes on only selected differentials. The number of plants and apparent 'escapes' (individual seedlings scoring zero, with no discolouration) for wheat and barley differentials in each of the three experiments is summarised in Table 4.3. The three experiments had a total of 30.6 and 28.6 % escapes for barley and wheat, respectively. When examining the proportion of escapes observed in each differential variety (Table 4.4), these ranged from 16 to 42 %, with Timgalen having the least escapes, and Sunlin having the most. The proportion of escapes for each isolate (Table 4.5) ranged from 1.7 to 57.9 % for barley (with 05050 displaying the least escapes, and A05#7 having the most) and 3.6 and 51.7 % for wheat (with A05#16 having the least and USQ09005a having the most).

Once escapes were removed from the data, all replicates for each differential × isolate combination were compared using t-tests to determine whether there were any significant differences among replicates or experiments (Appendix 4.0). Out of the 48 differential × isolate combinations, ten showed one replicate that differed significantly from the other replicates at the 0.05 level. No other significant differences were evident. For this reason, all data from the separate experiments and replicates were pooled for each differential × isolate combination in the data analysis.

Table 4.3 Summary of Experiments 1, 2 and 3, including the total number of wheat and barley plants scored and percentage of plants that scored zero (no discolouration, apparent escapes) in brackets. The total of these values (over all experiments) is given in the final column.

Experiment	Barley Escapes	Wheat Escapes
1	477 (21.2)	345 (31.0)
2	464 (27.8)	340 (27.6)
3	77 (42.9)	55 (27.3)
Total	1018 (30.6)	740 (28.6)

Table 4.4 Summary of the number of plants scored for each differential, and the percentage of apparent escapes (plants with no discolouration).

Differential	Total Plants	% Escapes
Timgalen	213	16.4
Leichardt	242	28.5
Sunlin	230	42.2
Franklin	234	26.1
Flagship	217	25.3
Tallon	234	19.7
Delta	256	26.6

Table 4.5 Summary of the number of wheat and barley plants scored for each isolate, and the percentage of escapes (plants that had no discolouration).

Isolate	Total Barley Plants	% Barley Escapes	Total Wheat Plants	% Wheat Escapes
A05#7	114	57.9	90	37.8
A01#32	118	11.9	76	18.4
A06#1	122	36.9	86	40.7
A05#16	120	5.0	84	3.6
O5050	117	1.7	89	15.7
SB61	115	3.5	86	22.1
HRS#10008	119	31.1	87	42.5
USQ09005a	116	48.3	87	51.7
Total	941	24.5	685	29.1

4.3.1.1. Disease Ratings of Differentials over the Three Experiments

Figure 4.1, which shows distribution of the SCI surface discolouration for the three experiments demonstrates that most individuals displayed discolouration of 20 percent or less, with a mean score of 10.2, 14.5 and 21.5 percent discolouration for Experiments 1, 2 and 3 respectively. Since the data is skewed to the left, both parametric and non-parametric tests were applied. The ANOVA (Table 4.6) indicated significant differences between the means of the three experiments at the 0.05 level, as did the independent samples Kruskall-Wallis test.

Table 4.6 ANOVA comparing the percentage of subcrown internode (SCI) discolouration between experiments (escapes excluded).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	12095.493[a]	2	6047.746	20.967	.000
Intercept	140261.385	1	140261.385	486.264	.000
Experiment	12095.493	2	6047.746	20.967	.000
Error	368058.453	1276	288.447		
Total	594023.000	1279			
Corrected Total	380153.945	1278			

a. R Squared = .032 (Adjusted R Squared = .030)

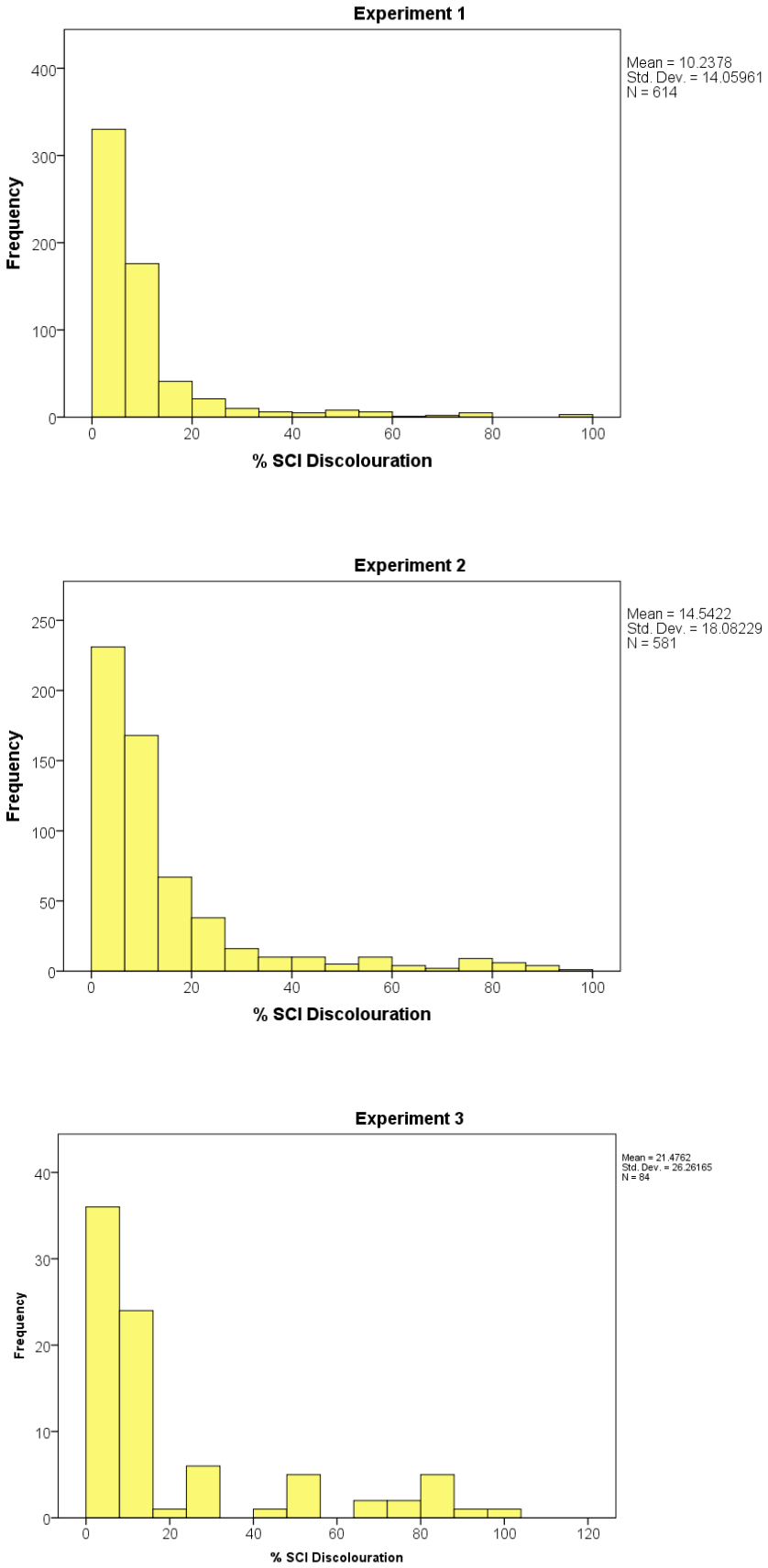


Figure 4.1 Histograms showing numbers of individuals with various percentages of discoloured subcrown internode (SCI) tissue in Experiments 1, 2 and 3 (escapes are excluded).

Timgalen, which is used as a susceptible standard in field trials and was rated as very susceptible (up to 100% discolouration) in earlier preliminary seedling trials (Sheedy and Reen, 2010), scored an average discolouration between 19 and 55 percent for the three experiments, the highest scores of all the differentials. The level of disease symptoms increased in four differential varieties (Timgalen, Sunlin, Franklin and Delta) from Experiment 1 to 3. However, although the average discolouration increased by approximately 36 % from the first to third experiments for Timgalen, the increases observed in Sunlin, Franklin and Delta between the first and final experiments were no more than approximately 10 %. Three varieties (Flagship, Tallon and Delta) displayed consistently low disease symptoms below a mean of 15 % discolouration for all three experiments. Of these three differentials, the barley variety Flagship is rated susceptible to CRR as an adult plant in the field. The barley varieties Tallon and Delta are rated moderately resistant and resistant respectively, to CRR in the field. A minimal spread of data was observed, even among the previously tested wheat varieties shown to exhibit differential reactions to CRR. However comparisons were still made among groups ratings of differentials and isolates, but with the escapes removed.

Data for the differentials in Experiments 1 and 2 was analysed (excluding data for the apparent escapes) with mean, standard error and maximum percentages of discolouration for each differential shown in Table 4.7. As was demonstrated in Figure 4.2, wheat differential Timgalen had the highest average SCI discolouration, followed by Leichardt and Sunlin. The barley differentials had very low average discolouration not exceeding 10.6 %. Even the susceptible genotypes in field tests displayed low disease scores. The standard error was relatively low for all differentials. Three susceptible genotypes (Timgalen, Janz and Franklin) displayed maximum discolouration ratings 90% or higher, however high maximum ratings were also observed in three of the resistant genotypes (Leichardt, Sunlin and Tallon).

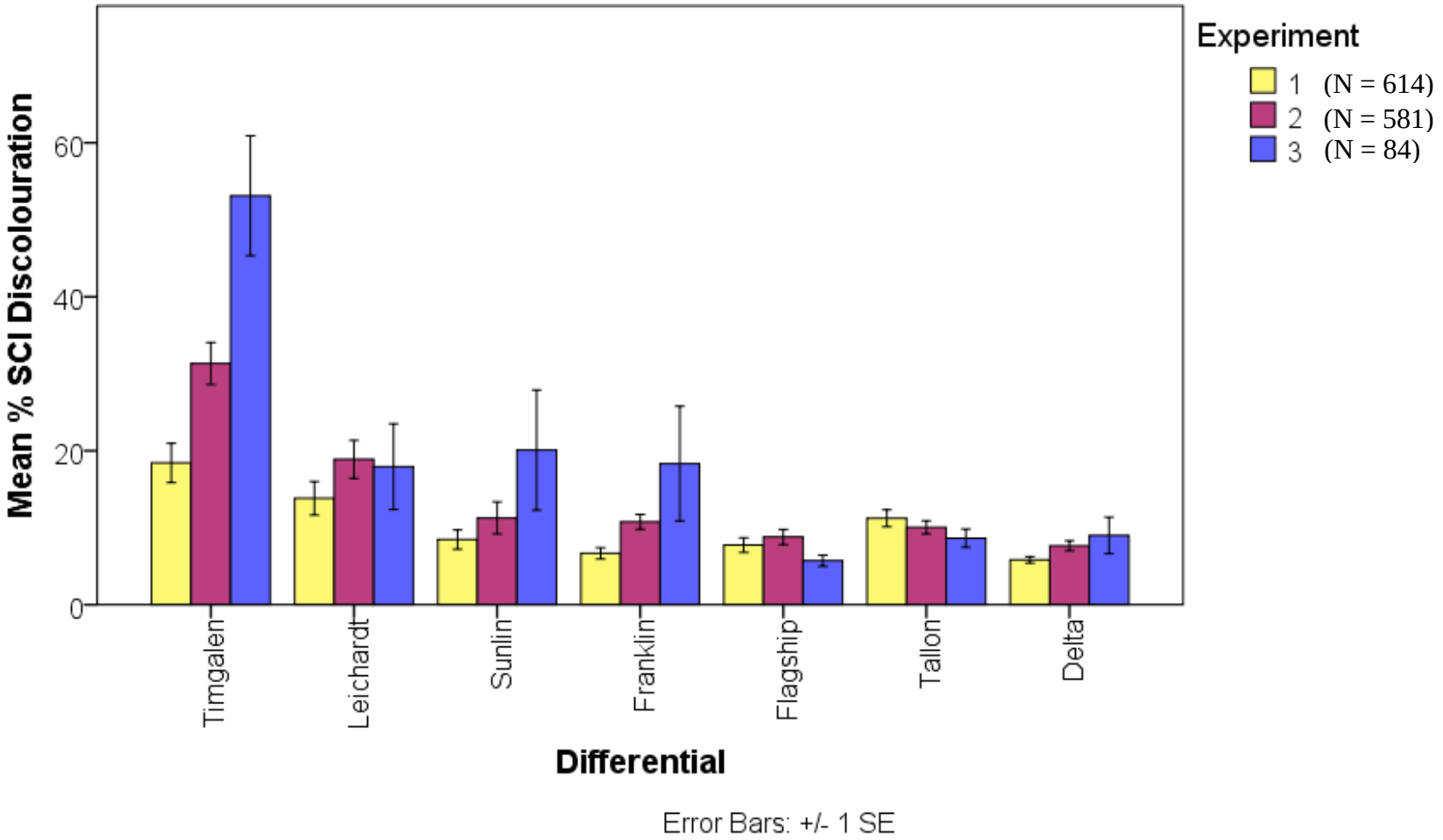


Figure 4.2 Mean percentage of discolouration on the subcrown internode (SCI) surface of each of the wheat and barley differentials in Experiments 1, 2 and 3 (escapes are excluded). The three experiments are indicated in each colour. Number of plants in each experiment is indicated next to the legend.

Independent samples t-tests (last row in Table 4.7) that compared mean SCI discolouration over Experiments 1 and 2 showed that only Timgalen and Leichardt differed significantly from each other and all the other differentials. The SCI discolouration was compared between differentials using ANOVA (Table 4.8), which showed a significant difference at the 0.05 level. A non-parametric independent samples Kruskall-Wallis test found the distributions of the SCI discolouration were significantly different among differentials.

Table 4.7 Statistical results for the subcrown internode (SCI) tissue discolouration of each differential for Experiments 1 and 2. Escapes (scores of 'zero') are excluded from this analysis. The 'Group t-test comparison' indicates groups that differ significantly from each other at the 0.05 level. Columns with the same letter do not differ significantly from each other at this level.

Statistic	Timgalen	Leichardt	Sunlin	Franklin	Flagship	Tallon	Delta
Mean	25.17	16.49	9.77	8.64	8.26	10.70	6.69
Std. Error of Mean	1.93	1.67	1.18	0.62	0.68	0.72	0.37
Maximum	95	90	83	45	50	72	25
Group t-test comparison	a	b	c	c	c	c	c

Table 4.8 ANOVA comparing percentage subcrown internode (SCI) discolouration between differential varieties.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	44728.32[a]	6	7454.72	32.61	.000
Intercept	177035.94	1	177035.94	774.32	.000
Differential	44728.33	6	7454.72	32.61	.000
Error	271618.11	1188	228.63		
Total	498037.00	1195			
Corrected Total	316346.44	1194			

a. R Squared = .141 (Adjusted R Squared = .137)

4.3.1.2. Disease Ratings of Isolates over Three Experiments

The average SCI discolouration induced by each isolate on the barley differentials (shown in Figure 4.3a) indicate that the barley SB isolates 05050 and SB61induced the highest levels of discolouration. The isolates A06#1 (barley CRR), USQ09005a (wheat SB) and A05#7 (barley CRR) produced the lowest level of symptoms on the differentials. The individual isolates differed significantly in SCI discolouration induced at the 0.05 level among at least two isolates, as demonstrated by the ANOVA (Table 4.9), and supported by the non-parametric Kruskall-Wallis test at the 0.001 level. This was despite lower levels of discolouration induced on barley differentials than on wheat, and no significant differences observed among symptoms induced on barley differentials. The average CRR discolouration induced on the wheat differentials (Figure 4.3b) was more severe than on the barley differentials. Isolates inducing the highest level of disease on wheat were A05#16 (wheat CRR) and A05#7 (barley CRR). Isolates inducing the lowest discolouration on the wheat differentials were the barley SB isolates HRS#10008 and 05050, and the wheat SB isolate USQ09005a. The SCI discolouration induced by each isolate on

the wheat differentials was significantly different at the 0.005 level in the ANOVA (Table 4.10). This was supported by the non-parametric Kruskall-Wallis test at the 0.001 significance level.

A summary of the statistics of SCI discolouration induced by each isolate on the barley and wheat differentials is given in Table 4.11, listing the mean, standard error and maximum percentage of subcrown internode discolouration on each species of differentials. The mean discolouration values induced by the CRR isolates were much lower on the barley differentials compared to the wheat differentials. The mean discolouration values induced by the SB isolates on the wheat and barley differentials differed by a maximum of 3.1 %. T-test comparisons showed the isolate A06#1 caused significantly lower levels of disease on the barley differentials than most other isolates except USQ09005a and A05#7. Five isolates did not cause infection levels which were significantly different from each other. On the wheat differentials, CRR isolates A05#7 and A05#16 induced significantly higher disease levels than all other isolates except A06#1.

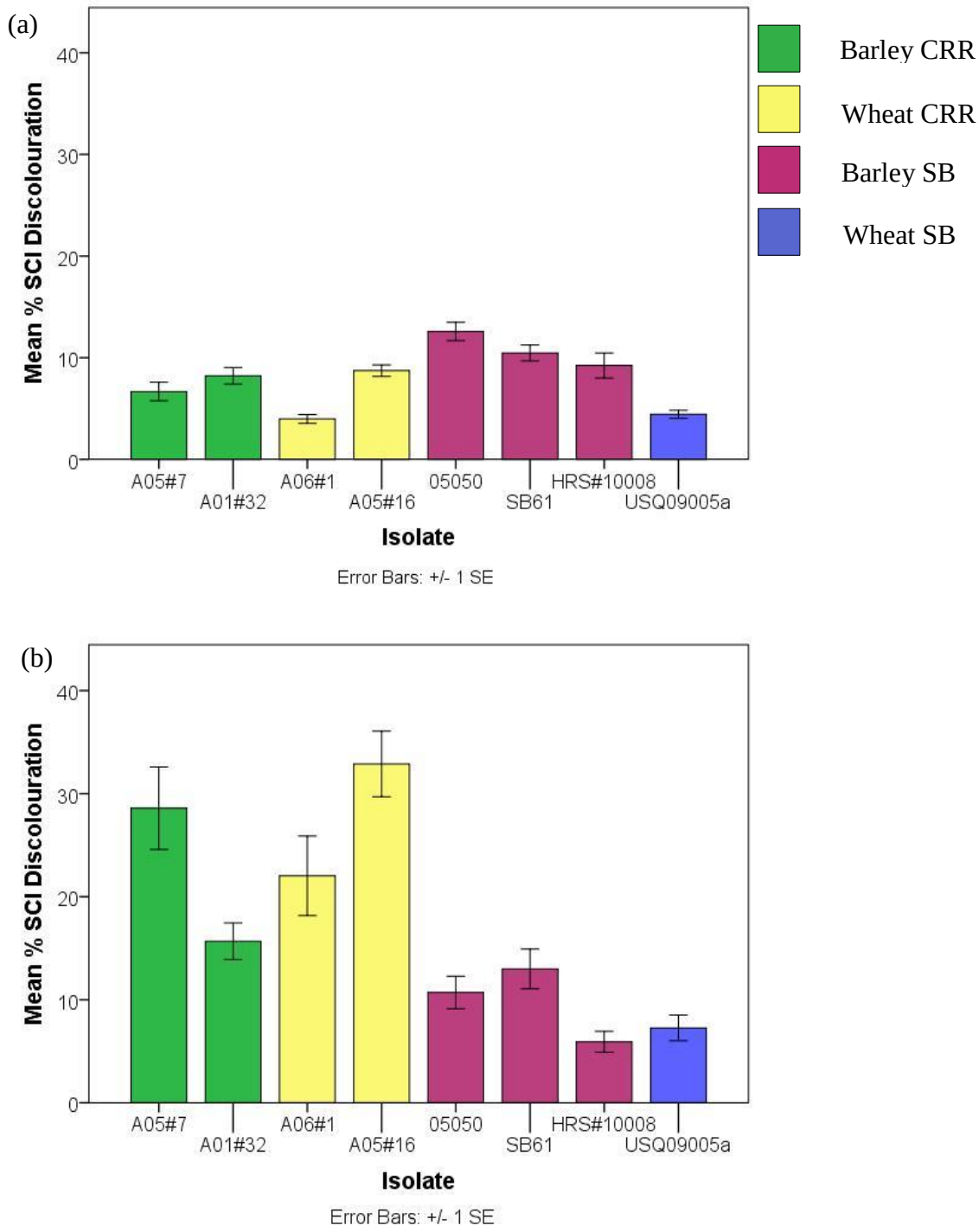


Figure 4.3 Average percentage of subcrown internode (SCI) discolouration induced by each isolate in experiments 1 and 2 for individual isolates (escapes excluded), on (a) barley differentials, and (b) wheat differentials.

Table 4.9 ANOVA comparing the mean percentage of subcrown internode (SCI) discolouration among individual isolates on the barley differentials in experiments 1 and 2.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	310.676[a]	50	6.214	1.456	.022
Intercept	2589.332	1	2589.332	606.853	.000
Percentage SCI Discol	310.676	50	6.214	1.456	.022
Error	4881.244	1144	4.267		
Total	29108.000	1195			
Corrected Total	5191.920	1194			

a. R Squared = .060 (Adjusted R Squared = .019)

Table 4.10 ANOVA comparing the mean percentage of subcrown internode (SCI) discolouration among individual isolates on the wheat differentials in experiments 1 and 2.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	318.360[a]	43	7.404	1.733	.004
Intercept	1422.695	1	1422.695	333.026	.000
Percentage SCI Discolouration	318.360	43	7.404	1.733	.004
Error	1879.689	440	4.272		
Total	11484.000	484			
Corrected Total	2198.050	483			

a. R Squared = .145 (Adjusted R Squared = .061)

Table 4.11 Comparison of Experiments 1 and 2 results (percentage of subcrown internode (SCI) discolouration) for each isolate. Original infection type (spot blotch (SB) and common root rot (CRR)) and host (wheat and barley) from which isolates were collected are indicated along the top of the table. The letters in the 't-test comparison' row indicate which isolates differ significantly in the level of discolouration induced on each set of differentials. Individuals with the same letter do not differ significantly at the 0.05 level. Escapes (scores of 'zero') are excluded from this analysis.

		Barley Differentials				Wheat Differentials			
Isolate	**Original Host and Infection Type**	**Mean**	**Std. Error**	**Maximum**	**t-test Comparison**	**Mean**	**Std Error**	**Maximum**	**t-test Comparison**
A05#7	Barley CRR	5.75	1.09	50.00	a,c,d,g	28.59	4.00	95.00	a
A01#32	Barley CRR	7.63	0.73	45.00	a,b,f,i	15.68	1.77	55.00	bc
A06#1	Wheat CRR	3.97	0.43	17.00	c	22.02	3.86	90.00	ab
A05#16	Wheat CRR	8.98	0.60	40.00	b,d,f	32.88	3.19	95.00	a
05050	Barley SB	12.94	0.92	60.00	e	10.71	1.57	75.00	bc
SB61	Barley SB	10.73	0.70	37.00	e,f,h	12.99	1.93	75.00	bc
HRS#10008	Barley SB	9.02	1.25	72.00	b,g,h	5.92	1.02	45.00	c
USQ09005a	Wheat SB	4.72	0.40	12.00	c,i	7.26	1.25	50.00	cd

4.4 Discussion

Disease levels in tests performed in the current experiments were considerably lower than levels observed in previous experiments (Sheedy and Reen, 2010). Wheat variety Timgalen displayed consistently high levels of up to 100 percent SCI discolouration in these previous preliminary tests. However in the current experiments, disease levels were inconsistent and apparent escapes appeared frequently. The barley varieties used in the current tests were selected based upon their differential reactions to CRR in adult field screens, and hence it was unknown how they would react to CRR in glasshouse seedling trials. These barley varieties exhibited consistently low reactions to CRR in all three experiments. It is evident that in the current experiments, the reaction induced by isolates from wheat and barley induced opposite reactions on the SCI tissue of differentials, whereas in the study of leaf reactions to isolates in Chapter 3 these differences were not observed among isolates from the two hosts.

Alternative methods of inoculum production were used to investigate other root diseases of various crops. These methods involved colonisation of material that was kept whole for soil inoculations. For example, Flett (1994) in an investigation of *Phytophthora clandestina* virulence in clover, prepared inoculum by use of vermiculite as a solid substrate for fungal colonisation. The vermiculite was placed in a suitable broth for *P. clandestina* growth and used as inoculum after 2 weeks by incorporating into pasteurised potting mix. A different approach was taken by Knudsen *et al.* (1995) in which barley seeds were directly inoculated with fungal spores suspended in gelatine, and seeds were planted in pots of sand to enable germination and growth. In Syrian studies of CRR (Arabi and Jawhar, 1999; Arabi and Jawhar, 2001), inoculum was prepared by combining an adjusted concentration of spore suspension with peat, and seeds were inoculated directly with this mixture. This inoculation method along with the method by Knudsen *et al.* (1995) could reduce the potential for escapes, since all germinating seedlings are likely to make contact with fungal spores or mycelia. All of these methods could potentially be adapted for assessment of *B. sorokiniana* isolate virulence on wheat and barley. Additionally, the conditions in the growth chamber during experimentation, such as temperature, light period and humidity could be adjusted to optimise infection of roots.

Sheedy and Reen (2010) found in their preliminary tests that banded rather than dispersed inoculum was most effective in symptom production, without prematurely killing plants. An alternative to inoculation of barley and wheat grain, as performed in the current experiments, could be adaptation of a method used by Elad *et al.* (1980), in which fungal spores were scraped from agar plates and combined with soil at a specific w/w ratio. Duffy and Defago (1997) developed a method for production of an abundant supply of fungal propagules. This method proliferated fungal tissue in broth, and centrifuged the fungal biomass before it was blended and combined with sterilised soil substrate (Duffy and Défago, 1997). The soil mixture could be used as banded inoculum in CRR testing and would cut down on the time required for inoculum production.

In discussions with QDAFF staff, it was suggested that fresh inoculum should be prepared for each experiment, as inoculum viability can deteriorate over time once ground to powder (Cassandra Percy, *pers comm.*). In the experiments reported here,

the same inoculum was used for all experiments due to the long process of inoculum production (8 to 9 weeks in total), and time restrictions. The period of inoculum storage ranged from 25 weeks for Experiment 1, to 4 years for Experiment 3. In order to produce more comparable and consistent results, fresh inoculum should be used, possibly in higher proportions than previously produced. Inoculum preparation methods used elsewhere in the literature (as discussed above) would take much less time than the method used in the current experiment, and could serve as a way to further reduce seedling screening time.

The bulk of research investigating pathogenicity of *B. sorokiniana* has examined infection response of leaves from wheat and/or barley, which were discussed in detail in Chapter 3 (Bashyal et al., 2010; Duveiller and Altamirano, 2000; Ghazvini and Tekauz, 2007; Knight et al., 2010; Meldrum et al., 2004; Persson et al., 2008; Valjavec-Gratian and Steffenson, 1997b). Much research conducted to investigate root reaction to the pathogen has been conducted in the field, or in the glasshouse using soil from the field (Burgess and Griffin, 1968; Conner and Atkinson, 1989; Duczek, 1989; Grey and Mathre, 1984; Kokko et al., 1993; Piening, 1973; Tinline et al., 1988; Wildermuth et al., 1992). A limited number of studies have investigated reaction of barley to single spore isolates of *B. sorokiniana*, however two of these examined the disease response induced by mixtures of single spore isolates, rather than individual isolates (Arabi and Jawhar, 1999; Arabi and Jawhar, 2001). A study by Duczek (1984) examined the reactions of barley SCIs to individual single-spore isolates of *B. sorokiniana*, however all of these were originally isolated from SCIs, and comparisons were only made among geographic origins. The purpose of the current experiment was to determine whether a rapid screening method for CRR previously developed by Sheedy and Reen (2010) could be used to assess the ability of individual isolates to cause root infections in wheat and barley.

Very limited research has been conducted to investigate the relationship between CRR and SB. To our knowledge, no studies have investigated the co-occurrence of the two diseases in the field. One study by Duveiller and Altamirano (2000) investigated the relationship between SB, CRR and black point in wheat, by conducting a glasshouse inoculation study. *B. sorokiniana* solates from tissue showing each symptom were inoculated onto leaf tissue of the spring wheat cultivar Ciano T-79 and the number of lesions per leaf and infection severity were assessed. The group found that the number of lesions per leaf differed significantly according to the tissue of origin, however there was no significant difference in the disease severity of each group of isolates.

Due to insufficiencies of the testing method, comparisons made among disease levels induced on the wheat and barley differentials are preliminary only. In the current experiment, host specificity was not evident on either barley or wheat differentials. In the previous chapter examining infection response of wheat and barley leaves to various isolates, host specificity was observed among spot blotch and common root rot isolates. However it is difficult to draw conclusions from the results in the current chapter, as tests were preliminary, and disease was induced on seven differentials by eight isolates only. Further development of the methodology in future may allow for testing of a larger sample of isolates on a larger collection of differentials.

Little research has been conducted comparing ability of SB and CRR isolates to cause infections in root tissue. Almgren *et al.* (1999) compared the ability of 13 isolates, primarily collected from leaf tissue, to cause CRR and SB infections. They found that isolates were most virulent on leaf tissue, and less virulent on roots. Arabi and Jawhar (2007b) tested a collection of 22 *B. sorokiniana* isolates from barley (five originating from root tissue, and 17 originating from leaf tissue) on the roots of three barley differentials. Experiments were undertaken in the glasshouse, and seedlings were rated for CRR susceptibility after 7 weeks, based on SCI discolouration. Despite inclusion of isolates from both tissues, the level of disease induced among isolates of different origins was not compared statistically.

In the current study, tissue specificity was strongly evident when isolates were tested on the wheat differentials, with wheat and barley CRR isolates inducing a significantly higher level of disease than wheat and barley SB isolates. These differences were found to be significant in both parametric and non-parametric tests. Tissue specificity was also observed in the SB study in Chapter 3. Evidence indicates that although isolates tend to be tissue-specific, low levels of infection can occur on alternate tissues. Since tests in the current chapter were essentially a preliminary trial of potential seedling trial methodology, much work remains to be done before we can be confident of assessing CRR phenotypes in seedlings. Limitations of the methodology in its current state of development are highlighted by a number of factors. These include the significant level of infection escapes, high variability between disease scores on individual seedlings within host genotype × isolate interactions, low overall infection severity compared to field results and a failure to discern between disease reactions on most genotypes.

In future, it would be beneficial to test some alternate methods of inoculation used in studies discussed earlier (Flett, 1994) in order to identify a fast screening method for assessing *B. sorokiniana* isolate virulence wheat and barley roots. In this process, barley varieties should be screened for differential seedling reactions to CRR infection, and determine whether adult plant resistance in the field is expressed in seedlings in the glasshouse. It would also be informative to determine infection level of rated SCI tissue, by use of quantitative PCR (qPCR) (see Chapter 6) and histological examination. Escapes should be assessed to determine whether they are colonised and infected by the fungus, but remain symptomless.

Chapter 5: Development of more robust markers for adult plant resistance to spot blotch on barley chromosome 3H

5.1 General Introduction

In this chapter, the genomic interval responsible for adult plant resistance to spot blotch on barley chromosome 3H is specifically investigated. In barley, the most consistently detected resistance QTL are located on chromosomes 3HS and 7HS. Adult plant resistance located on chromosome 7H has been part of a significant study based in the USA (Drader and Kleinhofs, 2010) but fine mapping of the 3H region has proven difficult, due to a lack of polymorphic DNA in the region, therefore the 3H region is the focus of the current study.

Adult plant resistance to spot blotch has been detected at statistically significant levels on chromosome 3H in North American doubled-haploid (DH) populations Dicktoo/Morex (Bilgic et al., 2005) and Calicuchima-sib/Bowman (Bilgic et al., 2006). In Australian studies this region has contributed adult plant resistance in four doubled haploid (DH) populations (VB9524/ND11231-12; TR251/Gairdner; ND11231-11/WI2875-17; WPG8412-9-2-1/Lindwall) (Bovill et al., 2010). All of these populations appeared to have resistance in the same region on chromosome 3H. Adult plant resistance has also been detected on chromosome 7H at significant levels in the same four DH populations by Bovill *et al*. (2010), in three DH populations (Harrington/Morex; Dicktoo/Morex; Harrington/TR 306) by Bilgic *et al.* (2005), in a single DH population (Calicuchima-sib/Bowman) by Bilgic *et al.* (2006) and in one DH population (Steptoe/Morex) by Steffenson *et al.* (1996). Of these, three studies appear to have detected both adult and seedling resistance in the same region of 7H (Bilgic et al., 2005; Bovill et al., 2010; Steffenson, 1996), while Roy *et al*. (2010) also identified this chromosome region in seedling trials of 318 wild barley accessions from the Wild Barley Diversity Collection, developed at the University of Minnesota. A single DArT marker (bPb-1068) had a significance level of P=0.006, with 3.6% of the phenotypic variance explained.

Spot blotch can be controlled to an extent by a variety of methods, including fungicide application (Arabi and Jawhar, 2007a; Couture and Sutton, 1978) and crop rotation (Acharya et al., 2011; Wildermuth and McNamara, 1991), however the most effective and efficient mode of control is the use of genetically resistant varieties (Ibeagha et al., 2005; Joshi et al., 2004; Roy et al., 2010). Resistance may be in one of two forms: qualitative or quantitative resistance. Qualitative genetic traits are controlled by one or a few genes and can be classified into distinct classes, such as presence or absence of awns in wheat (Poehlman, 1987). Quantitative traits in contrast, are controlled by multiple genes, which may act additively or epistatically, and possess a range of phenotypes that cannot be classed into distinct groups, for example height (Poland et al., 2008). Regions controlling quantitative resistance are referred to as quantitative trait loci, or QTL. Qualitative resistance may seem like a preferable method of control, however this kind of resistance is rapidly overcome by a single gene mutation in the pathogen. Quantitative resistance is generally more stable as it requires multiple mutation events to be overcome (Poland et al., 2008). Both forms are widely applied in breeding programmes and frequently

used in combination where this is possible. The mapped regions associated with spot blotch resistance in barley are considered to be quantitative trait loci.

A first step in the process of QTL detection is the identification of markers at appropriate genomic intervals, which are then checked for polymorphism between parents. Markers that are polymorphic are then screened across the entire population and mapped in the interval under the study (Darvasi and Pisante-Shalom, 2002). The phenotypic assessment of disease resistance within the population can then be used to identify QTL that contribute to resistance against the pathogen of interest. A number of researchers have identified QTL associated with spot blotch resistance in barley and wheat (Bilgic et al., 2005; Bilgic et al., 2006; Bovill et al., 2010; Castro et al., 2012; Kumar et al., 2010; Lillemo et al., 2013; Roy et al., 2010; Steffenson, 1996). Both crops are discussed, however it should be noted that spot blotch of wheat is not a significant problem in Australia.

Fine mapping (i.e. construction of a linkage map with markers closely linked to the trait of interest) is necessary to make breeding of lines for inclusion of identified resistance more efficient. If flanking markers used in a breeding programme are not linked tightly enough to the trait of interest, there is a risk of crossover events occurring during meiosis, which can ultimately unlink one or both of the markers from the trait. This makes the markers useless for the purpose of selection due to development of breeding lines which possess marker(s) of interest but not the QTL (false positives), or exclusion of lines which do possess the QTL, but not the flanking markers (false negatives). Mapping of closely linked markers is completed with the use of large mapping populations, and markers such as SNPs or ESTs which occur more frequently in the genome. Current flanking markers for this region (bPb3865 and Bmag0919) based on the study by Bovill et al. (2010) are 2.2cM apart. Although these markers are closely linked, markers can sometimes be useless when there is no polymorphism between parents when using other genotypes, therefore it is useful to have more markers at hand, and where map structure varies markedly between cultivars. The fine mapping of this region could also facilitate the identification of candidate genes, enabling more detailed study of the mechanisms of this disease resistance locus. The larger mapping population of ND24260-I x Flagship is examined in this study rather than the TR251 x Gairdner population mapped by Bovill *et al.* (2010) due to a higher likelihood of crossover events and hence a greater chance of finding polymorphic markers. Additionally, different DArT markers were mapped to the ND24260-I x Flagship population than the earlier mapped population.

A map has been constructed with Diversity Arrays Technology (DArT) markers in an ND24260-I/Flagship DH population with 334 lines in a study by Hickey *et al*. (2011). The aim of the current study was to discover markers more closely linked to the adult spot blotch resistance QTL on chromosome 3H by mapping onto the ND24260 x Flagship population. For this purpose EST markers developed by Sato *et al*. (2009) and PCR-based markers based on SNP sequences from a study by Szucs *et al*. (2009) were designed and tested.

5.2 Methods

5.2.1 Plant Material

The mapping population used in this study was a barley DH population of 334 lines produced by Jerome Franckowiak at the QDAFF Hermitage Research Facility, Warwick, from a ND24260-I × Flagship cross. A molecular map of this population using DArT markers had earlier been constructed by Hickey, *et al.*(2011). The line ND24260-I (ND19869-I//ND17274/ND19119) is a North Dakota two row breeding line with superior resistance to spot blotch. Flagship (Cheiftan/Barque//Manly/VB9104) is a high quality two-row barley malting variety susceptible to this disease.

5.2.2 Field Trials

A single spot blotch field trial was run in duplicate by staff at the Queensland Department of Agriculture, Forestry and Fisheries (QDAFF) in 2010 at Redlands Research Station in Cleveland. Plants were inoculated with the commonly used SB61 isolate and rated by Greg Platz and Lee Hickey at the adult stage on a 1-9 scale (with 9 being most severe). The trial was designed by Susan Fletcher (Leslie Research Centre, QDAFF) with two replicates using Immdesign software.

5.2.3 DNA Extraction

Seeds were surface sterilized, and three seeds of each line were placed in individual wells containing 1% agar. Seeds were germinated at room temperature in the dark for ~ 8 days. Approximately 200 mg leaf material was harvested for each, and homogenised in buffer solution containing lead pellets using a FastPrep-24 (MP Biomedicals^{TM}). A small number of lines (20 in total) were not able to be germinated, hence DNA was extracted from whole seeds. DNA was extracted using a Wizard Genomic DNA extraction kit (Promega). DNA concentration was determined with an Implen Nanophotometer and diluted to a concentration of 25 ng/uL.

5.2.4 PCR Markers from a Previous Spot Blotch Mapping Study

Previously, a DArT map was constructed for the ND24260 × Flagship population in which a total of 634 polymorphic DArT markers were used. A total of 105 markers mapped to chromosome 3H (Hickey et al., 2011). As an initial step in the current study, seven Polymerase Chain Reaction (PCR)-based markers most closely linked to adult plant spot blotch resistance in the study by Bovill *et al.* (2010) were tested on both parents of the ND24260 × Flagship population. These were the following PCR markers: bPb-3865, GBM1159, Bmag919, HvLTPPB, HVGLTP4X3D0001, Ebmac705 and scssr10559. The bPb-3865 was a DArT marker converted to a PCR-based marker by Bovill *et al.* (2010) to assist ease of genotyping. The remaining six of these markers are SSRs, and are therefore co-dominant markers. Polymorphic markers were then screened across the whole population. PCR was performed in a 10 µL reaction with the following mix: 1 µL template DNA, 0.2 U BIOTAQ ^{TM} Red DNA Polymerase, 1 µL 10x reaction buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 2.5 µM of each primer (F and R). PCR conditions were: 7 mins at 95^{o}C, followed by 35

cycles of 94ºC for 30 secs, 55ºC for 30 secs, and 72ºC for 30 secs, with a final extension at 72ºC for 10 mins. PCR fragments were resolved on polyacrylamide gel with a Gelscan 3000, at 1200 V for 55 mins, or until markers had run through, with ethidium bromide used as the intercalating agent.

5.2.5 Markers from Barley Mapping Studies

In order to find markers used previously in mapping studies, but not yet mapped to the 3H region associated with spot blotch resistance, the MapSearch function at CCG CMap Live (http://ccg.murdoch.edu.au/cmap/ccg-live/) was used, with a Steptoe × Morex map (Wenzl et al., 2006) as the reference map. Ten maps were aligned and compared in this function, including the Bovill *et al.* (2010) TR251 x Gairdner map, five consensus maps (Varshney *et al.* 2007, Rostoks *et al.* 2005, Wenzl *et al.* 2006, Marcel *et al.* 2007, Close *et al.* 2009), a SNP and DArT map of a DH OWB dominant x OWB recessive population (Szuchs *et al.* 2009), a high-density transcript linkage map of a Haruna Nijo x H602 DH population (Sato *et. al* 2009) and an SSR and DArT map of a Barque-73 x CPI71284-48 DH population with 90 lines (Hearnden et al. 2007). Two studies with markers covering the area of interest were selected (Sato et al., 2009; Szucs et al., 2009), as these markers had not previously been screened for polymorphism or association with SB resistance, either in the barley populations in the study by Bovill *et al.* (2010), or in the ND24260 × Flagship population. The Sato *et al.* (2009) map was a transcript linkage map based on the genotyping of 93 DH lines from the cross Haruna Nijo x H602. The Szuchs *et al.* (2009) map was based on genotyping of 93 DH lines of a Wolfe Dominant x Wolfe Recessive population (OWB). PCR-based primers, which were previously designed based on expressed sequence tag (EST) sequences of cleaved amplified polymorphism (CAP) and single nucleotide polymorphism (SNP) markers (Sato et al., 2009) were selected from around the region associated with spot blotch resistance by comparing the anchor markers from this study with other maps. New primers were designed based on sequence data of SNPs from the study by Szuchs *et al*. (Szucs et al., 2009). Primers for these markers were designed using Primer3 input version 0.4.0. Software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm). A total of 15 primers were selected from these two studies, which were located in close proximity to the region associated with the spot blotch resistance found by Bovill *et al*. (2010). The primers and their sequences are listed in Table 5.1.

Table 5.1 PCR primers based on EST marker sequences of CAP and SNP markers (Sato et al., 2009) and SNP data only (Szucs et al., 2009).

Primer	Fwd Primer	Reverse Primer	Marker Type	Study
k06994	5'ACCAACAACCACACTGACGA	5'GTGGCATACAAAGCTGCTCA	EST (CAP)	Sato et al 2009
k03021F	TTGGGAGCATCCTTCATTTC	CTACCGCTCCAGAGTCCAAG	EST (CAP)	
k07026F	AAGCGCAACGAATCAAAGTT	TCCGATGTTGCTGTCTGTGT	EST (CAP)	
k08330F	GGGAGTGAAGGAACAACCAA	AGCCCCTGCATCTCCTATG	EST (CAP)	
k02683F	AGTGGTCGATCAGCGAATCT	CATCATCTCCGGCTTAGCTC	EST (CAP)	
k01008F	AACGTACAGCAAACTCCCGT	GACAAGCAAGCTACGTGCAG	EST (CAP)	
k00069F	GCTATAGGCAGAAACGACGG	CTGCAGCTACTCAGCTCGTG	EST (SNP)	
POPA3_0113F	GAAGAAGGGAGGAGGTGTCC	TTCAGGGCCAATTTCATAGC	SNP	Szucs et al 2009
POPA2_0172F	GGAAACGTGAGGGTGACAGT	AAGGAGGACGGCAAAAATCT	SNP	
POPA2_1410F	CAGAGGCGGCTTACAAATTC	TGCAGCTCAAGCATGTTACC	SNP	
POPA1_0565F	TTGGTTGGCCGATGTATTTT	TATGTCGTGTGGTGGATGGT	SNP	
POPA2_0742F	GGGATCAGCACCGTGTACTT	TAGCAGCCAGCCTAAATCGT	SNP	
POPA2_0982F	TTCACTGATCGACCACTTGC	GTAGTGAATGACGCGAGCAG	SNP	
POPA2_0552F	TTTTGAGGTCTTGGGTCGTC	GTGCATGGGTCGAGAGAGTT	SNP	
POPA2_0556F	CAGTGGCTGAACGAGATTGA	TGGAGCATCATCAAGTTTGC	SNP	

5.2.6 Linkage Map Construction

Detailed genotypic data for markers mapped by Hickey *et al*. (2011), was provided by Jerome Franckowiak (QDAFF). Linkage analysis of markers was carried out manually within MapManager QTX (Manly et al., 2001) using the "links report" function.

5.2.7 QTL Detection

Field data from the 2010 spot blotch trial was provided by Jerome Franckowiak (QDAFF). Marker regression was carried out within the programme MapManager QTX. Simple Interval Mapping (SIM) and Composite Interval Mapping were carried out in Windows QTL Cartographer (Version 2.5_011), using Model 6 with a 10 cM window and a 2 cM walk speed.One thousand (1000) permutation tests at 2 cM intervals with a 0.05 significance level were conducted to determine significance thresholds for QTL detection.

5.3 Results

5.3.1 Anchoring Markers Associated with 3H Spot Blotch Resistance QTL in a TR251 x Gairdner population to the ND2420-I x Flagship Population Map

Of the seven markers screened from the previous spot blotch mapping study conducted by Bovill *et al*. (2010), two were not polymorphic (GBM1159; scssr10559) in this population, and were excluded completely. A map was constructed with MapChart 2.2 (Voorrips, 2002), which is presented in Figure 5.1. The map includes markers from the previous mapping study by Hickey *et al*. (2011), and newly mapped markers chosen from Bovill *et al*. (2010).

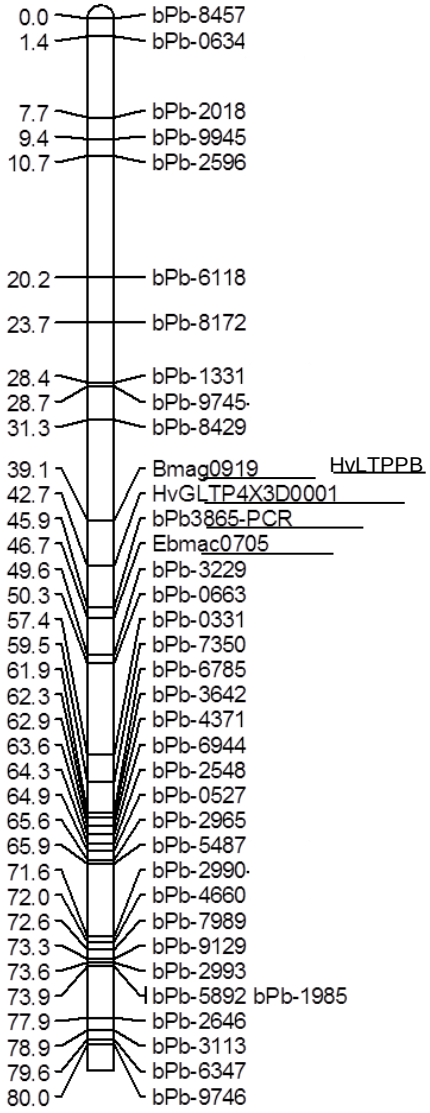


Figure 5.1 Genetic linkage map of chromosome 3HS for the doubled haploid population ND24260 x Flagship. The PCR-based markers from the Bovill *et al.* (2010) mapping study are underlined.

5.3.2 Phenotypic Analysis

The mean and standard deviation of the 2010 'ND24260-I x Flagship' phenotypic data were calculated (Table 5.2). Since the statistical tests for QTL detection assume normal distribution of the data, a histogram of the SB ratings for the field trial is also presented (Figure 5.1).

Table 5.2 Summary of the data from the ND24260-I x Flagship field trials (SB rating according to the scale of Fetch and Steffenson 2009) conducted by the Hermitage Research Station in 2010.

	2010
Average	6.82
Std dev mean	1.00
Median	7.03
n	676
Flagship mean	8.09
n (Flagship)	2
ND24260-I mean	4.69
n (ND24260-I)	2

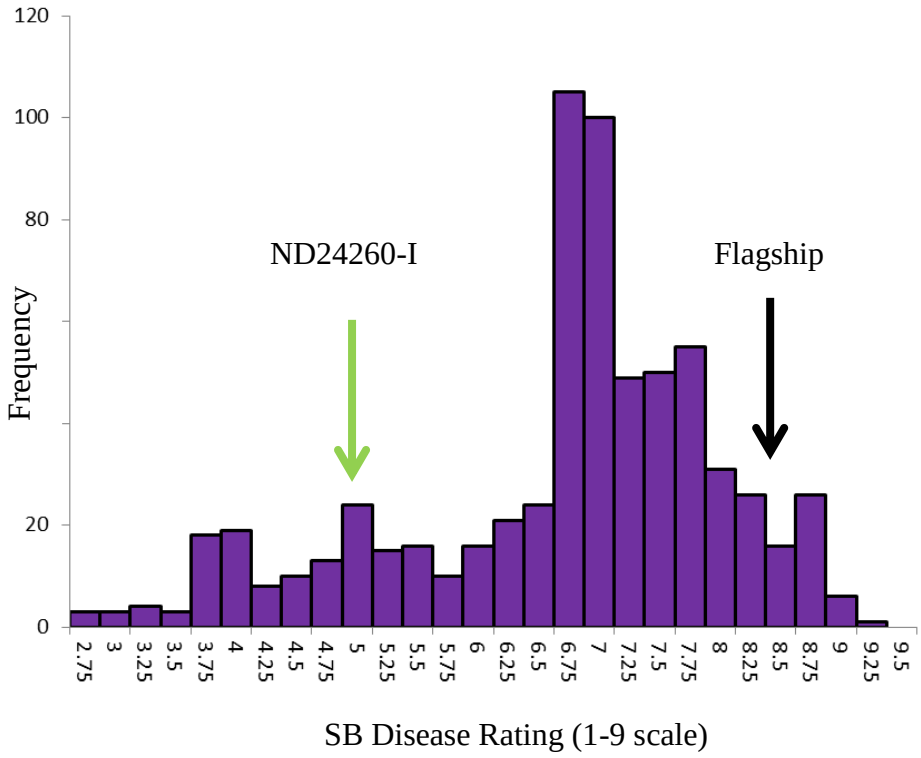


Figure 5.2 Histogram of SB rating (1-9 scale of Fetch and Steffenson 2009) of the population 'ND24260-I x Flagship' for the 2010 field trial. The parental means are indicated by the arrows.

5.3.2 Markers from the Two Barley Mapping Studies

Fifteen markers selected from the two barley mapping studies (Sato et al., 2009; Szucs et al., 2009) were tested against the two parents in PCR amplification and run on polyacrylamide gel. None of the markers were polymorphic when tested against the two parents.

5.3.3 QTL Detection

The marker regression was carried out to identify which markers on 3H were most likely to be associated with the trait of interest. The likelihood ratio statisitic (LRS) is an indication of the likelihood of a QTL or trait being linked to a particular molecular marker (Collard et al., 2005) and was calculated for each marker on chromosome 3H. The markers from the Bovill *et al*. (2010) study Ebmac0705 and HvLTPPB were found to explain the highest level of phenotypic variance (18%), and were inherited from ND24260-I, as indicated by the additive regression coefficient.

Simple interval mapping (SIM) was conducted within the programme Windows QTL Cartographer, which analyses the likelihood of intervals between adjacent pairs of markers being associated with a particular phenotypic trait, rather than analysing association of individual markers. Composite interval mapping (CIM) was conducted within the same programme, and this method combines the calculations of the likelihood of individual markers, as well as marker intervals, being associated with a particular trait, which gives the most accurate location of QTL.. Results of only CIM are presented here in Figure 5.3, since a single significant peak was detected by both methods at the same position. The single significant peak detected above the minimum suggestive threshold (LOD 3.0) in CIM, was detected at marker bPb-3229, adjacent to the 5 markers mapped from the Bovill *et al.* (2010) study . The QTL was contributed by parental line ND 24260-I and had a LOD value of 24.57, with 19 % of the phenotypic variance explained. A more detailed map of the region containing the QTL is presented in Figure 5.4.

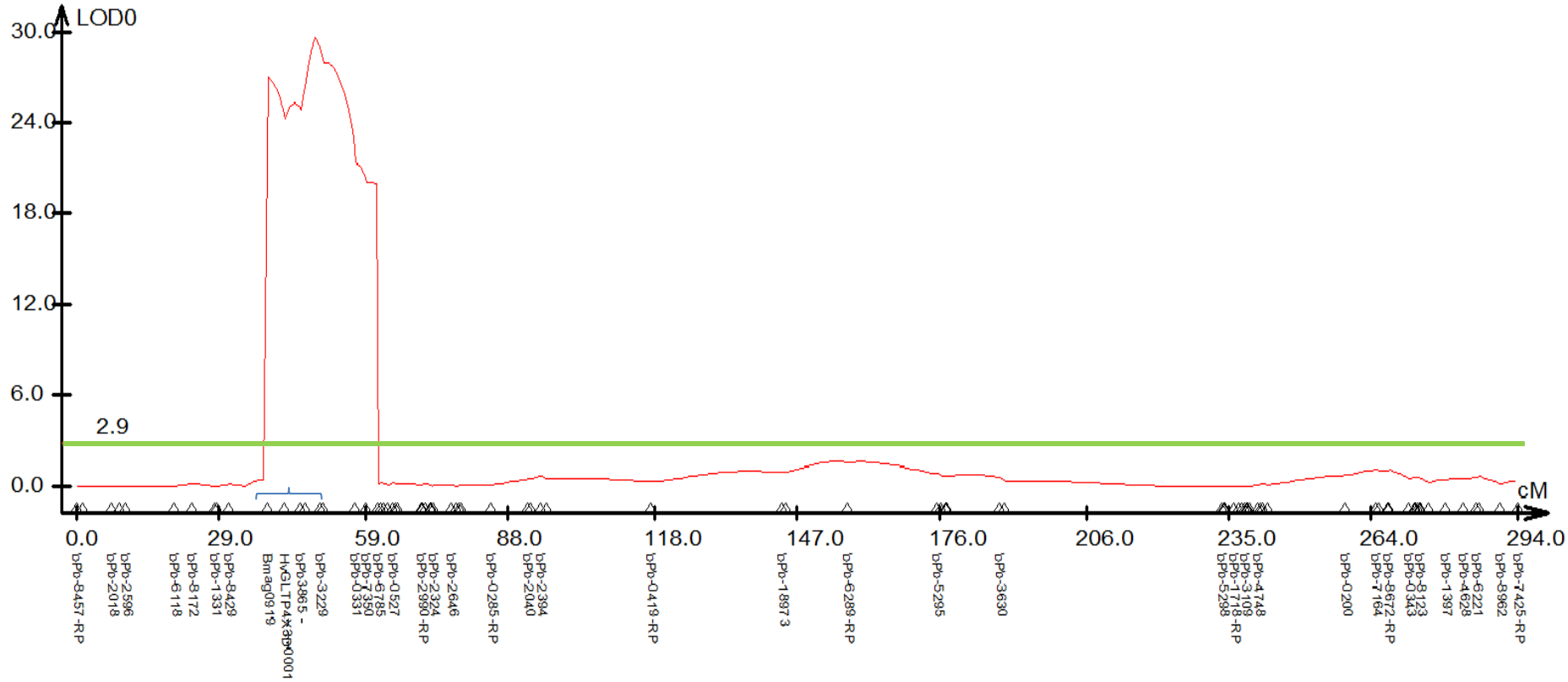


Figure 5.3 Composite Interval Mapping (CIM) profile of Chromosome 3H produced within the programme QTL Cartographer using data from the 2010 field trial of the ND24260 x Flagship population. The horizontal green line indicates the threshold for suggestive QTL. Markers added in this study are indicated with the blue bracket.

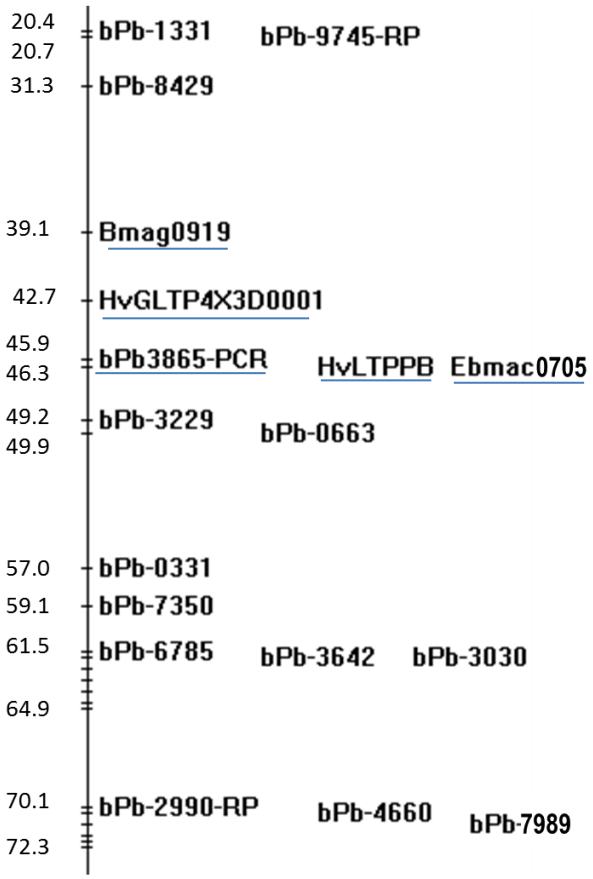


Figure 5.4 Detailed map of the 3HS region containing the spot blotch resistance QTL, and the markers from the study by Bovill *et al.* (2010), which are underlined. The centimorgan distances along the chromosome are indicated to the left of the vertical line, and the markers to the right.

To determine the predictive power of some markers within the interval for SB resistance, a selection of the most resistant and susceptible lines in the population was made from the phenotypic data, with the cut-off value for resistant genotypes being a maximum infection response of 4, and the cut-off value for susceptible genotypes being a minimum infection response of 8. The presence of a QTL was inferred by presence of flanking markers detected in CIM. For the 3H QTL, the closest markers were bPb-3229 and HvLTPPB which were 3 cM apart, and the closest markers for the 7H QTL were bPb-7990 and bPb-2533 (results are presented in Table 5.2). The bPb-2533 marker was most closely linked to the 7H QTL, and the bPb-7990 marker was 26.3 cM away, thus in the case of crossovers between the two markers, bPb-2533 was used to determine the likely parental allele possessed by the line. Of the ten resistant lines, all possessed the resistant parental alleles for the 3H region, five possessed both resistant parental alleles for the 7H region, and five possessed the resistant allele of the closely linked bPb-2533 marker, but not the resistant allele of the bPb-7990 marker. Of the 46 susceptible lines, 27 possessed none of the resistant parental alleles for either the 3H or 7H region, eight possessed both resistant parental alleles in the 3H region, five possessed at least one resistant parental allele in the 7H region, four did not have the resistant parental allele in the 3H region but had insufficient data to determine the presence of alleles in the 7H region, and one did not possess the resistant parental allele in the 7H region but had insufficient data to deermine alleles present in the 3H region. None of the susceptible lines had resistant parental alleles for both QTL regions. The original aim of this study was to increase the closeness of useful markers to the SB resistance gene, however this was unsuccessful.

Table 5.3 Lines from the ND24260 x Flagship population with the highest (> 8) and lowest (<4) infection responses (IR, given as a rating according to the method of Fetch and Steffenson (1999)) in the 2010 seedling trial. Overall spot blotch (SB) reactions are designated as susceptible (S) or resistant (R) for each line. Crosses (X) alone indicate the presence of a QTL assumed by presence of two closely linked flanking markers in spot blotch resistance regions on chromosomes 7H and 3H. Question marks (?) indicate insufficient genotypic data. A cross with a question mark in the 7H region indicates presence of only one flanking marker (bPb-2533, the most closely linked marker to the QTL).

Line	SB Rating	SB Reaction	3H	7H
DH031	3.05	R	X	X?
DH024	3.15	R	X	X?
DH093	3.17	R	X	X
DH095	3.19	R	X	X
DH153	3.73	R	X	X
DH115	3.76	R	X	X?
DH297	3.90	R	X	X
DH084	3.91	R	X	X
DH148	3.96	R	X	X?
DH276	4.00	R	X	X?
DH275	8.03	S	X	
DH067	8.04	S		
DH220	8.05	S		?
DH026	8.05	S		
DH158	8.07	S	X	
DH186	8.11	S		
DH020	8.15	S		
DH300	8.17	S		?
DH043	8.17	S		
DH036	8.20	S	X	
DH162	8.20	S		
DH364	8.23	S		
DH247	8.27	S	X	
DH037	8.28	S	X	
DH210	8.30	S		X?
DH121	8.33	S		
DH385	8.36	S		X?
DH237	8.36	S		
DH323	8.36	S		?
DH381	8.40	S	X	
DH080	8.41	S		X
DH077	8.47	S	X	
DH122	8.47	S		?
DH191	8.48	S	X	
DH011	8.49	S		
DH305	8.49	S		
DH133	8.55	S		
DH101	8.57	S		
DH209	8.63	S		
DH339	8.67	S		
DH119	8.82	S		X
DH106	8.82	S		
DH373	8.83	S		
DH358	8.84	S		
DH211	8.85	S		
DH041	8.86	S		
DH257	8.86	S		
DH241	8.88	S		
DH268	8.89	S	X	
DH360	8.92	S		
DH013	8.92	S		
DH286	8.94	S	?	
DH232	8.99	S		
DH251	9.02	S		
DH240	9.11	S		X?
DH005	9.25	S		

5.4 Discussion

The primary aim of this study was to find new markers which map to the barley chromosome 3H region associated with spot blotch resistance, in order to produce flanking markers closer to the resistance gene. None of the new markers screened on the ND24260 × Flagship population from either of the mapping studies by Sato *et al*. (2009) or Szucs *et al*. (2009) were polymorphic, however, several other important results were obtained, including mapping of SB QTL in a new mapping population (ND 24260-I x Flagship), the 3H interval as expected was found to be important in this new population, and 5 new markers (those from the Bovill 2010 study) were added in this interval.

Resistance in the ND24260 x Flagship population was detected in the same region as the four populations examined in the study by Bovill *et al*. (2010). The flanking markers most closely linked to the 3H QTL were detected in all ten lines displaying the highest level of resistance to spot blotch. The 7H resistance QTL was also detected in five of these resistant lines, and five others possessed the most closely linked bPb-2533 marker, but not the more distant bPb-7990 marker. None of the susceptible lines possessed both QTL, although 14 of the 46 susceptible lines did have at least one of the markers associated with either the 3H or the 7H QTL, three of which possessed the bPb-2533 marker only. These results suggest that resistance is most effective when both QTL are present

The QTL detected in the same region on chromosome 3H as previously detected by Bovill *et al*. (2010) was highly significant, with a LOD of 24.57, and explained 19 % of the phenotypic variance. This is broadly consistent with the previous study by Bovill *et al*. (2010), in which the 3H QTL explained between 13 and 25 % of the phenotypic variance among the four populations in the field. The 7H resistance QTL, although located in the same region for both adult and seedling resistance, is most effective at the seedling stage with LOD scores ranging from 15.4 to 27.5 in the three populations screened by Bovill *et al.* (2010). Additionally, both the 3H and 7H resistance QTL are effective against only some isolates. This was observed in Chapter 3 in which ND B112, a primary source of SB resistance in commercial six-row barley (Gubis et al., 2010), had varying reactions to different isolates of *B. sorokiniana*, ranging from a score of 0 to 6. The action of the two QTL is clearly additive as demonstrated in Table 5.2, with the most resistant genotypes possessing both genes and the least resistant genotypes possessing neither, or only one of the 7H or 3H QTL. Hence the work in this chapter has confirmed the importance of the 3H region, particularly in the presence of the 7H resistance loci, in confering adult plant resistance to spot blotch under Australian conditions. The original aim of this chapter was to find markers more closely linked to the region of interest, however no new markers were identified, and the new interval is quite large despite the population being bigger, possibly due to DArT marker effects, and therefore fine mapping was unsuccessful.

A number of options could be taken as a next step for further fine mapping of the chromosome 3H spot blotch resistance region. Firstly, DH populations are not the most appropriate for fine mapping, as this population is derived from a single meiosis (F1), meaning only low opportunities for all possible recombinants to appear despite the larger size of the population. Other populations which may be used in future work may include recombinant inbred lines (RILs) and near isogenic lines

(NILs) populations, which are more suitable as they enable more opportunities for recombinants and hence a higher likelihood of identifying markers which are polymorphic between parents. Association mapping or nested association mapping may also be appropriate in identifying a greater number of polymorphisms and hence a denser genetic map, as these methods utilise natural polymorphisms occurring over several generations {Hall, 2010 #346}(Deschamps *et al.* 2012, Hall *et al.* 2010, Yu *et al.* 2008).

DArT sequences from the study by Hickey *et al.* (2011) to design primers for PCR, which is a faster method for marker genotyping than DArT mapping, and could potentially show different polymorphisms to the original DArT markers. Additionaly, SNP sequence data from the study by Szucs *et al*. (2009) could be used to design primers producing shorter fragments, which could then be used in high resolution melt analysis. An alternative option would be to map the region of interest in multiple populations. A second large barley doubled haploid population has recently become available from QDAFF (ND24388 x NRB06059). Two adult resistance screens were run in 2011 and 2012, but this population was not screened with the markers due to shortage of time. The advantage of screening multiple populations is that non- polymorphic markers in the first population may be polymorphic in the second, and hence able to be mapped.

Next-generation sequencing could also be utilised in fine mapping. Sequencing of the barley genome has proven difficult in the past due to a high level of repetitive sequences (Muers, 2013), however recently the International Barley Genome Sequencing Consortium published sequence data for the entire genome of barley cultivar Morex (The International Barley Genome Sequencing Consortium, 2012). Next-generation sequencing is a term used to describe several new sequencing techniques, which are an improvement on earlier methods based on Sanger sequencing (Shendure and Ji, 2008). These new techniques enable sequencing of much longer reads, and a greater degree of automation of the sequencing process. Procedures vary in data aquisition methods, but all have similar underlying principles (see Section 1.12.1). The process involves preparation of DNA libraries by random fragmentation of DNA, followed by ligation of adapters to the fragments, enabling spacial separation of individual fragments on either a planar surface or beads. These fragments (or reads) can be amplified several-fold, and subjected to a sequencing method (Ansorge, 2009). As an example, the Illumina sequencing technique involves the use of fluorescently-labelled NTPs, with each specific base (A, T, G or C) emitting a light detected at different wavelengths. These NTPs are additionally ‘reversible terminators’, allowing addition of only one base at a time in each PCR cycle. At the end of each base addition, a photo is taken, and it can be determined which base was added by the wavelength of the light emitted (Ansorge, 2009).This procedure allows analysis of several reads in parallel, and is a relatively fast process. The genotyping-by-sequencing approach enables marker discovery and genotyping of a population to occur in a single step, and is robust across a number of species (Poland Rife 2012). This method targets a specific region of interest for sequencing and utilises software to identify polymorphisms in the population. With these methods in mind, the mapping population used in the current study could be sequenced in order to more finely map the 3H spot blotch resistance region.

Two recent studies utilised sequencing methods for high density mapping of the barley genome. Mayer *et al.* (2011) used a novel method which incorporated multiple methods including next-generation sequencing and chromosome sorting. This research group assigned approximately 86% of the barley genes to individual chromosome arms, and produced 470 Mbp of high quality sequence data which mapped to chromosome 3HS. The second study by Poland *et al.* (2012) used a two-enzyme genotype-by-sequencing method to produce polymorphism data, and mapped over 34 000 SNPs and 240 000 tags onto the Oregon Wolfe Barley reference map. Sequence data from both studies could also be used in future high density mapping of the 3H SB resistance region.

Chapter 6: Analysis of black-pointed barley seeds for presence of the fungal pathogen *Bipolaris sorokiniana* using q-PCR

6.1 General Introduction

The grain defect black point (BP) is a significant constraint in both wheat and barley, and is highly undesirable in both the flour milling and malting industries. The defect appears as a brown to black discolouration at the embryo end of grain, and in severe cases can extend along the ventral crease. In wheat, the discolouration can affect flour quality, gives a greyish colour to bread, and undesirable black spots when milled for semolina (Lorenz, 1986; Maloy and Specht, 1988; Rees et al., 1984). In the barley malting industry, BP is undesirable due to the assumption it is colonised with fungus, which can lead to beer gushing or fungal toxin release and staling of beer. Barley varieties with >10 % discoloured grain are downgraded to feed quality, which sells at a lower price (March et al., 2007) . The defect has also been observed in some studies to cause a decrease in germinability (Hudec 2007).

A great deal of research into the cause of BP has been carried out, although much of it has focussed on the defect in wheat, not barley. The cause has not yet been conclusively determined in either plant species, although several researchers attribute the disease to the fungal pathogen *Bipolaris sorokiniana*, which causes spot blotch and common root rot of wheat and barley. So far, research investigating the cause of BP has included microscopy studies examining fungal colonisation of seeds from the field with and without BP symptoms, field experiments investigating effect of fungicide and soil nutrient treatments on BP symptoms, and a physiological study investigating artificially induced symptoms in the absence of fungal species.

In addition to *B. sorokiniana*, other fungal pathogens and saprophytes named as causative agents of BP include most frequently *Alternaria alternata*, and less frequently *A. infectoria*, *Epicoccum nigrum*, *Cladosporium herbarum*, *Stemphyllium botryosum*, *Fusarium spp.*, *Curvularia spp.*, and *Chaetomium spp*.(Conner and Davidson, 1988; Ellis et al., 1996; Lorenz, 1986; Moschini et al., 2006; Williamson, 1997). However, each study has obtained different results with regards to levels of individual fungal species in BP and symptomless grain, and fungal isolates have also been obtained from apparently healthy seeds (Maloy and Specht, 1988). Environmental conditions in which the defect is most prevalent are rainfall during seed maturation (Maloy and Specht, 1988), high humidity, and temperature extremes (Mak et al., 2006; Walker et al., 2008), which are also conditions conducive to fungal growth, factors which confound research investigating the cause of BP. Williamson (1997) also successfully produced BP symptoms in wheat grain *in vitro* in the absence of any fungal species, by exposing seeds to hydrogen peroxide, a compound produced by cells during periods of high stress. Furthermore, Koch's postulates have not been able to be satisfied.

The occurrence of BP in only some grains of individual heads (Malaker and Mian, 2010) seems to contradict the theory of fungal colonisation being the cause of BP. In three separate studies of wheat, the middle spikelets on heads had a higher incidence of BP than those at the top and bottom of head (Ellis et al., 1996; Hoyle, 2000; Huguelet and Kiesling, 1973). Two of these studies (Hoyle, 2000; Huguelet and

Kiesling, 1973) also found that central florets in each spikelet, which normally produce smaller grains, had lower incidence of BP.

A number of other factors affecting the occurrence of the defect have been investigated, including grain size, nutrient availability and stress. However, some of these factors were found to have inconsistent effects between experiments within studies and also between individual studies. For example, grain size in some studies was found to be significantly higher in grains displaying BP symptoms (Ellis et al., 1996; Hoyle, 2000; Lorenz, 1986) and in other studies, BP seeds had significantly lower weights (Clarke et al., 2004; Huguelet and Kiesling, 1973). Application of foliar fungicides has not been consistently effective in controlling levels of BP. Although application of fungicide at ear emergence can reduce BP levels, it has been found to increase the defect when applied early before ear emergence (Dimmock and Gooding, 2002; Ruske et al., 2003; Wang et al., 2002). Ellis *et al.* (1996) found in one year of experiments, foliar fungicide application increased levels of BP, but in an experiment in the following year, the same treatment decreased BP levels. It is thought that fungicides that protect the supply of grain assimilate, increase grain size and therefore levels of BP (Clarke et al., 2004).

Application of nitrogen fertilizer likewise was found to reduce levels of BP in wheat (Clarke et al., 2004; Ruske et al., 2003; Sisterna and Sarandon, 2005).. Higher thousand grain weights have been associated with higher BP levels (Cromey and Mulholland, 1988; Fernandez et al., 2000; Maloy and Specht, 1988). In contrast, Fernandez and Conner (2011) found an increase in the level of BP symptoms with increased nitrogen fertilizer, and suggested this may be due to a thicker leaf canopy, and therefore production of the ideal microclimate of high humidity for the production of BP symptoms.

In spite of evidence to the contrary in studies investigating BP in wheat, many researchers still believe BP to be caused by fungal pathogens, in particular *B. sorokiniana*. The current study aims to investigate whether there is a relationship between the level of *B. sorokiniana* present in black point affected seeds and the level of black point by utilizing a more rigourous approach to analysis, quantitative PCR. This method provides an objective analysis of the quantity of *B. sorokiniana* DNA, which is used as an indicator of fungal biomass. This method of analysis has been used in similar studies investigating correlations between symptom and pathogen. Knight *et al.* (2012) utilized qPCR to analyse association between the level of tissue discolouration in wheat plants displaying crown rot symptoms, and the level of Fusarium pseudograminearum present in the tissue. The current study will be the first to investigate BP in Australia using this method.

6.2 Materials and Methods

6.2.1 Field Trial design

A field trial was conducted for both SB and BP on a doubled haploid (DH) barley population derived from a cross of Flagship × ND24260-I. This population was selected for the trial because the parents are known to have differential resistance to both SB and BP. The trial was conducted in 2010 (planted on 28/06/2010) at Redland Bay Research Station, Queensland as part of a separate study. The trial was

a partially replicated (p-rep) design (Cullis et al., 2006), using Immdesign software. Two plots were planted, one sprayed with the fungicide Tilt at 250 mL per ha on four occasions (4th August, 24th August, 13th September and 28th September 2010, this was the BP trial), and one unsprayed (SB trial), with two replicates per treatment. A total of 48 lines were tested in duplicate for the BP trial. For the SB trial, 229 lines were run in duplicate and a single replicate of 107 additional lines were tested in either rep 1 (54 lines) or rep 2 (53 lines). The trial was not inoculated, but naturally infested with *B. sorokiniana*. Seed was harvested on 29th November, 2010.

6.2.2 Trial Sample Collection

Barley seed was sampled from seed bulks of already harvested seed of ten randomly selected lines in the ND 24260-I × Flagship DH population, which were DH003, DH064, DH123, DH139, DH174, DH189, DH277, DH289, DH329 and DH372. Seed was also sampled from varieties Gairdner (Onslow/Tas83-587) and Tallon (Triumph/Grimmett). Tallon is susceptible to both spot blotch (Johnston and Mackay, 1991) and BP (Sulman et al., 2001b), while Gairdner is more resistant to BP (Hadaway et al., 2005) and susceptible to spot blotch (Bovill et al., 2010). In order to obtain barley seeds showing a complete spectrum of BP symptoms, a random selection of seeds from the 10 doubled haploid lines and two varieties were sampled from both fungicide treated and untreated plots. For each DH line and variety from the sprayed plots, 30 seeds each of BP affected and unaffected seeds were collected. For the unsprayed plots, BP free seeds were difficult to identify, thus 30 BP seeds only were collected from each line or variety. Due to this difficulty, seeds from unsprayed plots were used only in the fungal colonisation study in the next section (6.2.3). All other sections compared BP and BP free seeds of the sprayed plots only.

6.2.3 Fungal colonisation and collection of isolates

A random sample of seeds was selected to test for the presence of fungal colonisation. Twelve seeds from seven lines and one variety (DH003, DH174, DH189, DH277, DH289, DH329, DH372 and Gairdner) were selected from the unsprayed BP group; five seeds from 3 lines and one variety (DH 174, DH277, DH372 and Tallon) were sampled from the fungicide sprayed BP group and 15 seeds from 6 lines and both varieties (DH 003, DH064, DH139, DH189, DH277, DH329, Gairdner and Tallon) were selected from the BP free, sprayed group. All seeds were surface-sterilised for 5 seconds in 70 % ethanol, then 2 minutes in 5 % NaOCl, and rinsed 3 times in milliQ water. Six of the seeds from the BP unsprayed group were separated into seed and husk before surface-sterilisation. Whole seeds, hulled seeds and their husks were placed on moist filter paper in sterile petri dishes (one dish per genotype/symptom combination), sealed with parafilm and incubated at room temperature under normal day/night light conditions. After two to four days, seeds were observed for fungal colonisation. Species of fungi present were identified and recorded, and single-spore isolates were taken for each species from each petri dish. Single spores were initially placed on potato dextrose agar (PDA) for 2 to 3 days until mycelial growth was visible, and then transferred to fresh PDA. Isolates were placed in an incubator in the dark at 24°C for 7 days until sufficient growth was obtained for DNA extraction.

6.2.4 AFLP analysis of *B. sorokiniana* isolates from black-pointed seeds

In order to identify any genetic patterns specific to isolates from different groups of seed (BP or symptomless), all 19 *B. sorokiniana* isolates harvested from seeds of sprayed plots were subjected to AFLP analysis, following the protocol described in Section 2.2.4. Polymorphic and monomorphic markers were scored as binary data.

6.2.5 Rating of black point symptoms

Ten BP seeds per line or variety and disease state (BP or BP free) from the sprayed plots were rated according to the percentage of discolouration due to BP (Figure 6.1). Seeds were classified as 'BP free' when no dark brown to black discolouration was evident at the embryo end of the seed (Figure 6.2a). BP seeds (Figure 6.2b) included any seed with this discolouration present. First the complete seed with husk attached was rated for discolouration, then the husk was removed and set aside, and the internal seed surface was rated. Seeds and husks were dried in the oven at 60^{o}C overnight, after which dry weights were recorded for each. Individual seeds and husks were ground to powder using a tissue lyophyliser. Dry weights of ground seed material were remeasured after grinding, due to losses during sample transfer.

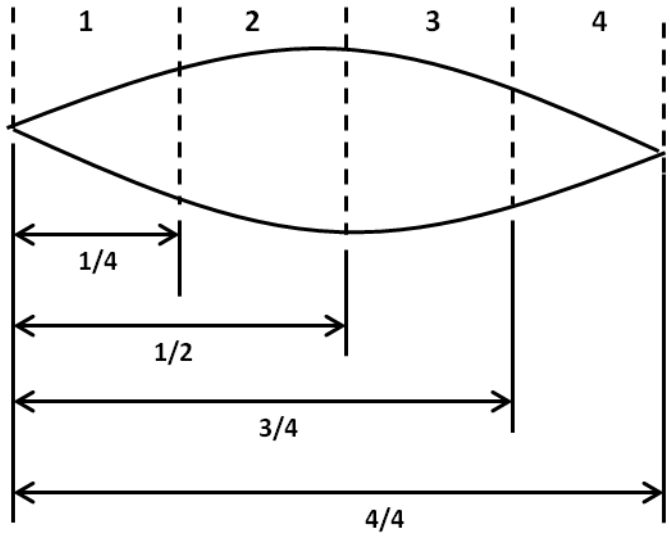


Figure 6.1 BP seed rating scale, according to the percentage discolouration of the seed.

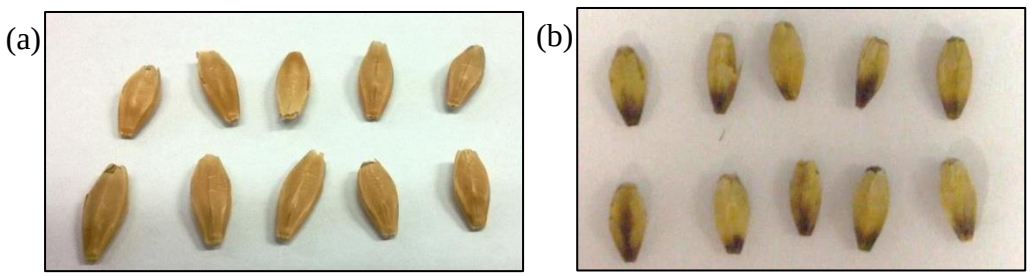


Figure 6.2 Barley grains (husk attached) (a) free from BP symptoms, and (b) affected with BP showing discolouration at the embryo end of the grain.

6.2.6 DNA extraction

DNA was extracted from fungal isolates, hulled seed and husk tissue using the cetyl trimethyl ammonium bromide (CTAB)-based method of Saghai-Maroof *et al.* (1984). For seeds and husks, all ground tissue was subjected to DNA extraction. For seed DNA extraction, the method was modified by increasing centrifuge speed to 14 000 rpm for 10 mins to ensure DNA was not lost when pouring off isopropanol. Extracted DNA was quantified using an Implen Nanophotometer (Integrated Sciences).

6.2.7 Design of species-specific primers

As discussed in Chapter 2, species-specific primers for identification of *B. sorokiniana* were previously designed and tested by Matusinsky (2010), but were found to be inappropriate for that purpose in Australian isolates. For this reason, new species-specific primers were required to be identified for use in qPCR, and five DNA sequences of the pathogen available on Genbank (Table 6.1) were screened for similarity with other species using the nucleotide BLAST function on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi? PROGRAM= lastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Since no highly similar sequences were identified in other fungal genomes for these regions, primers were designed using Primer3 software. Sequences for these primers are presented in Table 6.1.

All four primer pairs were tested on a number of *B. sorokiniana* isolates from the black-pointed seed tissue as well as a large collection of isolates used in a previous study, totalling 182 isolates, to check for consistency within *B. sorokiniana*. A further 36 isolates of other species were also tested with the primers, (*Fusarium graminearum*, *F. crookwellense*, *F. scirpi*, *F. semitectum*, *F. proliferatum*, *F. compactum*, *F. poae*, *F. equisetum*, *Pyrenophera teres* f. *teres*, *P. teres* f. *maculata*, supplied by P. Davies, University of Sydney) along with the other species isolated from BP seeds (*Alternaria alternata* and *Epicoccum nigrum*), which were identified with the assistance of A. Martin (USQ) and R. Fowler (QDAFF).

Barley-specific primers designed previously by Jill Petrisco based on the elongation factor 1 alpha (EF1α) were used as a check for individuals that were negative for the PPT1 primers. All primers were supplied by Integrated DNA Technologies.

Table 6.1 The *B. sorokiniana* sequences and the primers designed for testing species-specificity. The barley specific EF1a primers designed by Jill Petrisco are also included.

Region	Genbank Accession	Forward Primer	Reverse Primer
ITS	DQ337383	5'ATCTCTTGGTTCTGGCATCG	5'TAAGGCGAGTCTCCCAGAAA
NPS6	HQ830032	5'CCTCGATCTCCTACGCTACG	5'TCTGAAACTTCGCCTCAACC
PPT1	HQ830035	5'GATTCGACGGCTGGATAGAC	5'CGTGATGCTGAGTTGCTTG
MAT-2	AF275374	5'TTTGGCACAACCTCTCTCCT	5'CTTCTGGAGCCGTAGTCGTC
EF1α	L11740	5’ACGTGGCTGTCAAGGAT	5’CTGGGCAGTGAAGTT

6.2.8 Distance-based clustering analysis

A similarity matrix was constructed using the Dice coefficient (Dice, 1945) in the Qualitative Data programme within the NTSYS-pc version 2.20f software package. Cluster analysis of matrix data was performed with NTSYS-pc using the unweighted pair-group method with arithmetic mean (UPGMA, Sneath and Sokal, 1973) provided in the SAHN programme. A dendrogram was constructed using TREE PLOT.

6.2.9 q-PCR Analysis

All samples of DNA extracted from seed and husk material were subjected to q-PCR analysis using a Rotorgene 6000 series. Standards were diluted into six five-fold serial dilutions of pure genomic *B. sorokiniana* and barley (0.002 ng to 5.0 ng per uL) DNA, which were initially tested in triplicate and then included in duplicate in every run. DNA concentrations of *B. sorokiniana* and barley were initially determined spectrophotometrically using a nanophotometer (Implen). The *B. sorokiniana* isolate BP#8, which was isolated from a BP seed acquired in the process outlined in Section 5.2.3, was used for the *B. sorokiniana* standard curve. The barley variety Bowman was used for the barley standard curve. 'Bowman' DNA was used as a negative control in the *B. sorokiniana* detection q-PCR, and likewise 'BP#8' was used as a negative control in the barley detection q-PCR runs. The barley primers were used as a check only, to determine if results giving a negative result for *B. sorokiniana* DNA were accurate, or due to poor quality DNA. 'No template controls' (NTCs) were also included in every assay. Each unknown seed and husk sample was tested at least once in duplicate, with a selection of samples duplicated across separate PCR assays. The husk DNA was undiluted when run in the assays, and the seed DNA extract was diluted ten-fold, due to its high concentration.

Each q-PCR reaction contained the following reagents: 1 µL template DNA, 0.25 U of IMMOLASE ™ DNA Polymerase, 2µL 10x reaction buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 10 µM of each primer (F and R) and 30 µM of the fluorescent dye Syto9 (Life Technologies). PCR conditions were: hold at 95ºC for 7 mins, followed by 40 cycles of 95ºC for 15 secs, 55ºC for 20 secs, and 72ºC for 20 secs.

6.2.10 Statistical Analysis of qPCR Data

The number of cycles for each q-PCR run was set at a maximum of 40 to discriminate between positive and negative results, and thermocycling was completed in less than 2 hours. Threshold cycle (Ct) was recorded as the cycle at which fluorescence level exceeded background fluorescence. The slope (M) was calculated as the change in Ct divided by the change in log input (DNA quantity). Efficiency of amplification (E) was given as the proportion of total DNA amplified per cycle, so for a 100 % efficient reaction, this is 1. Results were presented as $y = Mx + B$, where M is the slope and *B* the intercept. The amount of detected *B. sorokiniana* and barley DNA of each sample was recorded, and total ng DNA per mg tissue was calculated for each. The data generated in qPCR analysis was used to calculate the proportion of *B. sorokiniana* DNA per ng of total DNA extracted (ng/ng), and the amount of *B. sorokiniana* DNA per mg of seed tissue. These values were used in data analysis using the programme IBM SPSS Statistics 21.

6.3 Results

6.3.1 Fungal colonisation

Individual seeds were sampled from seed bulks harvested from Redland Bay Research Station in 2010, from fungicide sprayed and unsprayed plots. When seeds were surveyed for fungal colonisation (Table 6.2), all seeds whether black-pointed or black-point free, fungicide treated or untreated, were colonised with fungus. Only three species of fungus were observed; in order of frequency these were *Bipolaris sorokiniana*, *Alternaria alternata* and *Epicoccum nigrum*. Individual spores of each fungal species from each petri dish were isolated and recorded. Of the BP unsprayed seeds, ten were only colonised by *B. sorokiniana*, and two were also colonised by *A. alternata* and *E. nigrum*. Four of the BP sprayed seeds were colonised only by *B. sorokiniana* and one also had *A. alternata* present. In the group of BP free, fungicide sprayed seeds, nine were colonised by only one species (six by *B. sorokiniana* only, and three by *A. alternata* only), three were colonised by *B. sorokiniana* and *A. alternata*, and two were colonised by *B. sorokiniana*, *A. alternata* and *E. nigrum*.

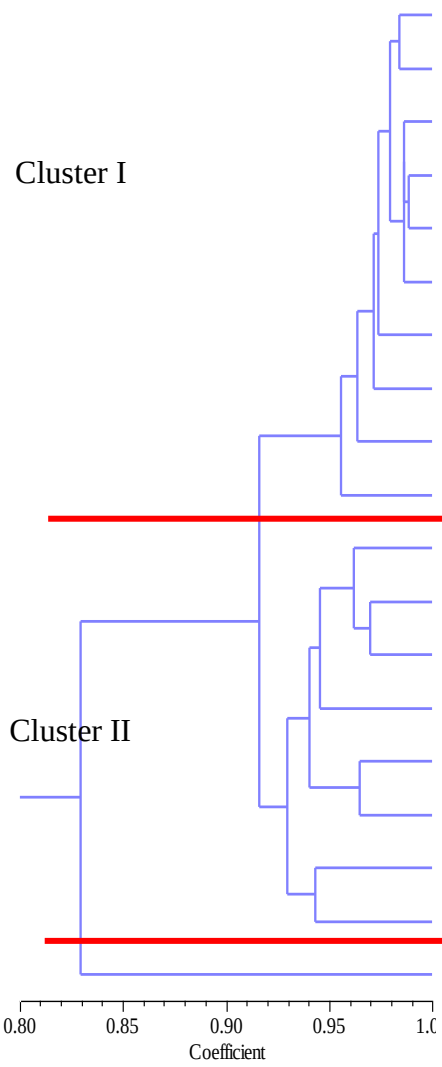
6.3.2 AFLP Analysis

The aim of AFLP analysis was to investigate whether any genetic clustering can occur for isolates from BP and BP free seeds. In this preliminary investigation, a total of 291 markers were scored (181 monomorphic and 110 polymorphic). Both monomorphic and polymorphic markers were included in the cluster analysis (Figure 6.3). This revealed two clusters of isolates, which nevertheless were 93 % similar. In addition one outlier was present with respect to both these clusters, isolate B#35-2 from cultivar Gairdner. This isolate was from the same cultivar and disease state (BP free) as isolate BP#35-1, but these were not from the same seed. Some isolates from the same variety and disease state clustered separately (BP#30-1, BP#30-2 and BP#30-3) and several from seeds of different genotypes and disease states were very similar. Isolates sampled from seeds of the same variety were highly similar in some cases, but none were genetically identical. The level of similarity among isolates is high (>82% for all isolates). Isolates of BP seeds did not cluster separately from isolates of BP free seed materials.

Table 6.2 Fungal species observed on BP and BP free seeds of fungicide treated and untreated plots. Three species were observed: *Bipolaris sorokiniana*, *Alternaria alternata* and *Epicoccum nigrum* (E). Some seeds were kept whole (WS) for colonisation tests, some were separated into seed (S) and husk (H) before surface sterilisation. Isolates were collected of each species observe

Line	Black point (BP) of Black Point Free (F)	Sprayed (S) or Unsprayed (US)	Seed	Species	No. Isolates Collected
DH003	BP	US	WS	B	1
	BP	US	H	B	1
	BP	US	S	B	1
DH174	BP	US	WS	B	2
DH189	BP	US	H	B	1
DH277	BP	US	H	B	1
	BP	US	S	B	1
DH289	BP	US	H	B	1
	BP	US	S	B	1
DH329	BP	US	WS	B	2
	BP	US	H	B	1
	BP	US	S	B	1
DH372	BP	US	WS	B	1
	BP	US		A	1
	BP	US		E	1
Gairdner	BP	US	H	B	1
	BP	US	S	B	1
	BP	US	WS	A	1
	BP	US		E	1
DH174	BP	S	WS	B	2
DH277	BP	S	WS	B	1
DH372	BP	S	WS	B	1
	BP	S		A	1
Tallon	BP	S	WS	B	1
DH003	F	S	WS	A	1
DH064	F	S	WS	A	1
DH139	F	S	WS	B	3
DH189	F	S	WS	B	3
	F	S		A	2
	F	S		E	1
DH277	F	S	WS	B	1
	F	S		A	1
DH329	F	S	WS	B	1
	F	S		A	2
	F	S		E	1
Gairdner	F	S	WS	B	2
	F	S		A	1
Tallon	F	S	WS	B	2
	F	S		A	1

Key	
Abbreviation	**Item**
BP	Black Pointed
F	Black Point Free
S	Sprayed
US	Unsprayed
WS	Whole seed
H	Husk
S	Hulled Seed
B	*B. sorokiniana*
A	*A. alternata*
E	*E. nigrum*



Isolate	Line/Variety	Black point (BP) or Black Point Free (F)
BP#17-1	DH174	BP
BP#30-2	DH189	F
BP#17-2	DH174	BP
BP#22	DH372	BP
BP#28-2	DH139	F
BP#35-1	Gairdner	F
BP#31	DH277	F
BP#28-1	DH139	F
BP#28a	DH139	F
BP#32	DH289	F
BP#19	DH277	BP
BP#24	Tallon	BP
BP#36-2	Tallon	F
BP#36-1	Tallon	F
BP#30-1	DH189	F
BP#34	DH382	F
BP#30-3	DH189	F
BP#33	DH329	F
BP#35-2	Gairdner	F

Figure 6.3 AFLP cluster analysis of *B. sorokiniana* single spore colonies isolated from BP and BP free seeds. All isolates are from fungicide treated plots.

6.3.3 Comparison of DNA Extraction Among Seed Weight Categories

DNA was extracted from the complete seed tissue for each individual seed. The amount of total DNA extracted per seed (Figure 6.4) increased significantly from the 1 to 10 mg seed category to the 21 to 30 mg category. In contrast the total DNA extracted from seeds weighing more than 30 mg was not significantly different from the quantity extracted from the 21 to 30 mg seeds. Based on this data, the calculated proportion of *B. sorokiniana* DNA (ng *B. sorokiniana* per total DNA extracted) was used for all data analysis.

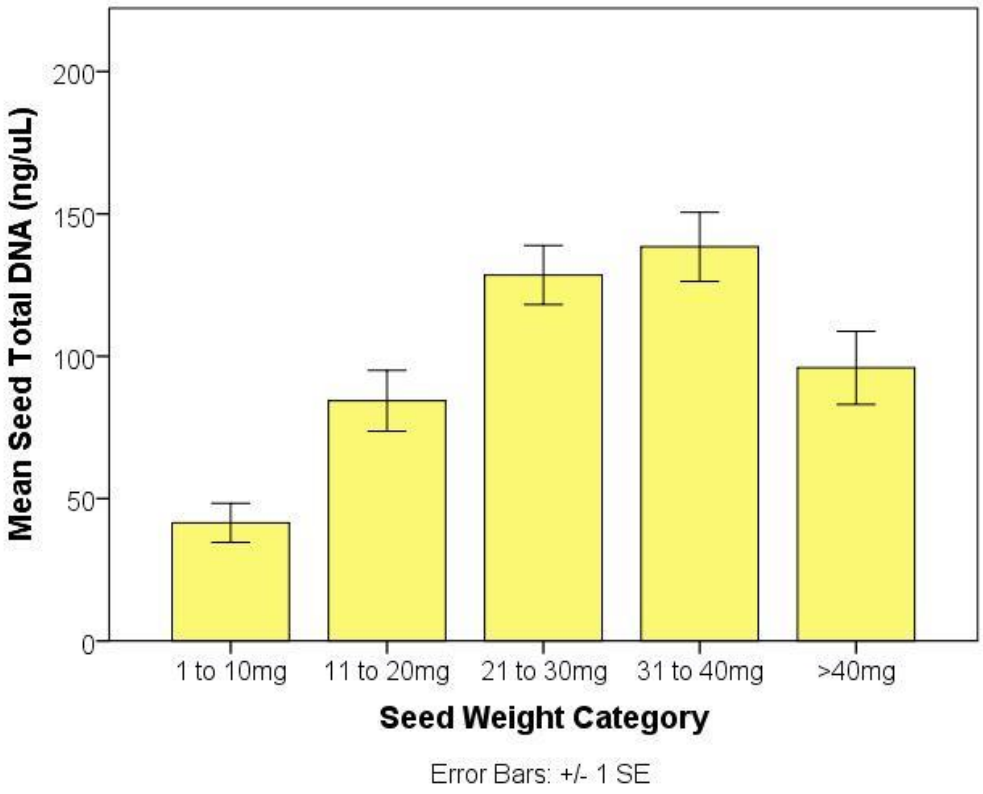


Figure 6.4 Level of total DNA extracted from individual seeds in each seed dry weight category.

6.3.4 Species-Specific Primers

All data for primer specificity tests are recorded in Appendix 6.0. The primer pair with the highest proportion of false negatives was MAT-2 (42 %), and the two markers with the lowest proportion of false negatives were ITS1 (5 %) and PPT1 (5 %). Since ITS1 scored a very high proportion of false positives in the alternate species test (59 %) and PPT1 scored only 2 false positives, this primer pair was selected for use as the species-specific primer pair. Most importantly, isolates of *A. alternata* and *E. nigrum* collected from seeds in the colonisation tests, tested negative to the primer pair. *F. pseudograminearum* and *F. poae* were the two species which gave a positive result for the PPT1 marker. Although both species can be isolated from grains of grasses (Akinsanmi et al., 2004; Summerell et al., 2011), they were not observed during the examination of seeds for species colonisation, and so the PPT1 primers were deemed suitable for use.

6.3.5 Black point seed visual discolouration rating

The total number of samples per level of discolouration for each tissue (husk and seed) is presented in Table 6.3. The discolouration level which appeared most frequent in both seed and husk tissue was 1 to 25 % browning, and only the internal seed surface occasionally showed a level of discolouration greater than 50 %. It was also observed that among the 120 seeds with BP free husks, the seed surface beneath the husk in 21 seeds had discolouration to some degree (not more than 25 %). Among the 114 seeds showing BP symptoms on the husks, 37 had an internal seed surface showing no discolouration. When the level of *B. sorokiniana* DNA detected by qPCR in the hulled seed was plotted in a scatter graph against the levels of DNA detected in the husk (Figure 6.5) a very low regression level was observed, with an R^2 value of 0.003, not significant at the 0.05 level.

Table 6.3 Total numbers of individuals in each tissue discolouration category for husk and hulled seed tissue.

Percent Discolouration	Number Lemmas	Number Seeds
0%	120	138
1 to 25%	68	79
25 to 50%	46	9
51 to 75%	0	4
76 to 100%	0	4
Total	**234**	**234**

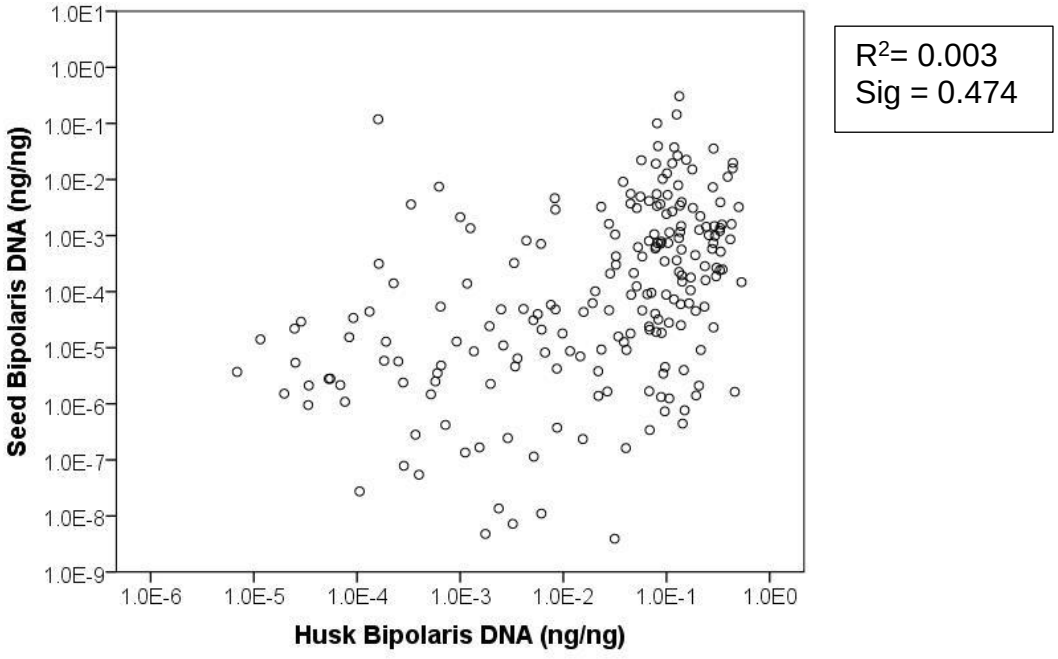


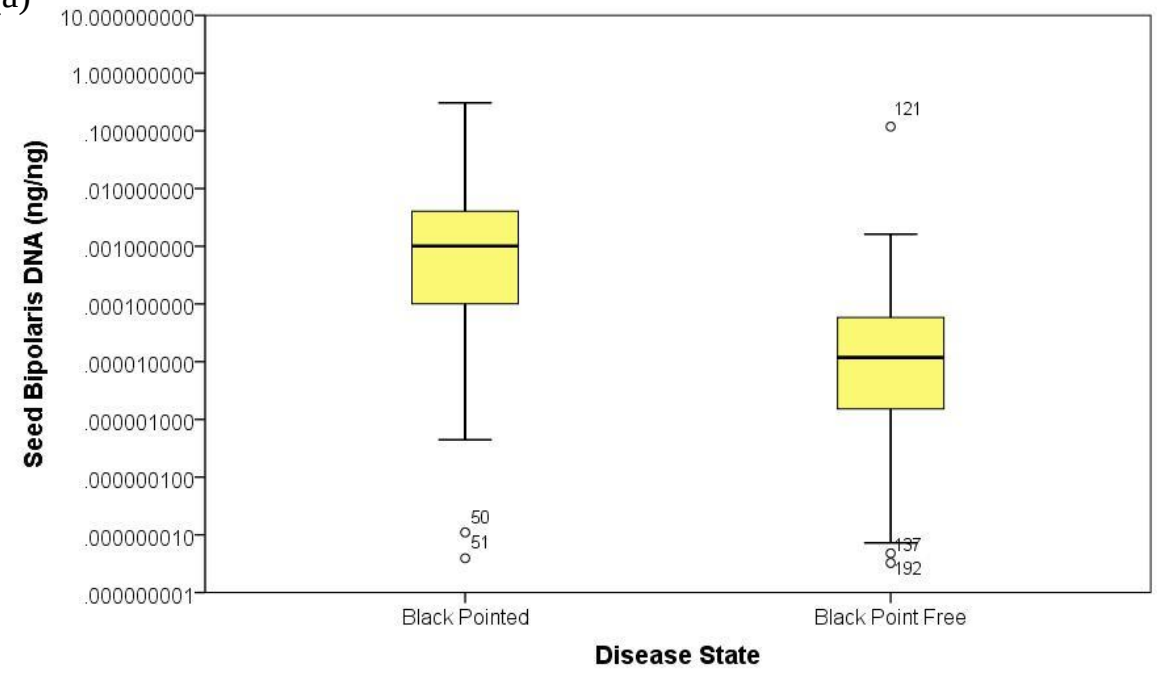
Figure 6.5 Scatterplot comparing the level of *B. sorokiniana* detected in husk tissue to the level of *B. sorokiniana* detected in hulled seed tissue. The regression coefficient (R^2) and significance of regression are shown in the legend.

6.3.6 q-PCR of Individual Seeds

Linear regression correlations between Ct and known DNA quantities were high for both *B. sorokiniana* ($R^2 > 0.96$, $y = -3.4x + 29.0$, $E = 0.98$) and barley ($R^2 > 0.91$, $y = -2.193x + 25.786$, $E = 0.95$). Once q-PCR was completed, calculations were made for the amount of *B. sorokiniana* DNA (ng) per ng of total DNA extracted. The q-PCR data was divided into individual categories (i.e. black-pointed and black-point free, fungicide treated plots only). Data was checked for normality, and none of the q-PCR datasets for each category showed a normal distribution; all were skewed significantly to the left. Statistical transformations failed to give a normal distribution, and as a result, both parametric and non-parametric tests were used to analyse statistical differences. Occasional missing data points were due to loss of samples either during the process of separating seed from seed coat, or during the grinding and extraction process.

Bipolaris sorokiniana levels present in seed and husk tissue were compared (for fungicide sprayed plots only) using various statistical analyses. When comparison was made between BP and BP free seed and husk (Figure 6.6) tissue using box plots, both the BP seeds and husks suggested a higher level of *B. sorokiniana* DNA compared with the black-point free seeds. An ANOVA of the data (see Table 6.4) showed this difference was significant at the 0.05 level for seed tissue, and the 0.001 level for the husk tissue. The non-parametric independent samples Mann-Whitney U analysis supported this significant difference at the 0.001 level for both tissues. Examination of scatter plots of the seed data (Appendix 6.1) showed a single outlier in the BP category which was above 0.3 ng fungal DNA/ng total DNA. All other values for the sprayed plots were below 0.15 ng/ng.

(a)



(b)

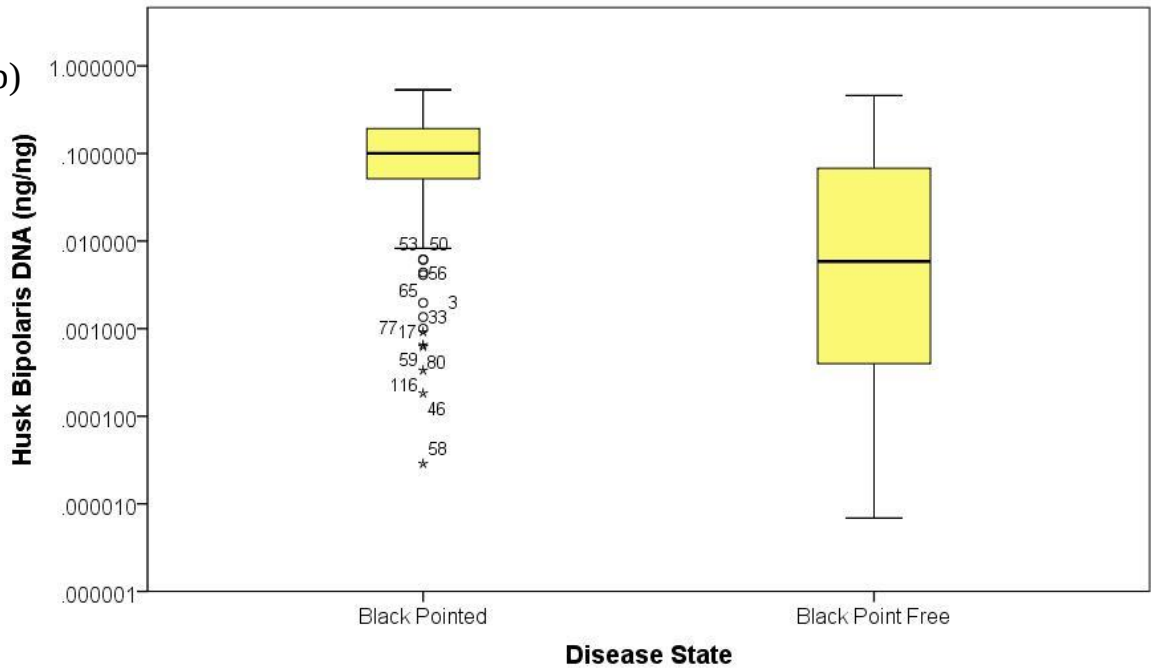


Figure 6.6 Level of *B. sorokiniana* DNA (ng fungal DNA per ng total extracted DNA) detected in BP and BP free (a) hulled seed tissue, and (b) husk tissue.

Table 6.4 ANOVA comparing level of *B. sorokiniana* detected in BP and BP free hulled seed and husk tissue (sprayed plots only).

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Seed Bipolaris DNA (ng/ng)	.003[a]	1	.003	4.653	.032
	Husk Bipolaris DNA (ng/ng)	.468[b]	1	.468	41.750	.000
Intercept	Seed Bipolaris DNA (ng/ng)	.006	1	.006	8.411	.004
	Husk Bipolaris DNA (ng/ng)	1.805	1	1.805	160.933	.000
Disease State	Seed Bipolaris DNA (ng/ng)	.003	1	.003	4.653	.032
	Husk Bipolaris DNA (ng/ng)	.468	1	.468	41.750	.000
Error	Seed Bipolaris DNA (ng/ng)	.137	204	.001		
	Husk Bipolaris DNA (ng/ng)	2.288	204	.011		
Total	Seed Bipolaris DNA (ng/ng)	.146	206			
	Husk Bipolaris DNA (ng/ng)	4.677	206			
Corrected Total	Seed Bipolaris DNA (ng/ng)	.140	205			
	Husk Bipolaris DNA (ng/ng)	2.756	205			

a. R Squared = .022 (Adjusted R Squared = .018)
b. R Squared = .170 (Adjusted R Squared = .166)

The bar chart comparing the level of *B. sorokiniana* DNA detected in seed tissue for each hulled seed discolouration category (Figure 6.7a) showed a higher level of fungal DNA for the seeds with the highest levels of discolouration (>25 %). The bar chart comparing the level of fungal DNA in the husk tissues for each discolouration category (Figure 6.7b) showed a significantly lower level of *B. sorokiniana* DNA in the husks with no discolouration. The ANOVAs comparing these groups (Tables 6.5 and 6.6) showed a significant difference between two or more of the discolouration groups for both seed and husk tissues. The non-parametric independent samples Kruskall-Wallis test also showed significant differences in the level of *B. sorokiniana* DNA for both seed and husk tissues (at the 0.05 significance level). Pairwise comparisons with t-tests show that only the >25 % discolouration category differed significantly in its level of *B. sorokiniana* DNA, and for the husks only, the 'no discolouration' category differed significantly in its level of *B. sorokiniana* DNA.

The level of fungal DNA present in hulled seed and husk tissue was compared for the dry weight categories (Figure 6.8). The lowest seed dry weight category (1 to 10 mg) had a significantly higher level of *B. sorokiniana* DNA relative to total DNA than the seeds weighing more than 10 mg. On the other hand, no significant differences were observed in the level of fungal DNA present for the four dry weight categories of husk tissue.

The average grain weight was compared for each BP rating category (Figure 6.9), which displayed a lower grain weight for the highest BP discolouration category of >25 %. An ANOVA (Table 6.7) showed significant differences at the 0.005 level among two or more groups, a finding supported by the non-parametric Kruskall-Wallis test. Pairwise comparisons with Mann-Whitney U tests showed only the >25 % discolouration category was significantly different.

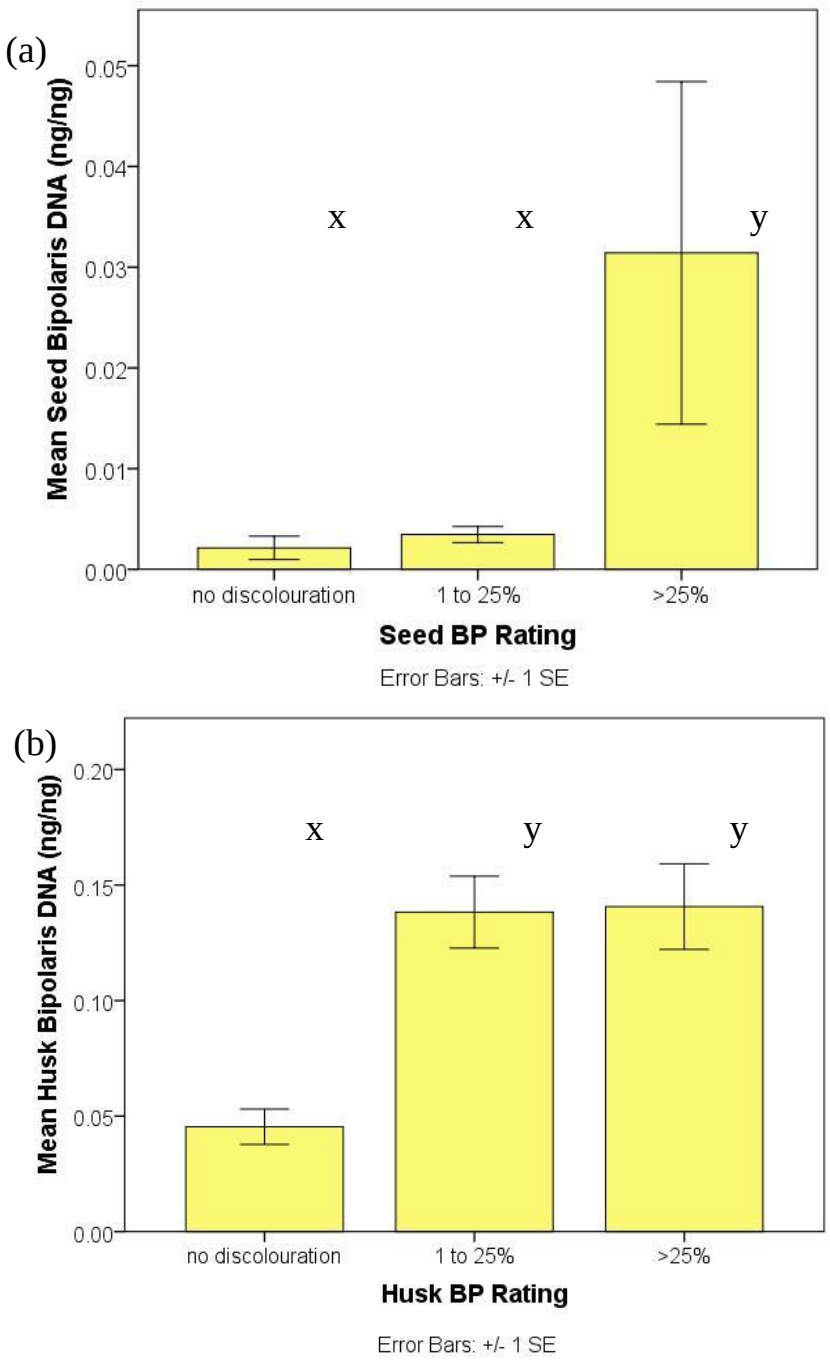


Figure 6.7 Level of *B. sorokiniana* DNA (ng fungal DNA per ng total DNA extracted) in each (a) seed and (b) husk BP discolouration category. Groups with the same letter above the bar are not significantly different at the 0.05 level.

Table 6.5 ANOVA comparing the level of *B. sorokiniana* DNA present in seed tissue among the three seed discolouration categories (0%, 1-25%, >25%).

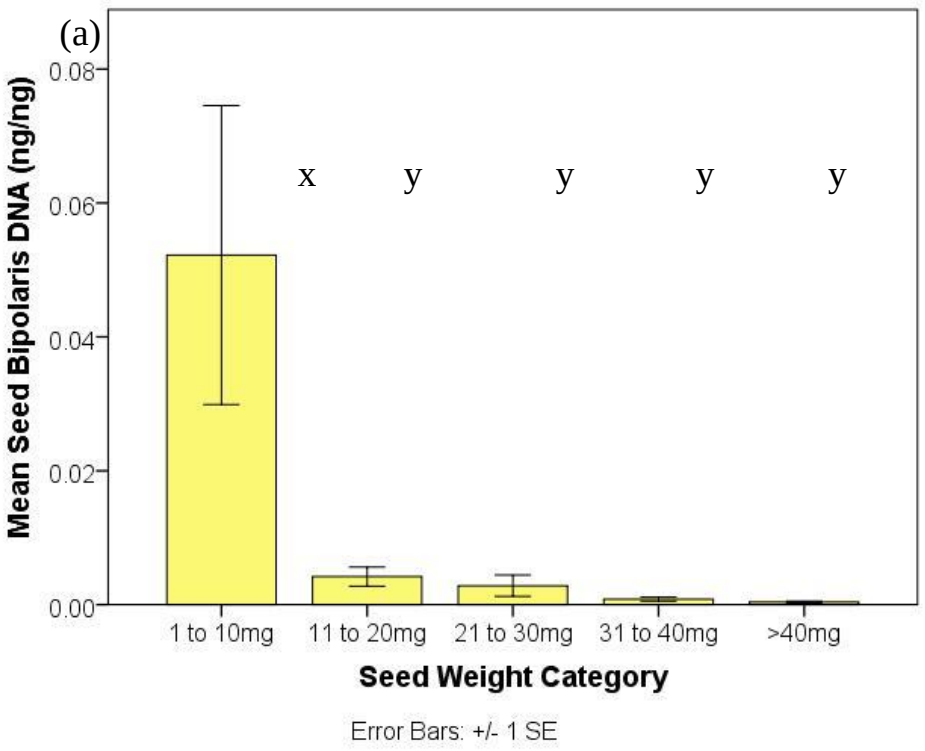
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.015[a]	2	.007	12.855	.000
Intercept	.019	1	.019	32.885	.000
Seed Rating	.015	2	.007	12.855	.000
Error	.126	223	.001		
Total	.146	226			
Corrected Total	.141	225			

a. R Squared = .103 (Adjusted R Squared = .095)

Table 6.6 ANOVA comparing the level of *B. sorokiniana* DNA present in husk tissue among the three husk discolouration categories (0%, 1-25%, >25%).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.470[a]	2	.235	21.258	.000
Intercept	2.221	1	2.221	200.684	.000
Husk Rating	.470	2	.235	21.258	.000
Error	2.335	211	.011		
Total	4.699	214			
Corrected Total	2.806	213			

a. R Squared = .168 (Adjusted R Squared = .160)



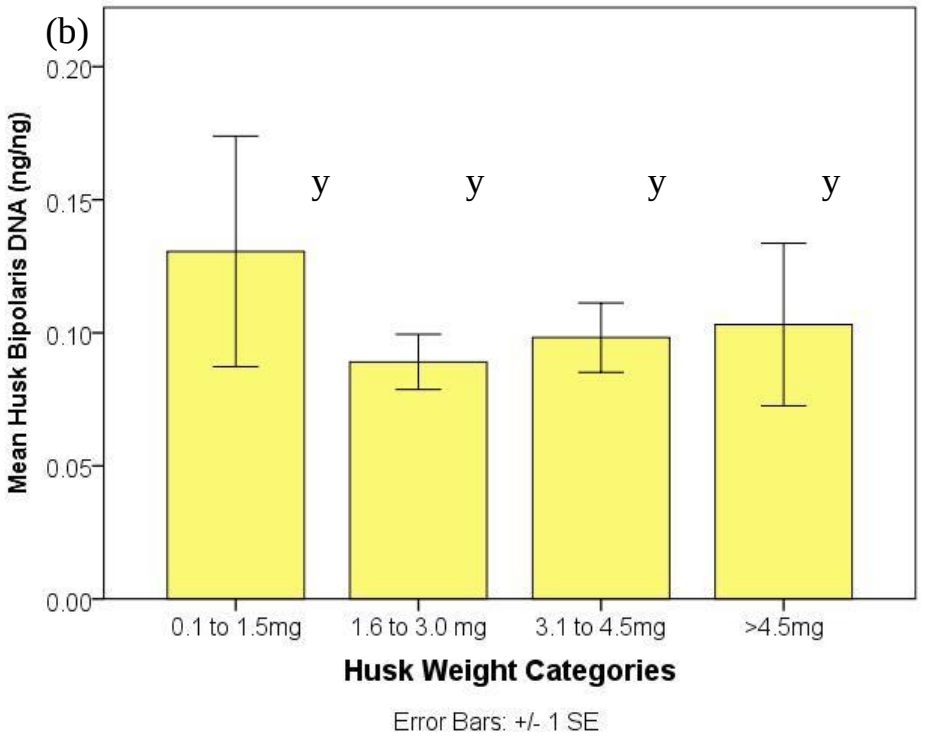


Figure 6.8 Level of *B. sorokiniana* DNA (ng *B. sorokiniana* DNA per ng total DNA extracted) in each (a) seed and (b) husk dry weight category. Groups with the same letter above the bar are not significantly different at the 0.05 level.

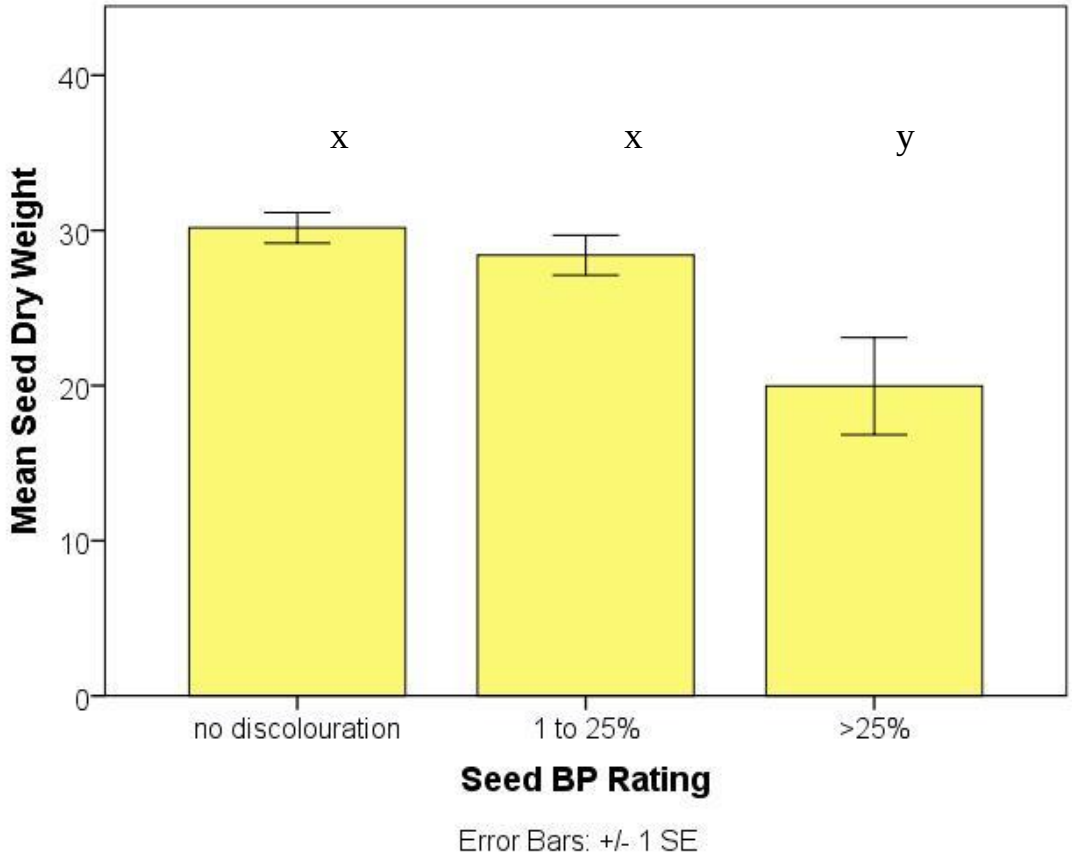


Figure 6.9. Comparison of the average seed weights for each seed BP rating category (sprayed plots only). Groups with the same letter above the bar are not significantly different at the 0.05 level.

Table 6.7 ANOVA comparing the average seed dry weights among the seed discolouration categories.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1836.527[a]	2	918.264	6.637	.002
Intercept	87646.648	1	87646.648	633.450	.000
Seed BP Rating	1836.527	2	918.264	6.637	.002
Error	32377.165	234	138.364		
Total	230083.130	237			
Corrected Total	34213.692	236			

6.4 Discussion

This study utilised the method of quantitative PCR in order to estimate and compare levels of *B. sorokiniana* present in seeds with and without BP. This method has been used in other studies of pathogen-disease relationships (Knight et al., 2012; Moya-Elizondo et al., 2011a; Winton et al., 2002; Xu et al., 2008), but has not been previously used to investigate the relationship between BP and *B. sorokiniana*.

The cause of BP in wheat and barley is a much debated topic, and currently no single condition has been proven as the cause of the defect. Environmental factors such as humidity and extreme temperatures during grain fill were found to be major factors contributing to BP development. Although various saprophytic fungi have been isolated in numerous studies from BP seeds of wheat (Conner and Davidson, 1988; Cromey and Mulholland, 1988; Ellis et al., 1996; Huguelet and Kiesling, 1973; Lorenz, 1986; Malaker and Mian, 2010; Moschini et al., 2006; Rees et al., 1984; Sisterna and Sarandon, 2005; Toklu et al., 2008) and barley (Hudec, 2007; Zare, 2013), no single species was consistently isolated. Nevertheless, several researchers attribute the defect to specific fungal species, particularly *A. alternata* and *B. sorokiniana*. A number of these researchers refer to the review by Kumar *et al.* (2010) to support the hypothesis that *B. sorokiniana* is the causal agent of BP, however the authors of that review provide no evidence for their claims. Research in this area has proven difficult due to BP developing in conditions also conducive to fungal growth. *B. sorokiniana* appeared most frequently in the barley seeds in the current study; however it appeared equally in all seeds, not only in those displaying BP. Results from studies in wheat have also been in agreement with the current study, observing fungal colonisation in seeds displaying BP, and also symptomless grains (Conner and Davidson, 1988; King et al., 1981; Rees et al., 1984).

In the preliminary AFLP study of the small collection of isolates, no apparent genetic differentiation was observed between isolates from BP and BP free seeds. The lack of differences among these two groups of isolates is likely due to the low number analysed. A larger collection could be analysed in future to investigate whether any real genetic differences exist among isolates from seeds with and without BP symptoms. Not surprisingly, the level of genetic diversity in the isolates examined was relatively low with all but one isolate being >92 % similar, as compared with the population study in Chapter 2 (Figure 2.2, >72 %). This would be expected since the population study examined fungal isolates from across major regions of the Australian cereal belt and sampled from a range of host tissues and genotypes. The isolates in the current study were collected from a plot naturally infested with *B. sorokiniana*, therefore the isolates could be expected to have greater genetic differences than isolates from plots inoculated with a single spore isolate. However, since field inoculations with *B. sorokiniana* are completed regularly with a single source isolate (SB61) at Hermitage Research Station where this particular field trial took place, several of these isolates might be expected to be related to this source.

Among the 120 seeds (from the sprayed plots) with BP free husks, 21 displayed a low level (< 25 %) of discolouration on the covered tissues of the seed testa. Among the 114 seed with husks displaying BP symptoms, 37 showed no visible discolouration of the seed coat tissues inside the husk. The scatter plot comparing the

levels of *B. sorokiniana* in seed tissue against the level of *B. sorokiniana* detected in husk tissue showed an insignificant correlation. These results indicate that the discolouration level is not always the same for the husk and the internal seed surface, nor is the level of fungal colonisation in the husk related to the fungal level on the internal seed surface. Although the internal seed surface is protected to an extent from fungal colonisation by the husk, higher levels of discolouration can still occur internally compared to externally in some cases. This is an additional indication that fungal colonisation is not the root cause of the defect.

In the current study when comparing the level of *B. sorokiniana* DNA measured by qPCR in seed with BP compared with BP free seeds, the BP seeds did contain on average, a significantly higher level of fungal DNA. Additionally, the level of *B. sorokiniana* in seed tissue was significantly higher in the >25 % discolouration category compared with seeds displaying lower levels of discolouration, and in husk tissue the 'no discolouration' category had significantly lower levels of *B. sorokiniana*. However these are not normally distributed populations and it is apparent that a small number of outliers within the sampled population have significantly higher fungal levels, and most individuals in the BP and BP free categories and each discolouration category (0 %, 1-25 % and >25 %) contained a range of values which were similar among all groups. The few outliers with higher levels of seed discolouration that show significantly higher fungal DNA levels suggests that the highest BP levels are associated with higher fungal loads. It is possible that BP seeds expressing higher levels of discolouration may have a higher level of susceptibility to *B. sorokiniana* infection. Considering other fungal species were also isolated, but at lower frequencies from seeds across the treatments, a similar q-PCR study in future, including investigation of the levels of multiple fungal species, would help to clarify whether general rather than specific fungal colonisation is enhanced by the development of more extreme BP symptoms.
Alternative factors studied in association with BP levels in grains include grain size, nitrogen application (Clarke et al., 2004; Ellis et al., 1996), fungicide application (Dimmock and Gooding, 2002; Ellis et al., 1996) and water availability (Cromey and Mulholland, 1988; Fernandez et al., 2000; Maloy and Specht, 1988). A number of studies have found that larger grains are more susceptible to BP than smaller grains (Cromey and Mulholland, 1988; Dimmock and Gooding, 2002; Lorenz, 1986; Rees et al., 1984) and some of these studies have also found that location of the grains on the head and within spikelets is significant. Basal and apical spikelets and grains located in the middle of the spikelet, which are generally smaller in size, are least affected by BP (Dimmock and Gooding, 2002; Ellis et al., 1996; Hoyle, 2000). Nitrogen fertilizer has been found in some studies to decrease occurrence of BP, which is thought to be due to nitrogen increasing grain numbers and thus decreasing individual grain size (Clarke et al., 2004). Some studies on the other hand, have found a greater prevalence of BP in smaller grains (Huguelet and Kiesling, 1973; Malaker and Mian, 2010), and likewise in this study there was a negative relationship between BP rating and average seed weight (Figure 6.8a). Due to conflicting results of these independent studies, the case for grain size playing a role in the level of BP observed in grain is weak.

In conclusion, the results from the current experiment suggests there is no compelling evidence that colonisation of seeds by *B. sorokiniana* is a sufficient condition for production of BP symptoms under favourable environmental

conditions. This is particularly because of the ubiquitous presence of *B. sorokiniana* in clean seed and the lack of consistent differences in fungal load between BP and BP free seed across the majority of categories of infection intensity (Figures 6.6 and 6.7). Furthermore the ability of internal tissues to develop symptoms while the husk remains clean supports this contention. Studies by other groups which indicate that the position of spikes on the head influences the likelihood of BP development suggest that we should consider a physiological cause for this condition. There is clearly a need for further study of BP aetiology specifically in barley, since the structures of wheat and barley seeds are dissimilar. This would include investigation of positional effects within the head on BP development, as well as *in vitro* induction of BP in barley grains, and potentially whether systemic colonisation of the main stem, rachis, kernel and husk tissue.

Chapter 7: General Discussion

The purpose of the current research project was primarily to investigate population diversity among isolates of *Bipolaris sorokiniana*, and to determine whether host or tissue specificity exists among isolates, to enable more adequate targeted control of this damaging pathogen. Additionally, the known SB QTL on chromosome 3H was to be investigated in order to produce a denser map, with the eventual aim of future identification of candidate genes. Finally, we aimed to investigate whether a relationship exists between *B. sorokiniana* and the barley grain defect black point. This defect leads to downgrading of barley which would be otherwise used in the malting industry.

The purpose of this final chapter is to outline the main findings of each study, addressing each of the proposed hypotheses in Chapter 1. Results are compared to previous studies investigating population diversity and virulence of *B. sorokiniana*, and additionally the studies examining spot blotch adult resistance QTL on chromosome 3H and association of *B. sorokiniana*, if any, with the grain defect black point. The potential contributions to the more efficient and adequate control of the pathogen and possible effect on strategies for breeding programmes are identified. Finally, future directions for the research will be discussed.

7.1 Genetic Diversity

AFLP genotyping was completed on 126 *B. sorokiniana* isolates originating from various tissues primarily of the two hosts, wheat and barley, and various locations in Australia, in order to address the following hypothesis:

Hypothesis 1: There is a significant difference between the genetic fingerprints of *B. sorokiniana* isolates from different hosts and tissues.

Significant differences in the genetic fingerprints are evident in the current study most prominently between the populations of SB CRR isolates. Significant differences are evident among CRR and SB isolates from barley, and CRR and SB isolates from wheat. Additionally, significant differences were observed among the barley and wheat SB isolates, and among the barley and wheat CRR isolates. Therefore Hypothesis 1 has been accepted. This study attempted to address the shortcomings of the previous preliminary study by Knight *et al.* (2010) by including more isolates from barley roots and wheat leaves.

Previous investigations of the genetic diversity of *B. sorokiniana* isolates have primarily aimed to determine the diversity among SB isolates from a single host, either wheat or barley (Aggarwal et al., 2010). A genetic diversity study by Ghazvini and Tekauz (2012) examining AFLP patterns of *B. sorokiniana* isolates from various geographic locations investigated primarily the relationship between genotype and pathotype. The study included a majority of isolates from SB infections of barley hosts, but also included isolates collected from leaves of wheat, rye and oat. However these authors did not make comparisons of the genotypic fingerprints of isolates from different hosts. A limited number of studies have examined isolates from different tissues, however two of these have included mostly isolates from leaves, and a minimal number of isolates from other tissues, and have not made

comparisons of the genetic diversity among isolates from the different tissues (Arabi and Jawhar, 2007b; Zhong and Steffenson, 2001b). A study investigating genetic diversity of isolates from barley grains, stem bases and roots (Baturo, 2005) compared the RAPD fingerprints among isolates, but did not find significant differences among tissues of origin. The only analysis performed in this study was a distance-based cluster analysis (dendrogram) based on the results of only five RAPD primer pairs. No other statistical analyses were performed to compare the isolates collected from the three different tissues. The lack of statistical tests and low level of genotypic data are likely explanations for the lack of differences observed among the isolates from different tissues. If more RAPD markers had been tested, the model-based cluster analysis and AMOVA could have been used to detect more subtle differences between groups than visual observation of the distance-based cluster analysis or dendrogram.

Two separate studies by Knight *et al.* (2010) and Gyawali *et al.* (2012), were the most comprehensive (prior to the current study) to genetically compare isolates from different hosts and tissues. The first study (Knight et al. 2010) identified clear differentiation between isolates from SB and CRR infections, however included isolates from SB infections which were sourced from barley alone, and not wheat, and most (22) of the CRR isolates were sourced from wheat plants with only four coming from barley. The second study (Gyawali *et al.* 2012) was similar to the first but on a larger scale, and examined a total of 208 isolates from North Dakota, of which 76 were from barley leaves, 97 were from barley roots and 35 were from wheat roots. An AFLP analysis was performed with 127 loci scored, and differentiation was observed among both hosts and tissue types. The current study attempted to address the shortcomings of these previous studies by including more CRR isolates from barley and SB isolates from wheat, however there were still far fewer isolates in the collection from these sources, reflecting the relative occurrence and research attention given to these forms of the two diseases in Australia.

The species-specific primers used in the current study for identification of isolates in the Australian collection of 126 *B. sorokiniana* individuals included a previous primer pair designed by Matusinsky et al. (2010), and an additional four primers (two forward and two reverse) designed by our group based on the Brn1 locus. All six pairings of primers were found to be unsuitable for the purpose of species identification, as the rate of isolates negative for the markers was high for all pairs. Therefore it was necessary to screen alternative loci for the design of species specific markers for use in the qPCR analysis of Chapter 6.

Two additional hypotheses were addressed in this diversity study, both considering different aspects of the source of genetic diversity among *B. sorokiniana* isolates. These hypotheses were initially proposed by two international research groups, in Czech Republic and Brazil, respectively:

Hypothesis 2: There is a higher level of genetic variation among isolates from the same lesion from plants in the field than is expected in an asexual organism.

Hypothesis 3: Genetic variation among clonally propagated isolates can arise according to the genetic profile of the host plant.

Both hypotheses were tested to investigate whether these observations could be made among Australian isolates. Hypothesis 2 was proposed by Leisova-Svobodova *et al.* (2011) in their study of isolates collected from naturally infested barley fields in the Czech Republic. This group found in an analysis of molecular variance (AMOVA) that differences among isolates from within a single lesion on a barley leaf were 49.5% of the total variance, at a significance level of <0.001, which was considered to be too high to be explained by multiple infection sites merging to form a single visible lesion. In the current study, this hypothesis was tested in the Australian environment by collecting isolates from barley in a field inoculated with a single isolate, in an attempt to reduce the level of variation observed among isolates. It was found that genetic variation did exist among isolates from within a single lesion, but at a level of no more than 1%, much lower than that observed in the Czech study. These results suggest that there could be a low level of genetic variation of single spore isolates during infection in the field, however the occurrence of multiple infection points from environmental inoculum sources could still offer an explanation. Therefore Hypothesis 2 is not accepted.

A second study conducted in Brazil (de Oliveira et al., 2002) eliminated possible external sources of inoculum by conducting experiments in the glasshouse and inoculating leaves of three varieties of wheat with ten single spore isolates and comparing RAPD fingerprints of reisolated single-spore isolates. This group proposed Hypothesis 3 and found variation among isolates of the same original genetic source when reisolated from the different varieties. This hypothesis was tested in the current study in a similar manner, by the genetic assessment of *B. sorokiniana* reisolated from leaves of wheat and barley differentials after assessment of infection response in the glasshouse. In the current study, isolates were compared genetically using AFLP profiles, which is a more reliable method for genetic fingerprinting than the RAPD profiling used in the study by de Oliveira *et al.* (2002). Genetic variation was found to be present among Australian isolates, when genetically uniform sources were inoculated onto different wheat and barley genotypes. Differences among isolates from the same original source inoculated onto different genotypes were up to 2%, and there were differences of up to 1% evident among isolates from the same lesion. This suggests that isolates may undergo genetic changes according to the host of origin. That is, the genotype of the host may have an influence on the genetic re-arrangements of the pathogen during asexual reproduction. More testing of Hypothesis 3 using alternative genotyping methods is necessary before it can be accepted, and as yet has yet to be conclusively proven.

Suggested methods by which genetic variation could occur in the pathogen include parasexual recombination (Burdon and Silk, 1997; Poloni et al., 2009; Tinline, 1962), heterokaryon formation (Clutterbuck, 1995; McGuire et al., 2005), repeat-induced point mutation (Crouch et al., 2008; Galagan and Selker, 2004) and chromosomal rearrangements (Leisova-Svobodova et al., 2011; Zhong and Steffenson, 2007). Parasexual recombination occurs when two genetically diverse isolates make physical contact and recombination occurs outside of the sexual cycle. However, this process is believed to be rare due to the actions of the vegetative incompatibility (*vic*) genes, which prevent heterokaryon formation between individuals that differ at one or more *vic* loci (Clutterbuck, 1995). Although the process of parasexual recombination may be rare, a study by Chand *et al.* (2003) revealed that it is common for individual hyphal cells of *B. sorokiniana* to contain up

to six nuclei. Presence of multiple nuclei in a single cell may have an effect on the gene expression of these cells. Repeat-induced point mutation (RIPping) is a process which occurs when transposable elements are inserted into the fungal genome. Transposable elements can move around the host genome, and RIPping is believed to be a defence mechanism, whereby the transposon regions and the regions surrounding them in the genome are mutated severely over several generations to stop their destructive activity. This process has been investigated thoroughly only in the bread mould *Neurospora crassa*, in which it was found to occur during the sexual cycle (Galagan and Selker, 2004).

7.2 Ability of *B. sorokiniana* isolates to cause Spot blotch on Wheat and Barley

Chapter 3 investigated the following hypotheses:

Hypothesis 4: Host specificity does exist for isolates originating from both CRR and SB infections when inoculated onto wheat and barley leaf tissue.

Hypothesis 5: Tissue specificity is present in isolates sampled from SB and CRR infections of both hosts when inoculated onto leaves.

These hypotheses were tested by determining the virulence of 25 isolates from the population genetics study, following inoculation onto the leaves of 18 differential genotypes of wheat and barley. Host specificity was found to be present on both the wheat and barley differentials, with the isolates sampled from barley SB isolates causing the highest level of infection in the barley differentials, and the isolates sampled from wheat CRR isolates causing significantly higher levels of infection in the wheat differentials than the other three isolate groups, therefore Hypothesis 4 is accepted. The previous preliminary study by Knight *et al.* (2010) did not detect differences in the infection responses of isolates from wheat and barley. This may have been due to the fact that few wheat varieties were included in the differential set, and all wheat isolates were from CRR infections. Ghazvini and Tekauz (2007) found a certain level of host specificity in their study of isolates from leaves of wheat, rye, oat and barley on a set of barley differentials. Most of the 16 isolates from the three alternative hosts (wheat, rye and oats) had low virulence on the differential set, whereas most of the isolates from barley leaves were highly virulent on at least two (out of 12) of the barley differentials. Although evidence of host specificity was observed, *B. sorokiniana* can still cause low level infection in the alternate host, potentially increasing propagule load in the environment. This means that rotations between wheat and barley alone would not be a sufficient strategy to keep crop spot blotch levels down, and other crop species should be used in rotations.

Tissue specificity was found to be present in the current experiments, only when examining infection responses induced on the barley differentials by the isolates sampled from barley. The isolates originating from barley SB infections induced significantly higher infection responses than the isolates from barley CRR infections. Differences between barley root and leaf isolates were also observed in the genetic population study in Chapter 1, with isolates from each tissue type clustering separately in the dendrogram, and also differing significantly from each other in the Fst pairwise analysis (P=0.00000). In contrast when examining the infection responses induced on leaf tissue of the wheat differentials, the isolates sampled from wheat CRR infections induced a significantly higher level of infection than the wheat SB isolates. Again, significant differences were observed in the genetic study of Chapter 1, with significant differences observed among wheat aerial and basal isolates in the pairwise Fst analysis (P = 0.035). This is most likely due to SB of wheat being of no significance in the Australian environment as a result of lower humidity and temperature levels than are required for the pathogen to infect wheat leaves, whereas CRR of wheat is a significant disease constraint. Investigation of

tissue specificity of *B. sorokiniana* on wheat leaf tissue has not previously been investigated. From these results, Hypothesis 5 is accepted for barley only, but has not been proven in wheat.

7.3 Ability of *B. sorokiniana* isolates to cause Common Root Rot on wheat and barley

Two hypotheses were tested in this chapter examining virulence of *B. sorokiniana* isolates on root tissue:

Hypothesis 6: Host specificity exists for isolates sampled from CRR and SB infections of both wheat and barley when inoculated onto roots.

Hypothesis 7: Tissue specificity exists for isolates sampled from CRR and SB infections of wheat and barley when inoculated onto roots.

The primary aim of the common root rot pathogenicity tests was first and foremost to determine whether the assay tested by Sheedy and Reen (2010) could be used to assess virulence of *B. sorokiniana* isolates on root tissue of wheat and barley differentials. The assay was inadequate for this purpose, with a high number of escapes, and a high level of variability in the infection level within the plant genotype x isolate groups. In the limited time allowed at the end of the PhD for this experiment, it was not possible to further develop the method to produce more consistent results. A limited number of isolates (eight) from different host and tissue sources were tested on only eight differentials (four wheat and four barley genotypes). Some differences were observed among the disease reactions of the wheat differentials to the isolates with Timgalen showing significantly higher disease reactions than the other differentials, but the barley differentials had very low reactions to all of the isolates, making them less useful in the process of phenotyping isolates. Therefore all statistical analyses comparing the level of disease among isolate sources and differential species were performed with caution, and the hypotheses will need to be tested further using a more discriminating assay method before they can be accepted.

Despite the levels of infection in the current experiments being lower than those observed in previous preliminary work (Sheedy and Reen, 2010), limited evidence was found to support both hypotheses. A level of host specificity (Hypothesis 6) was observed when examining disease levels induced on the barley differentials only. The isolates sampled from barley SB infections induced significantly higher levels of infection on the barley differentials than all other isolate groups, and the isolates sampled from wheat SB isolates induced significantly lower levels of infection on SCI tissue of the barley differentials. Evidence for tissue specificity (Hypothesis 7) was also observed in the current experiments when examining the infection responses induced on the wheat differentials only, with the CRR isolates of both hosts causing significantly higher levels of infection than the SB isolates on the SCI tissue. Tissue and host specificity of *B. sorokiniana* has not previously been tested on root tissue of wheat or barley. If tissue specificity of isolates is found to occur in root tissue, as was observed in leaf tissue, this will mean that common root rot and

spot blotch control strategies will need to be assessed separately. However, as isolates originating from the two disease states are capable of causing low level infections in alternate tissues, the control strategies for each disease should be used together, to reduce the environmental propagule load. For example, crop species which are not susceptible to either disease state should be used in rotations, and in future we should aim to incorporate genetic resistance to both disease states into commercial varieties. This remains challenging particularly since resistance loci for each of these traits appear to be independent of those for the other trait (Bovill et al., 2010; Lehmensiek et al., 2010).

Previously, very limited work has been conducted examining virulence of *B. sorokiniana* on root tissue, with much of the experimentation being conducted in the field, or using soil from the field naturally infested with *B. sorokiniana* propagules (Burgess and Griffin, 1968; Conner and Atkinson, 1989; Duczek, 1989; Grey and Mathre, 1984; Kokko et al., 1993; Piening, 1973; Tinline et al., 1988; Wildermuth et al., 1992). Comparisons of isolate virulence in controlled glasshouse and laboratory conditions have been conducted previously, however two of these examined the level of disease induced by a mixture of isolates, and did not make comparisons among individual isolates (Arabi and Jawhar, 1999; Arabi and Jawhar, 2001). A study conducted by Duczek (1984) examined degree of SCI infection induced by individual isolates on a single barley cultivar, however only made comparisons among geographical locations.

The current study is preliminary in nature, and further work needs to be completed in identifying appropriate barley differentials. A faster method for the production of bulk inoculum is also desirable.

7.4 Spot blotch resistance QTL on barley chromosome 3H

Spot blotch can be controlled to an extent using methods such as crop rotation, and use of foliar fungicides (Acharya et al., 2011; Couture and Sutton, 1978) however the most efficient means of control is the use of adapted, genetically resistant cultivars. Two major resistance genes have been previously identified on chromosomes 7H and 3H consistently in various populations by a number of researchers (Bilgic et al., 2005; Bilgic et al., 2006; Bovill et al., 2010; Roy et al., 2010; Steffenson, 1996).

The purpose of the current study was to identify markers more closely linked to the SB adult resistance QTL identified on barley chromosome 3H:

Hypothesis 8: Fine mapping of the 3H barley region associated with spot blotch resistance can be completed using markers from other mapping studies.

This was to be completed by firstly mapping the markers associated with SB resistance in previous work (Bovill et al., 2010) in a large doubled haploid mapping population, followed by mapping markers (based on SNP and EST sequences) from other barley mapping populations in the same region. None of the new markers screened in this study were found to be polymorphic, however there are several options for future directions, which are discussed further in Section 7.6.

In previous work, fine mapping of the SB adult resistance QTL has proven difficult, due to a distinct lack of polymorphism in the region (Bovill et al., 2010). A number of recent studies have conducted mapping of the whole barley genome using various sequencing approaches (Sato et al., 2009; Szucs et al., 2009), which could be useful in providing information about polymorphisms in the 3H SB resistance region in the future.

7.5 Black Point of Barley

This study aimed to investigate evidence for a relationship between *B. sorokiniana* and the grain defect black point by use of quantitative PCR:

Hypothesis 9: Evidence does exist for a relationship between *B. sorokiniana* and the grain defect black point.

This was tested by examining *B. sorokiniana* levels (estimated by use of qPCR) in barley grains displaying black point symptoms, compared with grain free of black point symptoms. Species-specific primers were designed to amplify in *B. sorokiniana* DNA only, and a fluorescent dye was used to detect the quantity of DNA in each PCR cycle, which was used to calculate the original concentration of DNA in the plant tissue. A linear association between *B. sorokiniana* DNA content and symptom levels was limited to the range 1.12×10^{-6} to 169.1 ng of DNA per mg plant tissue for seed, and range 1.19×10^{-3} to 202.23 ng of DNA per mg tissue for husks. It was found that in the highest discolouration category, a small number of seeds and lemmas showed significantly higher levels of *B. sorokiniana*. Once these few outliers were removed, the seed and husk tissue, whether displaying minimal discolouration or a high level of discolouration, did not differ significantly in the average level of *B. sorokiniana* colonisation. This suggests one of two possibilities: either higher levels of the fungus may help to induce harsher black point symptoms, or greater levels of black point may encourage fungal colonisation and growth. Since only the levels of *B. sorokiniana* were investigated in this analysis, and other fungal pathogens were also observed in the seeds, future investigation of the levels of these other pathogens would be useful in determining whether generally high levels of fungal colonisation are associated with high levels of black point discolouration.

In a study of the defect in wheat, Williamson (1997) could not induce black point symptoms by inoculation with *Alternaria alternata*, and microscopic observation of infected grains found no association between hyphal density and the level of black point. Additionally, the symptoms could be induced *in vitro* in the absence of any pathogen, suggesting that fungal pathogens are not the cause of the defect. These tests should also be performed in barley grains to determine whether colonisation occurs more densely at the discoloured end of the grain, and whether the defect can be induced *in vitro*. In the current experiments, no compelling evidence was found to indicate that colonisation of barley grains is a sufficient condition to cause BP in suitable environmental conditions, therefore Hypothesis 8 remains unproven.

7.6 Future Directions

A number of future directions have been identified in the current study, which will further assist in developing a more effective control of the disease states of *B. sorokiniana*, and determining whether it plays a role in development of barley black point.

7.6.1 Genetic Diversity

The number of wheat SB and barley CRR isolates in the AFLP genotyping collection from this study needs to be increased, in order to determine more adequately whether genetic differences exist among both the host and tissue sources. In the current experiments, only five wheat SB and eight barley CRR isolates were included out of 126 isolates. As a starting point, some next-generation sequencing of isolates from each host and tissue source could be completed to identify whether there are genomic regions specific to the host or tissue. Since AFLP analysis only provides information about fragment lengths, it is useful for obtaining general data about isolate similarity in a population, but is limited. Sequencing of isolates originating from different host and tissue sources will provide information for the more efficient control of the two disease states using genetic resistance.

To determine the extent to which differentiation occurs among isolates from the same original source and inoculated onto different host genotypes, next-generation sequencing of the isolates shown to differ in this particular experiment could be completed to determine regions where these isolates differ. Additionally, isolates sampled from within the same lesion may also be sequenced to determine whether these do in fact differ from each other and to what extent. These investigations will provide information about the chromosome location and level of differentiation among isolates during the infection process.

From the sequence data obtained of the isolates, in future it should be determined whether Repeat-Induced Point mutation (RIPping) is occurring in individual isolates of *B. sorokiniana*, and whether this is a potential source of variation in the population. RIPping is a process which occurs in the fungal genome when transposable elements, which can move around the host genome, are introduced. The process modifies the region of the transposable element, and the region surrounding it by heavy mutation, reducing the deleterious effects of the element (Crouch et al., 2008). Duplicate sequences in the genome, such as transposable elements, are subjected to mutations where GCs are converted to ATs, and are susceptible to mutations for up to 6 generations (Crouch et al., 2008; Galagan and Selker, 2004). If this process is occurring in *B. sorokiniana*, it would introduce significant variation among individuals. Additionally, as previous research of the process in *Neurospora crassa* revealed that it only occurs during the sexual cycle, it would be essential to determine whether it occurs asexually in *B. sorokiniana*. If this high level of mutation is in fact occurring in the Australian population of *B. sorokiniana*, this will have implications on the control of the pathogen by means of genetic resistance as it indicates the pathogen may overcome major gene resistance more readily.

7.6.2 Ability of *B. sorokiniana* isolates to cause Spot blotch on Wheat and Barley

More CRR isolates should be tested from both wheat and barley on leaf tissue of a larger set of barley differentials to more adequately determine the ability of these to cause SB reactions. The differential set in the current experiments included ten barley differentials only. The effect of SB is known to be insignificant in wheat in Australia (Murray and Brennan, 2009) and was shown in the current experiments to be resistant to the disease. Therefore, expansion of the number of barley differentials in phenotypic assessment would enable the determination of susceptibility of the more popular barley varieties in Australia to isolates from a variety of host and tissue sources.

It should be determined whether seed-borne inoculum can cause SB and/or CRR in seedlings in order to identify whether this is a potential means of spreading the disease among tissues. It has been suggested that seed-borne inoculum can cause early root infection of plants (Al-Sadi and Deadman, 2010), which would also have implications on disease control. Since the leaf and seed isolates clustered together genetically in the cluster analysis in Chapter 2 (Figure 2.2), it is a possibility that seed-borne *B. sorokiniana* acts as a source of inoculum for root infection at germination.

7.6.3 Ability of *B. sorokiniana* isolates to cause Common Root Rot on Wheat and Barley

The common root rot assay tested in the current experiments was inadequate for the phenotypic assessment of *B. sorokiniana* isolates, due to a high level of escapes and a high degree of variation within differential x isolate combinations and within pots. Therefore the method must be further developed in several ways. An alternative method of inoculum production for CRR seedling trials should be determined. Ideally the method should take less time to produce the inoculum, and provide less opportunity for introduction of contaminants. A number of alternative methods have been identified from other root disease studies which could be trialled in future in the testing of *B. sorokiniana* on root tissue. For example, the use of soil as substrate for inoculum production (Duffy and Défago, 1997) or direct inoculation of seeds (Arabi and Jawhar, 1999; Arabi and Jawhar, 2001; Knudsen et al., 1995).

A wider set of barley lines with differential reactions to CRR at the seedling stage should additionally be identified for use in the seedling CRR screening. In the current preliminary study, four barley differentials were selected based on their reactions to common root rot at the adult stage. These differentials displayed much lower levels of infection than the wheat differentials (Figure 4.2), indicating that susceptibility to *B. sorokiniana* differs for plants between the adult and seedling stages.

Other approaches which may be useful in future investigations of isolate pathogenicity on root tissue could include microscopy and qPCR. As the SCI tissue is often used to determine the level of plant susceptibility to *B. sorokiniana* isolates, a microscopy study examining the level of fungal colonisation around the area of SCI discolouration may indicate whether the discolouration is strongly associated

with the growth of the pathogen or if there are also physiological causes. qPCR of SCI tissue could be used in conjunction with this to determine whether the level of discolouration is strongly associated with the amount of *B. sorokiniana* present in the tissue.

7.6.4 Spot blotch resistance QTL on barley chromosome 3H

Primers should be designed based on SNP marker data from the study by Szucs *et al.* (2009) which are suitable for using in High Resolution Melt analysis, in order to find markers more tightly linked to the 3H resistance QTL. This should be completed so that sequencing of the region may be executed and candidate genes identified. Sequences of the DArT markers used in the previous map of the ND24260 x Flagship population have recently become available (Hickey et al., 2011). These may be used to design primers for easier genotyping with these markers in future. The other large DH barley population that has recently become available (ND24160-I × Flagship) should also be tested for marker polymorphism and used in SB resistance fine mapping. Genotyping-by-sequencing should be utilised in the population used in the current study in order to identify a greater number of SNPs in the 3H region. Association mapping or nested association mapping could also be approaches used in the future to produce a higher density map.

7.6.5 Black Point of Barley

In future, experiments similar to those conducted by Williamson (1997) should be conducted with black pointed barley grains. Microscopy of BP and BP free seeds should be undertaken to determine whether fungal colonisation is denser at the discoloured end of the grain. Additionally it should be determined whether the defect can be induced *in vitro*, or whether inoculations of the head with *B. sorokiniana* can induce black point in barley. Other researchers have also found evidence of positional effects on the development of BP symptoms in wheat, with grains from the central floret showing less severe symptoms of BP than the outer florets, and grains from the top and bottom grains of the ear showing less severe BP symptoms than grains in the middle of the ear (Ellis et al., 1996; Huguelet and Kiesling, 1973). It would be beneficial to investigate whether this occurs in black point affected barley, as it could indicate a physiological defect, rather than a defect caused by fungal colonisation. An interesting study could be conducted more thoroughly examining genetic differences among isolates from BP and BP free seeds, to determine whether this affects the occurrence of symptoms in seed tissue.

7.7 Final Conclusions

Some significant goals were achieved in the course of these studies, with room for further investigation. Significant genetic differences among the SB and CRR isolates were observed. Observation of genetic differences among the same isolate when inoculated onto different host genotypes raises questions about mechanisms by which genetic differentiation occurs in this asexual species, and to what extent this occurs. Host and tissue specificity was observed when isolates from wheat and barley CRR and SB infections were tested on leaf tissue of wheat and barley varieties, and these findings will have implications on the disease management strategies such as crop rotations undertaken by winter cereal enterprises. The assay

used in testing pathogenicity of *B. sorokiniana* in seedlings will need further work before more detailed phenotypic analysis is carried out. Nevertheless some evidence of tissue specificity indicated. Testing of *B. sorokiniana* levels in BP and BP free seeds with qPCR provided no conclusive evidence for *B. sorokiniana* colonisation being a sufficient condition for the occurrence of BP.

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