Investigation of molecular and cellular aspects of cotton black root rot disease and the potential for biological control

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Abstract

The worldwide demand for high quality cotton has increased with time, and cotton production is now one of the most important rural industries in Australia. However, cotton growers face significant challenges in dealing with diseases that impact on crop yields. One of the most important diseases impacting cotton producers in Australia is black root rot disease, caused by the pathogenic fungi *Berkeleyomyces* spp. While a number of management practices have been suggested to help control this pathogen, there is not currently a complete solution to this problem. The main aims of this study were to gain a better understanding of the factors that affect disease severity, and explore the use of the bacterium *Azospirillum brasilense* as a biological control option for black root rot disease in cotton.

The distribution and abundance of *Berkeleyomyces* spp. in different soil samples from cotton farms (collected prior to planting) was investigated using quantitative polymerase chain reaction (qPCR). The incidence of the pathogen was higher in organically fertilised cotton fields compared to fields treated with mineral fertiliser. The relationship between crop rotation and pathogen abundance was also examined. Crop rotation with wheat and mung bean did not show any decrease in the soil load of *Berkeleyomyces* spp. However, soil properties, including iron and manganese levels, appeared to impact on the abundance of *Berkeleyomyces* spp. in soils.

The relationship between cotton cultivar, temperature and disease severity was also investigated, using *Berkeleyomyces rouxiae* BRIP40192 (a cotton isolate) and five cotton cultivars (Sicot 730, and the genetically modified cultivars Sicot 74BRF, Sicot 71BRF, Sicot 43BRF and Sicot 714B3F). Temperature had an influence on the proportion of root affected by black root rot lesions, and the water content of shoots,

supporting the findings of previous studies that indicate temperature is a significant factor impacting disease severity and, consequently, plant growth. At higher temperatures (25°C) soil collected from the rhizosphere for all genetic modified cultivars (Sicot 74BRF, Sicot 71BRF, Sicot 43BRF and Sicot 714B3F) showed a significant decrease in the levels of *B. rouxiae* BRIP40192 recovered, compared to recovery at lower temperature (20°C).

To better understand the processes involved in disease, *in vitro* pathogenicity testing was carried out to examine the ability of *B. rouxiae* BRIP40192 to perceive and respond to signalling molecules produced by the roots of cotton seedlings. The results demonstrated that cotton root exudates stimulate the directional growth of *B. rouxiae*, although the strength of this attraction varied between cotton cultivars. The inclusion of the bacterium *Azospirillum brasilense* in the test was able to prevent this directional growth and stop spore germination. This finding suggests that there may be a role for *A. brasilense* as a potential biological control agent for *B. rouxiae*. Interaction tests between *B. rouxiae* BRIP40192 and *A. brasilense* strains showed that that the two were not antagonistic to each other, although *A. brasilense* strains showed a high capability to interact with *B. rouxiae* BRIP40192 hyphae.

The interaction between *B. rouxiae* BRIP40192, *A. brasilense* and cotton was investigated, focusing on the role played by border cells of the cotton root. Cotton border cells of all cultivars tested failed to trap *B. rouxiae* BRIP40192, however, *A. brasilense* strains, proposed as a potential biocontrol organisms, showed a high level of interaction with border cells of the five cotton cultivars and with border cells from wheat (often used in crop rotation on cotton farms). The colonisation of border cells by *A. brasilense* may assist with the suppression of germination of *B. rouxiae* BRIP40192

spores and retard directional growth in the rhizosphere, thus protecting seedlings from infection.

The results of this study help improve understanding of factors, such as temperature, cotton cultivar and farm management practices, which influence the distribution and severity of black root rot disease in Australian cotton farms. The results of this study also suggest that *A. brasilense* could be considered as a potential biological control agent to block or suppress the growth of black root rot disease in cotton fields.

Certification of Dissertation

I certify that the ideas, experimental work, results, analyses, software 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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ENDORSEMENT

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Dedication

To

Prof. Lily Pereg memory

Hamid Abd Oun

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I wish to express my sincere thanks to Dr Mary McMillan since she was my co-supervisor and after she became my main supervisor when I lost my previous supervisor Prof. Lily Pereg who passed away during my study, for her outstanding continuous support, invaluable advice, her attempts to keep me on track and sane were second to none.

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Abbreviations

ANOVA	one-way analysis of variance
BRR	black root rot
CPS	capsular polysaccharide
DTPA	diethylenetriaminepenta acetic acid
EPS	arabinose sugar in exoploysacharide
IAA	indole acetic acid
ITS	internal transcribed spacer
LPS	lipopolysaccharides
MHC	moisture-holding capacity
NTC	no template control
OC%	soil organic compound
PAA	phenyl acetic acid
PCA	principal component analysis
PCR	polymerase chain reaction
PGPB	plant growth-promoting bacteria
qPCR	quantitative PCR
SAR	systemic acquired resistance

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Chapter 1: Literature review

1.1. Introduction

Cotton is an essential textile fibre crop grown in several countries globally. Australia is one of the top ten cotton producing countries worldwide (Most, 2017). Historically, during the 1800s, the cotton production in Australia was limited to a narrow geographical range, and increased during the 1900s. In Australian, cotton production now covers approximately 250,000 to 500,000 hectares annually. The peak of Australian cotton production had increased to 2,280,700 million bales in 2021 (225,950 cultivated ha), which makes it one of the major broad-acreage crops produced on the continent (Redfern and Twine, 2020). Cotton is grown in many rural and remote regions of New South Wales (65%) and Queensland (35%) due to the pattern of summer rainfall, type of soil and topography which make these regions suitable for cotton growing (Pyke and Finney, 2009). The fertilisation strategies used by Australian cotton growers include the use of nitrogen and phosphate-rich agrochemicals to boost cotton production (Maraseni *et al.*, 2010). One of the most significant challenges faced by Australian cotton producers are losses due to cotton pathogens (Pereg, 2013). Soil-borne pathogens are the most common cause of cotton seedling disease, resulting in yield losses as high as 705 bales/ha (Jhorar, 2004). There is therefore a pressing need to identify management options to reduce losses due to disease, allowing for low cost, environmentally-friendly and sustainable cotton production.

1.2. Black root rot disease in cotton

Black root rot (BRR) disease has a significant impact on the Australian cotton industry, causing massive yield losses from cotton fields (Kirkby *et al.*, 2013; Nehl *et al.*, 2004; Pereg, 2013). There are limited management options currently available for reducing the impact of black root rot disease (Pereg, 2013). Initially, BRR may be difficult to detect due to the first symptoms appearing below ground and being difficult to distinguish. Above ground symptoms include stunting or reduction in plant growth, delayed flowering or maturity, while below ground blackening of roots occurs and the number of lateral roots decreases (Allen, 2001). When above ground symptoms start to appear, plant survival and yield are already at risk (Bodah, 2017).

BRR is a seedling, soil-borne, filamentous fungal disease caused by the pathogens *Berkeleyomyces basicola* and *Berkeleyomyces rouxiae* (formerly both known as *Thielaviopsis basicola*) (Nel *et al.*, 2018). These pathogens have the ability to infect a wide range of plant species (Otani, 1962). The BRR pathogen was first reported in Australia in the 1930s in Queensland, as a sweet pea pathogen (Simmonds, 1966). Later, it was detected infecting other plants, including tobacco, bean and pine (Allen, 1990) and soybean (Mondal *et al.*, 2004). In cotton fields, the first detection was in 1989 in North-Western New South Wales (NSW) (Allen, 1990). In 2004, Nehl *et al.*, found that BRR pathogen (then identified as *T. basicola*) is widespread in cotton growing fields in NSW and Queensland, and should be considered as pandemic in Australia (Nehl *et al.*, 2004).

While BRR is widely distributed in Australia, there are limited options to control this pathogen. To date there have been no reports of specific cotton cultivars that are resistant to BRR disease. Wang and Davis (1997) tested twelve cotton cultivars and found that all of them were susceptible to BRR. Furthermore, the BRR pathogen is

difficult to eradicate from the soil, because of the persistent nature of the chlamydospore (Nehl *et al.*, 2004). There is therefore considerable interest in improving understanding of this pathogen and factors that influence disease severity, and in developing novel control measures to reduce the burden of disease.

1.3. The causative agent

The BRR pathogen was first described in the mid-1880s as *Torula basicola* (Berkeley and Broome, 1850). Since that time, the fungal pathogen has undergone complicated taxonomic augmentation, causing confusion in the literature (Nel *et al.*, 2019). Identification as *T. basicola* depended directly on the morphological characteristics of chlamydospores on the infected roots, and occasionally on the infected root symptoms (King and Presley, 1942).

The re-evaluation of the Ceratocystidaceae family of fungi resulted from LSU (nuclear ribosomal DNA large subunit), 60S (60S ribosomal protein RPL10) and MCM7 (mini-chromosome maintenance complex component 7) phylogenetic analysis by De Beer *et al.* (2014). Results supported the previous finding that *T. basicola* was related to the Ceratocystidaceae family, but had a separate genetic lineage, not related to *Thielvaiopsis* species. These results depended only on the analysis of a single isolate, and confirmation was required to make an acceptable taxonomic change (De Beer *et al.*, 2014; Nel *et al.*, 2018). Later work by Nel *et al.* (2018) used 41 isolates recently classified as *T. basicola* from 13 worldwide geographic regions. Based on phylogenetic analysis of six different gene region sequences, combined with fungal morphology, a new genus named *Berkeleyomyces* was introduced. *Berkeleyomyces* includes two highly similar sister species nominated as *B. basicola* and *B. rouxiae* (Nel *et al.*, 2018; Wingfield *et al.*, 2018). Much of the research carried out on BRR in Australia has taken

place prior to this reclassification into two sister species, and refers only to *T. basicola* as one strain. However, a recent study by Wilson *et al.,* (2021) carried out DNA sequencing of numerous BRR isolates from Australian cotton-growing regions, and concluded that all of these isolates were *B. rouxiae* and not *B. basicola*. It can be concluded that *B. rouxiae* is the most likely cause of BRR in cotton in Australia, although there is not yet enough research to rule out that *B. basicola* may also be present in the country.

Berkeleyomyces spp. are described as obligate hemibiotrophic plant pathogens (Hood and Shew, 1997; Mims *et al.*, 2000; Nel *et al.*, 2018). A hemibiotrophic fungal pathogen shares both biotrophic and necrotrophic phases in their life cycle (Gebrie, 2016). *Berkeleyomyces* spp. produce two types of asexual spores, as illustrated in Figure 1.1, during growth on solid media or in association with a host plant. The first type is a hyaline cylindrical liberated conidia (also known as phialospores or endoconidia) (Punja, 1993). The second type are larger, thick-walled, darker coloured spores called chlamydospores (also known as as macroconidia, arthoconidia or aleuriospore) (Alexopoulos *et al.*, 1996).

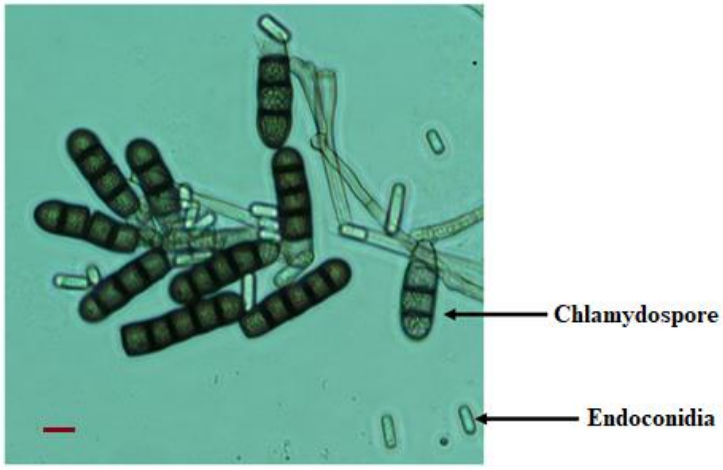

Figure 1.1: The two types of asexual spores produced by *Berkeleyomyces rouxiae* BRIP40192 when infecting cotton roots: endoconidia and chlamydospores (Image: this study). Bar= 10 µm

1.4. Berkeleyomyces spp. life cycle

1.4.1. Asexual life cycle

The *Berkeleyomyces* spp. asexual life cycle begins when suitable environmental conditions and availability of host root exudate occur (Coumans *et al.*, 2010; Coumans *et al.*, 2009). During the biotrophic stage, chlamydospores are induced to germinate in the rhizosphere (Hood and Shew, 1997; Pereg, 2013). Following germination a germ tube grows towards the root of a suitable host plant, and attaches to the root surface. The germ tube tip then starts to differentiate into an infection structure (an appressoria-like structure) (Baard, 1985; Hood and Shew, 1997; Mauk and Hine, 1988; Nan *et al.*, 1992), which has the ability to penetrate the plant root via a cylindrical infection peg (Mauk and Hine, 1988; Mims *et al.*, 2000; Punja *et al.*, 1992). More than one mode of root penetration has been recognised in *Berkeleyomyces* spp. The primary penetration site appears to be through host root hairs (Hood and Shew, 1997; Lindeman and Tousson, 1968), while penetration can also occur through wounds caused by other pathogens, through wounds caused by physical damage (Baard and Laubscher, 1985), via the epithelial cells, or through stoma (Pierre and Wilkinson, 1970). Direct colonisation has also described for some host plant species, when there is no clear appressorium-like structure formed (Christou, 1962; Wick and Moore, 1983). Following penetration, the fungi infects the host through a combination of intra- and intercellular colonisation (Punja *et al.*, 1992; Stover, 1950).

After a period of time, the necrotrophic phase begins. During this stage host cells are lysed to release nutrients, which are then utilised by the pathogen (Mims *et al.*, 2000; Pereg, 2013). A plenitude of chlamydospores is produced during necrotrophy, which are released into the soil, where they remain as an inoculum for the future infection of another host (Baard and Laubscher, 1985; Lindeman and Tousson, 1968;

Mosma and Struck, 2013; Pereg, 2013). The root's vascular tissue is usually not damaged during the infection (Walker *et al.*, 1998), so the plant host remains alive throughout (Hood and Shew, 1997; Mims *et al.*, 2000). This biotrophic and necrotrophic stages of the *Berkeleyomyces* spp. life cycle are illustrated in Figure 1.2.

Figure 1.2: The life cycle of the BRR causative agent, *Berkeleyomyces* spp., in cotton (Nehl, 2001)

1.4.2. Sexual life cycle

In 1876, *Thielvaiopsis basicola* was represented as the sexual form of the BRR pathogen (Nel *et al.*, 2018). However, there was little clarity concerning the sexual life cycle of this pathogen during that time. Attempts to initiate the sexual state of the pathogen using single spores were not successful (McCormick, 1925; Stover, 1950).

Further experiments were performed utilising multiple fungal spores and again no sexual state was observed (Johnson and Valleau, 1935). All the attempts to observe the expected sexual form of *B. basicola* and *B. rouxiae* led to the conclusion that *Berkeleyomyces* spp. reproduce only by asexual reproduction (Paulin-Mahady *et al.*, 2002).

More recently an ideal heterothallic mating system has been suggested, depending on mating genes located in two mating type loci observed in *Berkeleyomyces* spp. (Nel *et al.*, 2018). Nel *et al.* (2019) proposed a sexual reproduction cycle in *Berkeleyomyces* spp. that involves three main stages, however at this time there has not been further evidence collected to support this proposed mechanism.

1.5. Factors affecting *Berkeleyomyces* spp. incidence

The suppression or promotion of BRR disease, in soil infested with *Berkeleyomyces* spp., is affected by many abiotic and biotic factors such as temperature, soil moisture, pH, soil texture, levels of exchangeable ions, organic matter, soil microorganisms, plant species, and crop rotation. These factors influence radial growth, conidia production and germination of *Berkeleyomyces* spp. (Nehl *et al.*, 2004; Nel *et al.*, 2019; Pereg, 2013).

1.5.1. Abiotic environment

The severity of BRR disease of cotton is impacted greatly by soil temperature. The range of optimum growth temperature of *Berkeleyomyces* spp. in culture is between 20 and 30°C (Lucas, 1955). Soil temperatures below 24–26°C have been shown to

increase disease severity in a variety of hosts (Lloyd and Lockwood, 1963). However, survival rates of the fungus are greatest at temperatures between 10 and 18°C (Papavizas and Lewis, 1971). In general, cooler soil temperatures (below 26°C) increase the incidence and severity of *Berkeleyomyces* spp. infection in cotton (Rothrock, 1992).

Soil moisture content is an essential factor that affects the germinability of endoconidia and chlamydospores in soil. The relationship between high water content of soils and increased BRR severity was reported by (King and Presley, 1942). In moist soil, at 45-50% of the moisture-holding capacity (MHC), the germination of chlamydospores dropped rapidly, while intermediate reductions in germination were seen at 30% MHC, and no decline in germination at 15% MHC. At high MHC (45-50%) the endoconidia viability and numbers were also highly reduced (Papavizas and Lewis, 1971).

Soil pH is another factor that influences the incidence and severity of BRR disease. pH effects the solubility of ions in soil and is known to alter the activity of soil microbial communities (Kaufman and Williams, 1964). BRR disease caused by *Berkeleyomyces* spp. has been found to be promoted at pH ($CaCl_2$) values exceeding 5.6 and supressed at pH values less than 5.2 (Harrison and Shew, 2001; Meyer *et al.*, 1994).

Soil texture is another abiotic factor that has been correlated with the severity of BRR disease. Monfort *et al.* (2006) reported that populations of *Berkeleyomyces* spp. were lower in areas of a field with the greatest sand content (50.1 to 81.7% sand) compared with soil with lower sand content (22 to 50% sand). Jaraba *et al.* (2014) observed that root colonisation by *Berkeleyomyces* spp., and fungal reproduction and

survival rates, decreased in soil having 87% sand, in comparison with other artificial soil mixtures containing 53, 70 and 74% sand. In general, finer-textured soils have been found to increase the severity of BRR disease, compared with coarser-textured soils, in a number of cropping systems including soybean , tobacco (Rothrock, 1992) and cotton (Nehl *et al.*, 2004).

The level of exchangeable ions, such as calcium, aluminium, phosphate, ammonium and nitrogen in soils are considered an essential factor in suppression or promotion of *Berkeleyomyces* spp. growth in infested soils. Meyer *et al.* (1994) reported that high levels of aluminium suppressed infection by *Berkeleyomyces* spp. through the inhibition of spore germination and hyphal growth. It has also been reported that high levels of aluminium, combined with pH of <5.2, inhibited the production of fungal chlamydospores (Fichtner *et al.*, 2006). In another study aluminium was considered as fungistatic at low levels (> 0.5 meq of Al/100g of soil), and fungitoxic at high levels (1.1 meq of Al/100g of soil), where 1 meq=9 ppm of Al (Meyer and Shew, 1991). Similarly, ammonium also has a suppressive action on *Berkeleyomyces* spp., potentially by inducing the host plant to release a fungistatic compound (putrescine) (Harrison and Shew, 2001), and/or by direct action as a fungitoxic compound that penetrates and disrupts the cell membrane of the fungi (Candole and Rothrock, 1998). Moreover, high levels of phosphate or nitrogen in acidic soils have a suppressive effect on *Berkeleyomyces* spp. through the inhibition of spore germination and hyphal production (Delgado *et al.*, 2005; Meyer *et al.*, 1994). In regards to calcium, Meyer and Shew (1991) observed that a higher level of calcium in alkaline soil promoted the incidence of BRR disease.

Application of organic matter could impact on the incidence of soil-borne disease, based on its ability to induce suppression (Lazarovits, 2005). The application of green manure was observed by Rothrock *et al.* (1995) to control BRR disease. This action was suggested to be derived from supporting the growth of nitrogen fixing microbes that released ammonia during the decomposition of organic matter (Candole and Rothrock, 1998). Bonanomi *et al.* (2020) also reported that addition of compost to soils has a suppressive effect on *Berkeleyomyces* spp.. In contrast, Millner and Ja (1982) reported that sewage sludge increased BRR disease in beans, peas and cotton.

1.5.2. Biotic environment

Biotic environmental factors, such as soil microorganisms, plant species and crop rotation are all considered to be factors that have a role in suppressing or promoting the incidence of *Berkeleyomyces* spp. infections in cropping systems.

Some soil microbes can protect plants from infection, having the ability to supress pathogenic microorganisms (Almario *et al.*, 2014; Schlatter *et al.*, 2017). For example, Stutz et al (1989) demonstrated that *Pseudomonas fluorescens* has suppressive activity against BRR in tobacco. In addition, Paulitz *et al.* (2000) reported that *Berkeleyomyces* spp. are supressed by *Pseudomonas aureofaciens*. Moreover, the root colonisation of *Berkeleyomyces* spp. was inhibited by the action of *Paenibacillus alevi* strain K-165 (Schoina *et al.*, 2011), while Yi *et al.* (2010) showed that *Streptomyces hygroscopicus* Strain TA21 reduced the incidence of BRR disease of tobacco in glasshouse experiments.

Plant species can also influence the incidence of BRR disease. As pointed out by Pereg (2014), *Berkeleyomyces* spp. exhibit three modes of interaction with plants. Plants can be susceptible hosts (including cotton, bean and tobacco crops), in which the

pathogen infects the root and causes disease (Hood and Shew, 1996; Koenning *et al.*, 2004). Plants may also be non-susceptible hosts, where the pathogen infects the root without causing disease. Non-susceptible hosts include wheat (Pereg, 2014). Finally, plants may be non-hosts, with *Berkeleyomyces* spp. being unable to infect the root. Non-hosts include crops such as sunflowers (Delgado *et al.*, 2005; Tabachnik *et al.*, 1979). Rotating cotton with other non-host plants is one management strategy suggested to reduce the incidence of BRR disease, as discussed in the following sections.

1.6. Management strategies to control *Berkeleyomyces* spp.

BRR disease caused by *Berkeleyomyces* spp. has spread and become established in the soil, it is hard to control, because of the persistence of thick-walled chlamydospores (Mondal *et al.*, 2004). The term infection chain is used to describe how plant and other pathogens survive through interactions with hosts (Brown, 1997). Infection chains can be continuous, where pathogens keep infecting susceptible hosts in an uninterrupted manner, or discontinuous, where the infection chain is interrupted by periods where the pathogen survives outside of a host. These interruptions can be epiphytic (pathogen persists on the surface of its host and/or non-hosts without causing disease), saprophytic (pathogen survives on plant residue or organic matter present in soil) or resting stage (pathogen forms a resting structure, e.g. spores, that allow for survival independent of a host). These continuous and discontinuous infection chains are illustrated in Figure 1.3 (Brown, 1997). In regards to *Berkeleyomyces* spp. the persistence of chlamydospores limits the potential for control, as efforts generally result in a discontinuous infection thread, rather than in elimination of the pathogen.

Figure 1.3: Continuous and discontinuous infection chains of plant pathogens (Brown, 1997).

Accordingly, the best option to control disease is to prevent plants from becoming infected. There are a number of tools and management practices that can be used to reduce infection, falling broadly into the categories of cultural control, chemical control, breeding and genetic manipulation of plants, biofumigation and biocontrol.

1.6.1. Cultural control

In order to reduce the incidence and severity of BRR disease multiple cultural methods have been applied. According to Nehl *et al.* (2004) the use of crop rotation, timed planting, sanitisation and summer flooding have all been considered for agronomic benefit.

Crop rotation is considered as an effective means to reduce the incidence of BRR disease in cotton crops. Abawi and Widmer (2000) suggested that using corn or grain sorghum as rotational crops could help to reduce the incidence of disease caused by *Berkeleyomyces* spp., by preventing the reproduction of fungus. Nehl *et al.* (2004) pointed out that rotation with non-host crops, such as cereals, would help in limiting and delaying the build-up of the pathogen in soil. Hulugalle and Scott (2008) reported that a large proportion (~75%) of Australian cotton is grown in rotation with winter

cereals, such as wheat, or legumes, such as faba bean, and that the incidence of BRR disease was lower when grown with a wheat crop rotation, rather than in rotation with legumes. In one study rotation with wheat resulted in a 70% reduction in the severity of BRR disease in cotton fields after two years of rotation (Nehl *et al.*, 2004). The introduction of summer maize as a rotational crop in Australian cotton fields also showed a reduction in the severity and incidence of *Berkeleyomyces* spp., with improved lint yield and soil properties (Hulugalle *et al.*, 2020). Holtz and Weinhold (1994) observed that using monocots, a non-host crop, was able to reduce *Berkeleyomyces* spp. density in the soil, and therefore reduce the incidence of BRR disease. In general, crop rotation could reduce the incidence of BRR disease caused by *Berkeleyomyces* spp., however, extended rotation is not widely practiced by cotton growers in Australia (Toksoz *et al.*, 2009).

Management practices such as the application of fertilisers could also be adjusted to help control BRR infection. Application of organic amendments in the form of compost and manure have been shown to control *Berkeleyomyces* spp. effectively (Papavizas, 1968). The main action of mechanism of organic amendments involves the direct effect on biotic and abiotic properties of soil, such as affecting soil pH through the release of ammonia during decomposition (Tenuta and Lazarovits, 2004), the release of fungitoxic compounds like glucosinolates from Brassicaceae residues (Larkin and Griffin, 2007), and an increase in biomass of the microbial population, particularly in the rhizosphere, that have antagonistic activity against pathogens (Nelson, 2004). As reported by Rothrock *et al.* (1995), the application of green manure, combined with vetch (*Vicia villosa)* as a nitrogen fixing species, was able to control the incidence of *Berkeleyomyces* spp.. This action derived from the release of ammonia

during the decomposition of the organic residues, with *Berkeleyomyces* spp. showing a sensitivity to ammonia (Candole and Rothrock, 1998; Papavizas and Lewis, 1971).

As cooler temperatures (16- 20°C) are the favoured condition for increased incidence and severity of black root rot disease in cotton fields (Mauk and Hine, 1988; Rothrock, 1992), timing the planting of crops to avoid lower temperatures is also considered as a strategy to minimize the incidence of *Berkeleyomyces* spp. infections (Jhorar, 2004). Delaying sowing leads to reduced disease symptoms on the tap root, increased stand establishment and increased shoot dry mass of plants (Jhorar, 2004, 2004).

Summer flooding of crop lands has been recommended in Australia to assist with disease management (Allen *et al.*, 2011), as it is reported to reduce the severity of BRR disease effectively (Nehl *et al.*, 2000). Before planting, summer flooding helped to reduce BRR disease incidence by up to 98% in one study (Jhorar *et al.*, 2004). Although it may be effective, the use of summer flooding is restricted by cost, terrain, and water availability. Summer flooding also raises the challenge of dealing with the spread of pathogen through water runoff (Pereg, 2013).

The spread of pathogens between fields and/or farms can be minimised by following good hygiene practices. Practices involving the combination of sanitation such as “come clean, go clean”, proper plant care, trash management and machinery washing/disinfection are considered as essential to minimise and control the spread of *Berkeleyomyces* spp. (Daughtrey *et al.*, 1995). The commercial detergent Farm cleanse (Castrol) is commonly applied to decontaminate farm vehicles and machinery, as it is able to kill the fungal pathogen. This method is considered as a management tool to minimise the spread of pathogen among fields (Rourke, 2001).

1.6.2. Chemical control

Fungicides are commonly used to prevent or control fungal pathogens, and a number of different fungicides have been tested for efficacy against *Berkeleyomyces* spp.. Prinsloo *et al.* (1989) evaluated a combination of flusilazole and triadimenol as a sterol-inhibiting fungicide to control BRR disease in tobacco, and found that it inhibited the mycelial growth of *Berkeleyomyces* spp.. In addition they also tested a combination of flusilazole with benomyl, which also showed effective action against *Berkeleyomyces* spp., although less significant than the combination of flusilazole with triadimenol. However, both combinations showed phytotoxic effects when concentrations of flusilazole exceeded 0.4g a.i/m^2, or triadimenol exceeded 0.046g a.i/m^2. Triadimenol alone has also been used in cotton fields infested with *Berkeleyomyces* spp., and reduced the severity of BRR disease in some fields. However, triadimenol had a phytotoxic effect, delayed the emergence of plants by two days and slowed the growth of plants (Nehl *et al.*, 2004). Benomyl was also applied to cotton fields and reduced the severity of *Berkeleyomyces* spp. infection, with no apparent phytotoxic activity. However, a reduction in shoot growth was observed, and attributed to the density of *Berkeleyomyces* spp. in the soil (Hewajulige and Wijesundera, 2014; Nehl *et al.*, 2004). Toksoz *et al.* (2009) found that applying myclobutanil reduced root discoloration caused by *Berkeleyomyces* spp. by 36% compared to no fungicidal chemical treatment. Other fungicides such as azoxystrobin, fludioxonil and mefenoxam have been found to be ineffective against *Berkeleyomyces* spp. (Toksoz *et al.*, 2009).

A number of chemical compounds, while not directly fungicidal themselves, can be used to induce pathogen resistance in plants. The Systemic Acquired Resistance (SAR) pathways, which are based on the activation of plant defence mechanisms in response to pathogens, can be triggered by these compounds (Durrant and Dong, 2004;

Schneider *et al.*, 1996). One of the chemical inducers that stimulates SAR is acibenzolar-S-methyl (Bion), which has been shown to reduce BRR disease in cotton (Mondal *et al.*, 2004; Toksoz, 2008). Toksoz *et al.* (2009) observed that Bion was most effective as an inducer of host resistance when combined with the fungicide Triazole, and the combination was able to reduce the severity of BRR disease, in terms of both host colonisation and incidence of infection.

While a number of different chemical controls that impact BRR disease have been identified, there is no one compound that has been shows to completely manage this disease. In addition, the use of such chemicals raises issues around the potential negative effects on the environment, and the potential for the pathogen to develop resistance to these chemicals in the future. There is therefore a need to identify other non-chemical based control measures for this disease.

1.6.3. Biofumigation

Biofumigation of soil is considered to be an environmentally sustainable management strategy that can be used to control soil-borne pathogens and manage soil-borne disease (Pokharel, 2012). Biofumigation involves the cultivation of specialised cover crops, which are then mulched and incorporated into soil prior to planting of crops. Members of the Brassicaceae family, such as mustards, cauliflower and vetch have been grown as cover crops in rotation with a number of cash crops (Kirkegaard and Sarwar, 1998; Matthiessen and Kirkegaard, 2006), and can be used as biofumigants. These plants suppress the growth and spread of soil pathogens through the production of biocidal chemical compounds (De Boer *et al.*, 2005) such as Propenyl (Matthiessen and Kirkegaard, 2006). The suppressive effect of vetch, when used as a

biofumigant, on *Berkeleyomyces* spp. may also be due to the high level of ammonia that is released during degradation, as ammonia is considered as a toxic substance to this fungus (Candole and Rothrock, 1998). Allen (2001) also suggests that vetch decreased the severity of BRR disease through the release of ammonia when incorporated into soil. Vetch is not the only biofumigant tested against *Berkeleyomyces* spp.. A decrease in the severity of BRR symptoms on cotton roots has also been observed when mustard was used as a biofumigant (Henis and Chet, 1975; Smith and Kirkegaard, 2002). While biofumigation presents a potential mechanism to help control soil-borne disease a limitation of this strategy is concern from some growers that growing biofumigation crops, may increase the incidence of other plant diseases, such as *Fusarium* wilt, and raise issues with weed control (Nehl *et al.*, 2004; Pereg, 2014).

Thermal bio disinfestation is another method used to overcome the limitations of the biofumigation method. This method involves covering the soil surface with a polythene film to increase soil temperature after the green mass (vetch, mustard, or other biofumigation material) is incorporated into the soil. This combination of biofumigation plus covering the soil surface with polythene film increases the soil temperature by 1-3°C (Gurel *et al.*, 2019) . A soil-borne pathogen *Fusarium wilt (F. oxysporum)* are found to be susceptible against thermal bio disinfestation (Panth *et al.*, 2020; Shrestha *et al.*, 2014). In a field trial, Nehl *et al.* (2004) reported that the use of mustard, covered with polythene, reduced the population density of *Berkeleyomyces* spp. by 50% when compared with mustard used as a standard biofumigant. This was in contrast to vetch, where the non-sealed vetch appeared to decrease the severity of BRR by 25% more than when vetch plus a polythene seal was used. In general, the polythene cover reduced the seedling mortality by half, in both fumigated crops (Vetch and

mustard), however, the incorporation of biofumigated crops with solarisation to control the incidence of BRR disease needs more investigation.

1.6.4. Biocontrol

Biological control, or biocontrol, refers to the control of pests/pathogens through the use of other living organisms. A number of organisms, notably various species of bacteria, have been employed as biocontrol agents against a range of plant pathogens (Köhl *et al.*, 2019; Pal and Gardener, 2006). Biocontrol organisms can exhibit a number of modes of action against soil-borne pathogens, namely competitive, antagonistic, induction of host resistance or transmission of hypovirulence genes (Deacon and Berry, 1993).

A number of bacteria have been investigated as potential biocontrol agents that could be employed to supress BRR disease. The biocontrol agent *Paenibacillus alvei* strain K-165, applied as a seed coat, was tested for efficacy against BRR disease by Schoina *et al.* (2011). This strain was antagonistic to the BRR pathogen, and was effective in reducing the discoloration of roots, and number of hypocotyl lesions in cotton plants, thus contributing to reductions in secondary infections. Another biocontrol agent investigated in relation to BRR is *Pseudomonas fluorescens* CHA0 (Reddy and Patrick, 1992). This strain had a suppressive effect on *Berkeleyomyces* spp. infections in tobacco, when applied as a soil inoculant. *P. fluorescens* CHA0 has the ability to colonise the root of tobacco and synthesises several antifungal metabolites including hydrogen cyanide, 2,4-diacetylphloro- glucinol (Phl) and pyoluteorin (Plt), which induce plant resistance against the pathogen (Troxler *et al.*, 1997). *Pseudomonas aureofaciens* strain 63-28 has also been considered as a biocontrol agent. This

bacterium produces the compound furanone, which appears to have antifungal properties against *Berkeleyomyces* spp. (Paulitz *et al.*, 2000).

While there has been some promising research into biocontrol of *Berkeleyomyces* spp., this pathogen presents a number of challenges. As a soil-borne pathogen it raises additional challenges in terms of biocontrol, given that infection occurs in the rhizosphere. The rhizosphere is microbially active, and is a constantly changing environment. Use of a biocontrol agent in the rhizosphere is difficult as these agents need to be able to survive those conditions, and not be out-competed by existing rhizospheric microbes (Handelsman and Stabb, 1996; Whipps and Lumsden, 2001).

Even though these different control strategies exist/have been described, one of the issues is figuring out what really works is that there is little evidence of a direct correlation between soil pathogen load and disease severity (Allen, 2001; Allen *et al.*, 2011; Allen, 2007). Part of the reason for this is the reliance on plate culture as a means of assessment of soil load (Nehl *et al.*, 2004; Rothrock, 1992), and that different studies have focused on endoconidia as the infective status of the pathogen (Nehl *et al.*, 2004; Pereg, 2013). One way to avoid the short falls of plate culturing and the inconsistencies of determination from different spores is to assess and quantify the pathogen DNA in samples. More recently, molecular tools in the form of conventional polymerase chain reaction (PCR) techniques have been used to detect pathogens, with the aid of pathogen specific primers (Thalmann, 2008; Zhao *et al.*, 2009). Conventional PCR gives an indication of the presence or absence of a pathogen in a specific sample, while quantitative PCR (qPCR) represents a reliable method of determining the relative pathogen load in different samples. These techniques provide rapid, sensitive and relatively simple methods, requiring only 4-5 hr, to detect and quantify *Berkeleyomyces* spp. in environmental samples (Huang and Kang, 2010).

1.7. *Azospirillum* as a potential biological control agent against BRR disease

Azospirillum are diazotrophic (biologically N_2-fixing), free living microorganisms belonging to the α-subclass of Protobacteria, which includes a huge number of bacteria, such as *Rhizobium*, *Agrobacterium*, *Bradyrhizobium* and *Gluconacetobacter*. Members of this genus live in symbiotic relationships with plant roots, especially economically important crops such as wheat and rice. *Azospirillum* colonise plant roots and promote root hair development, lateral root appearance and increase root surface area. At a physiological level, *Azospirillum* effects production of growth promoting hormones (e.g. indole-3-acetic acid) and also alters the activity of specific enzymes in the glycolysis pathway and Krebs cycle. These morphological and physiological changes in the root lead to enhanced uptake of water and nutrient uptake by the plant (Bashan and Holguin, 1997; Bashan *et al.*, 2004; Hartmann and Baldani, 2006; Okon and Labandera-Gonzalez, 1994).

1.7.1. *Azospirillum* as a biocontrol

The use of plant growth-promoting bacteria (PGPB) as biological control agents for soil-borne diseases has been reviewed by Bashan and De-Bashan (2005), Compant *et al.* (2005) and Hassouna *et al.* (1998). Many *Azospirillum* species have been suggested to act as biocontrol agents against a range of plant-fungal pathogens. *A. brasilense* Sp245 showed an ability to inhibit the growth of the fungal pathogen *Rhizoctonia, in vitro* (Russo *et al.*, 2008). Siderophores produced by *A. brasilense* showed antifungal activity against *Colletotrichum acutatum, Alternaria* spp., *Bipolaris* spp. and *Fusarium* pathogens (López-Reyes *et al.*, 2017; Tortora *et al.*, 2011). An

auxin-like molecule called phenylacetic acid (PAA) in supernatant extracts from *A. brasilense* Sp7 and *A. brasilense* Sp245 showed inhibitory activity against *Fusarium oxysporum, Fusarium matthiolae, Neurospora crassa* and *Alternaria bassicicola* (Somers *et al.*, 2005). Resistance to the leaf spot pathogen *Pseudomonas syringae,* induced by *A. brasilense,* in *Arabidopsis thaliana* has also been recorded (Ramos Solano *et al.*, 2008). In addition, *A. brasilense* SBR, mixed with rhizobacteria strains *Azotobacter Chroococcun* ZCR, and *Klebsiella pneumoniae* KPR, exhibited biological control properties against sunflower root-rot disease complex caused by three fungal pathogens (*Fusarium solani, Macrophomina phaseolina* and *Rhizoctonia solani*) (El_Komy *et al.*, 2020). Somers *et al.* (2005) identified an auxin-like molecule from *A. brasilense* named phenyl acetic acid (PAA) with antimicrobial activity.

1.7.2. Ecological distribution

According to Bashan and Holguin (1997), *Azospirillum* spp. are widespread in the soil of different geographical regions across the world. The members of this genus have the ability to attach to roots of many different plant species, and there is no specific relationship between *Azospirillum* spp. and their host plants. *Azospirillum* spp. survive for a long time in the rhizosphere and transfer from root to root under the influence of attractant and repellent signalling molecules secreted by plant roots (Bashan and Holguin, 1994, 1997; El Zemrany *et al.*, 2006).

Azospirilla have been isolated from the roots of grasses of several genera in both tropical and subtropical-temperate regions (Tyler *et al.*, 1979). A survey of grasses collected from northern Australia indicated that 95% of *Azospirilla* isolated from the rhizosphere were *A. brasilense* (Weier, 1980). *Azospirillum* spp. isolated from the roots and rhizosphere of wheat collected from eastern Australia have been tested to explore the optimal pH for bacterial survival. Interestingly, some isolates were able to grow at

a minimum pH that was less than the pH of the soil from which they were first isolated (New and Kennedy, 1989). However, a considerable proportion of isolates had a minimum pH for growth that was higher than the pH of the soil from which they were isolated, suggesting that wheat roots provided an environmental niche protecting against soil acidity (New and Kennedy, 1989).

The ability of *Azospirilla* to adapt to environmental change has been well established (Steenhoudt and Vanderleyden, 2000). *Azospirilla* grown in liquid culture evolve a single polar flagellum, allowing them to swim, and exhibit chemotactic behaviour. If grown on solid or semisolid media, additional lateral flagella are developed for swarming motility. Under conditions unsuitable for growth, a morphological transformation occurs, with motile cells changing into cyst-like forms, which lack flagella and exhibit reduced metabolism (Steenhoudt and Vanderleyden, 2000). The bacteria stay in this relatively dormant form until the environmental conditions become suitable for growth. Undoubtedly, both types of growth are necessary for the survival of the *Azospirilla* (Pereg-Gerk, 2004). This ability to adapt to environmental changes is important in the consideration of *Azospirillum* as a potential biological control agent, as, as discussed previously, any biological control agent for BRR disease will need to be able to survive in the rhizosphere in order to be effective.

1.7.3. Flocculation and cyst formation in *Azospirillum* spp.

Flocculation and cyst formation are morphological and cellular transformations that occur in *Azospirillum* spp. in response to limitation of nutrients, or other environmental changes, such as an increase in oxygen level (Pereg, 2015). In flocculation, or floc formation, cells tend to attach to each other to form clumps

(Sadasivan and Neyra, 1985). During cyst formation, the vegetative cells transform into a resting cyst, and have the ability to survive adverse environmental conditions (Sadasivan and Neyra, 1987).

At present, very little is known about the genetic basis of flocculation and cyst formation in *Azospirillum* spp. Our understanding to date has been limited to only one regulatory gene identified in *A. brasilense* Sp7, designated as *flcA* (Pereg-Gerk *et al.*, 1998). *Flc*A has a role in regulation of flocculation, polysaccharide production and root colonisation. The deduced 215 amino acid sequence of *flcA* shows high similarity with members of the LuxR-UhpA transcriptional regulatory family. Most of these are response regulators, supporting the suggestion that *flcA* could be a part of a similar two-component regulatory system (Pereg-Gerk *et al.*, 1998).

Under conditions that limit the growth of *Azospirillum* the *flcA* gene is constitutively expressed, but gene expression is increased 3-4 fold under high ratios of calcium to nitrogen (Pereg-Gerk, 2004). A previous study by Sadasivan and Neyra (1985) suggested that high concentrations of fructose and low concentrations of nitrogen lead to optimal flocculation conditions. The relationship between expression of nitrogenase by *A. brasilense*, and its ability to flocculate, undergo morphological transformation into a cyst-like form, and ability to colonize wheat roots in hydroponic solution had been described. Results showed that mutant strains failing to flocculate exhibited a higher capacity for nitrogenase expression in comparison with wild-type strains (Pereg-Gerk, 2004). This study determined that nitrogenase expression in vegetative mutant strains of Sp7 and Sp245, impaired in flocculation and cyst formation, was higher than that in encysted wild-type strains when in interaction with wheat, despite reduced root colonisation by the mutants. In contrast, restoring the ability of the spontaneous mutant Sp7-S to form cyst-like structures and colonize roots

decreased the level of nitrogenase activity during interaction with plants, to levels similar to those exhibited by the wild-type Sp7. Tn5-induced *flcA* mutants expressed higher levels of nitrogenase than wild-type Sp7, but lower levels of nitrogenase activity than that of the spontaneous mutant Sp7-S, indicating that in Sp7-S there are other factors influencing the activity of nitrogenase enzyme during plant interaction (Gerk *et al.*, 2000).

Root exudates have been found to increase *flcA* expression (Pereg-Gerk, 2004). The expression of *flcA* in wild-type strains of *A. brasilense* Sp7 during the first 4-5 hours of growth increased when grown under suitable flocculation conditions, with *flcA* expression decreasing when flocculation is initiated. *FlcA* expression in parental strains was significantly lower than *flcA* expression in *flcA* Tn5-mutant strains Sp72001/2/4, which lack the ability to produce functional FlcA protein. The mutant strains failed to flocculate and the expression of *flcA* continued to increase during the growth period in comparison to the wild-type. For that reason, a likely interpretation is that the expression of *flcA* is negatively auto regulated by its own product (*FlcA*), either directly or indirectly. Direct regulation by the *FlcA* protein, or indirect regulation via influencing the expression of other genes, may have a role in the morphological transformation of *A. brasilense* from vegetative cell to cyst-like form (Pereg-Gerk, 2004; Valverde *et al.*, 2006).

1.7.4. Root Attachment and Colonisation by *Azospirillum* spp.

In order to successfully interact with plants, *Azospirillum* spp. need to survive in the soil and reach significant numbers on the plant root system (Steenhoudt and Vanderleyden, 2000). In the rhizosphere, plant root exudates create a nutrient gradient from the root to the surrounding soil. Bacteria move towards plant roots in order to

utilise root exudates as sources of carbon and energy. Motility and chemotaxis are two mechanisms that contribute to this process (Steenhoudt and Vanderleyden, 2000).

Bacterial motility and chemotaxis are necessary for successful interactions with plant surfaces for both pathogenic and symbiotic interactions. This ability is affected by soil conditions, bacterial species and type of plant (Broek and Vanderleyden, 1995). The importance of bacterial motility for *Azospirillum*-plant root interactions has been demonstrated using various *Azospirillum* mutants with altered chemotactic motility. These mutant strains display a significantly decreased ability to attach to root hairs of wheat, compared to the wild-type (Broek *et al.*, 1998). A mutant, impaired in both polar and lateral flagella formation (and therefore lacking motility) has been shown to exhibit decreased adsorption to wheat roots (Croes *et al.*, 1991). Purified polar flagella have the ability to bind to wheat roots *in vitro*, while under the same conditions lateral flagella fail to bind (Croes *et al.*, 1993). Recent studies demonstrated that a swarming mutant (Swa^- phenotype) of *A. brasilense* Sp245, which lacks the polar flagellum, also showed a decreased ability for adsorption to wheat roots (Shelud'Ko *et al.*, 2010).

Attachment of *A. brasilense* to the roots of wheat has been described as a two-step process. Each stage is distinct and fully independent of the other. Firstly, a rapid and weak adsorption (reversible) process takes place, which relies on a proteinaceous surface component of bacteria. This is followed by a strong anchoring (irreversible) phase, depending on calcofluor binding surface polysaccharides in the extracellular polysaccharide layer produced by *A. brasilense* (Michiels *et al.*, 1991). In *Azospirillum* spp. a fast adsorption and a strong anchoring to plant roots and soil particles was related to their ability to compete with other soil microorganisms (Michiels *et al.*, 1991).

The mechanisms of plant-root colonisation by *Azospirillum* spp. differ among members of this genus, depending on plant species, bacterial strain, environmental

conditions and other factors (Bashan and Holguin, 1997). These factors will lead to differences in the capacity of bacteria to colonise particular plants, different sites of colonisation and changing numbers of colonising bacteria. The majority of colonisation sites, for large numbers of plant species examined, lie at the site of root elongation, and along the root hairs. A network of fimbrilar material supporting root surface colonisation had been proposed as a feature of *Azospirillum* root colonisation (Bashan and Levanony, 1990). On the other hand, some *Azospirillum* spp. have the ability to colonise roots internally (Bashan and Levanony, 1990). Assmus *et al.* (1995) demonstrated that strain Sp7 was restricted to the rhizosphere, mainly in the root hair zone, while Sp245 presented at high numbers in the interior of the root hair zone. A previous study on colonisation of roots by *A. lipoferum* and *A. brasilense* showed that the bacteria are more numerous in the root hair zone than on the root tip, and that the bacteria are mainly found in the depressions between epidermal cells (de Oliveira *et al.*, 2002). The distribution and colonisation of *A. brasilense* Cd strain on wheat roots under saline condition has been studied. Bacteria grown under normal conditions were distributed along the whole root system, except the elongation zone, and colonised the *para*-nodules. Bacteria experiencing saline stress were principally localised at the root tips and to the lateral roots. It has been suggested that saline stress induces the synthesis of a different exopolysaccharide in *A. brasilense* (Fischer *et al.*, 2003). Colonisation has also been altered in stressed plants respsonding to treatment with the herbicide 2,4-Dichlorophenoxyacetic acid, with the majority of the bacteria found around the basal surfaces of the modified lateral root structure (Fischer *et al.*, 2000). Colonisation of strains of *A. brasilense* (Sp7, Sp245, Wa5) on different wheat root cultivars has also been evaluated. It appears that environmental factors, stress and crop cultivar (especially for wheat) have an influence on the pattern of colonisation of roots by these

strains. Generally, all strains colonised mostly the root tip in high numbers, however, strain Sp245 also colonised the cortical root zone and showed constant colonisation during the time of the experiments (Schloter and Hartmann, 1998) .

1.7.4.1. Chemical and physical factors effecting Azospirillum root attachment

Various chemical and physical parameters affect the physiological activity of microorganisms. Primary phase adsorption of *Azospirillum* to plant roots has been estimated by studying the cell surface charge and cell surface hydrophobicity of a numbers of *Azospirillum* strains. Results showed that *Azospirillum* spp. have a temperate cell-surface hydrophobicity and surface charge, less than known values for human bacterial pathogens, and this charge is higher in solid media than in liquid media (Bashan *et al.*, 2004; Castellanos *et al.*, 1997). The cell surface hydrophobicity and charge can be affected by treating the cells externally with physiochemical or enzymatic treatments (Castellanos *et al.*, 1997). Similar findings were reported by Fedonenko *et al.* (2001), where it was observed that lipopolysaccharide-defective mutants of *A. brasilense* Sp245 showed a considerable decrease in the hydrophobicity of the cell surface. These mutant cells also showed a decreased capacity for root attachment in comparison with the wild-type strain. The effect of pH and cations was also mentioned. Results showed that the presence of a low concentration (0.5 mM) of Ca^{2+} was sufficient to decrease the number of *Azospirillum* cells attached to wheat roots. In addition, a significant concentration of $(PO^4)^{3-}$ (above 50mM) reduced bacterial attachment in the culture medium. The effect of pH on the attachment of 10 strains of *Azospirillum* spp. revealed that, with one exception, strains collected from the rhizosphere or roots of wheat exhibited optimum adsorption at pH 6.0 or pH 7.0 (de Oliveira *et al.*, 2002).

1.7.4.2. Role of surface lipopolysaccharides in root attachment

Lipopolysaccharides (LPS), anchored on the external membrane of diazotrophic bacteria, mediate the direct contact of the bacteria with the host plants (Serrato, 2014). The role of extracellular polysaccharides, lipopolysaccharide-protein complexes, polysaccharide-lipid complexes, lipopolysaccharides, and O-specific polysaccharides produced by *Azospirillum* in bacteria-plant interactions has been studied in an attempt to understand the root attachment mechanisms of *Azospirillum* spp. (Skvortsov and Ignatov, 1998). Studies have shown that bacteria impaired in capsular exopolysaccharide production completely lost their capacity to attach to wheat germ agglutinin, and lost much of their capacity to adhere to the roots of wheat seedlings (Egorenkova *et al.*, 2001). A comparative study between wild-type *A. brasilense* Sp245 and mutants impaired in lipopolysaccharide production showed that the attachment ability of the mutants was less than that of the wild-type strains during the incubation period (15 min to 48 h) (Fedonenko *et al.*, 2001).

1.8. Role of roots in microbial-plant interactions

Plant environments are rich with microbes that can directly associate with plants in beneficial, neutral or pathogenic relationships. Additionally, microorganisms can affect plants indirectly by influencing their habitats. A greater understanding of the complicated nature of plant-microbial associations can lead to the development of new approaches to increase plant productivity in an environmentally friendly manner (Schenk *et al.*, 2012). Plant-microbial interactions involve signalling cascades, affecting the expression of numerous genes (Birch and Kamoun, 2000) and resulting in cellular responses. Abiotic factors such as drought, saturation with water, temperature, salinity and acidity of soil provide environmental signals that can induce or reduce these

interactions (Berg, 2009). A challenge in the future involves understanding the large-scale communication that may occur between different bacterial species, and inter-kingdom signalling molecules utilised in communication between plants and microbes (Hughes and Sperandio, 2008; Patel *et al.*, 2013), especially given the desire to use microbes as plant-growth promoters or biological control agents against plant pathogens.

The main function of the root is to anchor and support the plant, and to absorb water and nutrients (Abbott and Murphy, 2003). Root tips are the main part of roots involved in sensing environmental changes and controlling movement in soil (Hawes *et al.*, 2000). In the rhizosphere, roots exude different chemicals that are involved in communication with soil microorganisms. These include sugars, polysaccharides, aromatic acids, amino acids, aliphatic acids, sterols, phenolic, enzymes, other proteins, plant growth regulators and secondary metabolites (Badri *et al.*, 2009; Faure *et al.*, 2009). As discussed by Griffin *et al.* (1976), the majority of root exudates are carbon rich materials produce by border cells (originally known as sloughed root cap cells) and their associated products. Consequently, border cells play a crucial role in plant-microbial interaction, either by helping to prevent root infection, or through beneficial interactions (Hawes *et al.*, 2016; Hawes and Pueppke, 1987; Zhu *et al.*, 1997).

In the rhizosphere, microbial composition and abundance are influenced by the amount and composition of rhizodeposition (particularly, root exudate, mucilage) (Jones *et al.*, 2009). Root exudates have clear selective and supportive effects on specific microorganisms, enabling them to respond to, interact with, and finally colonise plant roots (Hartmann *et al.*, 2009). Mark *et al.* (2005) investigated the influence of root exudates from two different cultivars of sugar beet on gene expression of *Pseudomonas aeruginosa* PA01. Differential gene expression was observed while

establishing microbial-plant interactions in response to signal molecules produced from the root exudate. Such expressed genes involved some new unidentified genes related to microbial competition in the rhizosphere and genes associated with *P. aeruginosa* PA01 plant colonisation. *Bacillus amyloliquefaciens* SQR9 (isolated from the rhizosphere of cucumber plants) and *Bacillus subtilis* N11 (isolated from the rhizosphere of banana plants) have been used to investigate the effect of various root exudates and their organic acids on chemotaxis, biofilm formation and root colonisation (Zhang *et al.*, 2015). Both isolates were more effective in colonising their natural host (i.e., cucumbers and bananas respectively), than colonising other plants. Both cucumber and banana root exudates had a positive effect on chemotactic behaviour, and stimulated biofilm formation in *B. amyloliqufaciens* SQR9. *B. subtilis* N11 was attracted by both root exudates, and showed a significant increase in biofilm formation in response to banana root exudate. Citric acid isolated from cucumber root exudates induced chemotaxis and stimulated biofilm formation of *B. amyloliqufaciens* SQR9, whilst only inducing chemotaxis in *B. subtilis* N11. Fumaric acid isolated from banana root exudates induced chemotaxis and biofilm formation of *B. subtilis* N11, whilst influencing biofilm formation of SQR9, without chemotactic capability (Zhang *et al.*, 2015). Canavanine, a non-proteinogenic amino acid structurally homologous to arginine, secreted in legume root exudates acts as a signalling molecule in legume-rhizobium interactions (Cai *et al.*, 2009). Canavanine is toxic to many bacteria in the rhizosphere, since when incorporated during protein synthesis it can lead to abnormal protein production. However, the expression of *MsiA* in *Mesorhizobium tianshanense* and MsiA-like proteins in other rhizobial strains is induced by canavanine, and the proteins are responsible for bacterial resistance to canavanine. Moreover, resistance to canavanine is significant for root hair attachment and survival of rhizobial bacteria in a

rhizosphere containing high levels of canavanine (Cai *et al.*, 2009). These examples demonstrate the significant and complex roles that root exudates have in plant-microbe interactions.

The germination of *Berkeleyomyces* spp. spores of in response to root extracts (Patrick *et al.*, 1965), root exudates (Mathre *et al.*, 1966) and specific stimulatory substances such as natural lecithin, unsaturated fatty acids and unsaturated triglycerides Mathre and Ravenscr (1966), and Papavizas and Adams (1969) has been recorded. *Berkeleyomyces* spp. germination occurred in response to cotton root exudates and root extracts more than other plant (Mathre and Ravenscr, 1966; Rothrock and Nehl, 2000). In addition, the germination of cotton isolate *Berkeleyomyces* spp. induced by the non-host wheat has been recorded (Rothrock and Nehl, 2000). Coumans *et al.* (2010) found that root extracts from non-hosts (wheat, hairy vetch) and susceptible hosts (cotton, lupin) impact on the growth, colony and hyphal morphology and the proteome of *B. rouxiae* BRIP40192 (cotton isolate).

The successful colonisation of *A. brasilense* on plant roots is highly correlated with its ability to sense extracellular signalling molecules secreted by roots that trigger its movement, attachment and colonisation. Zhulin *et al.* (1988) mentioned that organic compounds, such as amino acids, saccharides, and organic acids, released by plant roots had a positive effect on stimulating chemotaxis in *A. brasilense. A. brasilense* Ab-V5 has also been shown to colonise maize in response to exudates from the roots of seedlings (Barbosa *et al.*, 2020). O'Neal *et al.* (2020) revealed that chemotaxis, flocculation, and root colonisation of *Triticum aestivum* (wheat) and *Medicago sativa* (alfalfa) roots by *A. brasilense* is under the control of metabolic attractants in the host plant rhizosphere. In considering *Azospirillum* as a potential biological control agent for the soil-borne BRR pathogen, it is important to consider these plant-microbe

interactions, and the impact of root exudates, as these factors could influence the ability of the bacteria to not only survive in the rhizosphere, but also to be attracted to areas of the rhizosphere where they may be effective in impacting pathogen growth.

1.9. General aims of this study

While BRR, caused by *Berkeleyomyces* spp., poses a significant challenge to Australian cotton producers, there are still many gaps in our understanding of this pathogen and the factors that influence its distribution and the severity of infection. Currently we have little information about how various farm management practices, including use of fertilisers and crop rotation, impact on the abundance of *Berkeleyomyces* spp. in soils. We also have little understanding of whether different cotton cultivars have more or less resistance to BRR disease, and thus whether changing cultivars could be one strategy to limit yield loss. To assess the impact of these different management strategies we need to ensure that we are using appropriate methods to assess pathogen abundance, and determine if there is a direct correlation between pathogen load in soil and disease severity. The identification of biological control agents against soil-borne pathogens, such as *Berkeleyomyces* spp., could assist in reducing economic losses faced by producers. Plant growth promoting bacteria such as *Azospirillum* have shown potential as biological control agents, however we do not yet know if these bacteria could be effective against *Berkeleyomyces* spp., and if they are, the mechanisms by which that control may take place.

Therefore, to address these gaps in knowledge, the general aims of this thesis are:

1. To use molecular tools to detect *Berkeleyomyces* spp. in soils from Australian cotton farms and assess the impact that soil physiological properties and farm management practices have on pathogen abundance (Chapter 2)

2. To investigate how growth temperature and cotton cultivar influence soil pathogen load and the severity of black root rot disease (Chapter 3)

3. To assess the potential for *Azospirillum brasilense* to act as a biological control agent for black root rot disease (Chapter 4)

4. To investigate the role of border cells in the plant-microbe interactions taking place between cotton, *A. brasilense*, and *Berkeleyomyces* spp. (Chapter 5).

Chapter 2: Effect of soil physiochemical, crop rotation and fertilisation on the abundance of *Berkeleyomyces* spp. in cotton farms

2.1. Introduction

Soil-borne fungal pathogens have a significant impact on plant growth in both natural habitats and in agricultural contexts (Otten and Gilligan, 2006). Black root rot (BRR) disease is one soil-borne disease that affects a number of commercially important crop species, including cotton, and is distributed globally. The causative agents are *Berkeleyomyces basicola* and *Berkeleyomyces rouxiae* (previously known collectively as *Thielaviopsis basicola*), which can infect a wide range of plant species (Nel *et al.*, 2018). In Australian cotton production, BRR is a serious threat to cotton growers due to its impact on plant establishment, resulting in yield loss. Since it was first reported in Australia over three decades ago, the pathogen has rapidly spread, resulting in significant losses for cotton producers (Nel *et al.*, 2019; Pereg, 2013).

A better understanding of the complexity of plant-pathogen-environment interactions could provide the basis to control infection, distribution and severity of BRR disease. *Berkeleyomyces* spp. can be easily distributed in soil via water movement, machinery, vehicles, animals and insects (Schippers, 1970; Wick and Moore, 1983). The pathogen can persist in the soil long-term via the production of spores, meaning that eradicating the pathogen is difficult once it becomes established in a soil. While *Berkeleyomyces* spp. spread easily and are now widely distributed in Australia, we do not yet have a complete understanding of the factors that determine whether the

pathogen will persist in soils, and why it may be more or less abundant in some soils compared to others.

Soil chemical factors appear to have a significant impact on the severity of BRR disease (Almario *et al.*, 2014; Meyer and Shew, 1991a, 1991b; Meyer *et al.*, 1989). For instance, release of high level of ammonia during the degradation of organic matter has been suggested to be toxic to the BRR pathogen (Allen, 2001; Candole and Rothrock, 1998). High levels of nitrogen and phosphate have also been linked to the suppression of fungal pathogens (Delgado *et al.*, 2005), specifically, the availability of these elements has been shown to enhance the development of cotton during the seedling stage, in the point at which it is most susceptible to infection (Bednarz *et al.*, 2000). Low availability of iron has also been linked to a reduced incidence of BRR disease (Ramette *et al.*, 2003). The level of these nutrients and other soil parameters can be influenced by farming practices, including the use of different fertilisers. Different types of soil amendments, such as poultry manure, bone meal and soymeal have been used to suppress soil-borne pathogen (Lazarovits, 2001). For example, Papavizas and Adams (1969) revealed that applying fatty acid, soybean protein, yeast extract, chestnut tannin, zein and lecithin as an organic soil amendmnet might inhibit *Berkeleyomyces* spp. by reduction the survival of spores in soil. Crop rotation has also been suggested as a potential method to reduce losses due to BRR disease. Crop rotation with non-host species such as corn or grain sorghum (Abawi and Widmer, 2000), winter cereal such as wheat (Hulugalle and Scott, 2008) have been shown to reduce the incidence of BRR disease. While these factors (soil chemistry, use of different fertilisers and crop rotation) are all suggested to have an impact on either pathogen abundance or disease severity, we do not yet have a good understanding of the interplay between these factors, and how they may differ across geographic regions. Therefore, there is a need

to further investigate these factors, and the link between pathogen abundance and disease severity, in the context of Australian cotton farms.

Investigation of the pathogen in the soil before, during and after planting, and during disease management, represents an important tool in mitigating yield losses due to disease (Huang and Kang, 2010). Historically *Berkeleyomyces* spp. have been detected in soil using a semi-selective medium to isolate and identify the fungus (Nehl *et al.*, 2004; Specht and Griffin, 1985). A carrot slice method may also be used (Heller, 2012). These methods are time consuming and labour intensive. More recently, molecular tools in the form of conventional polymerase chain reaction (PCR) techniques have been used to detect pathogens, with the aid of pathogen specific primers (Thalmann, 2008; Zhao *et al.*, 2009). Conventional PCR gives an indication of the presence or absence of a pathogen in a specific sample, while quantitative PCR (qPCR) represents a reliable method of determining the relative pathogen load in different samples. These techniques provide rapid, sensitive and relatively simple methods, requiring only 4-5 hr, to detect and quantify BRR pathogens in agricultural samples (Huang and Kang, 2010; Nakane and Usami, 2020). While species-specific primers have been developed for *Berkeleyomyces* spp., until now there is no literature reporting the use of these molecular tools to investigate the abundance of *Berkeleyomyces* spp. in Australian soils.

The aim of this study was to develop a better understanding of the factors that may influence the distribution and abundance of *Berkeleyomyces* species in Australian cotton soils. The influence that different long-term farm management practices (specifically the use of organic versus mineral fertilisation) and crop rotation may have on the abundance of *Berkeleyomyces* spp. was investigated using qPCR. The abundance of this soil-borne pathogen was then examined in relation to the soil physiochemical

parameters of the different sampling sites, to develop a deeper understanding of how environmental factors, such as soil chemistry, may impact on the pathogen abundance, and therefore severity of disease.

2.2. Methodology

2.2.1. Study site (cotton fields)

Cotton growing areas of New South Wales (NSW) and Queensland (QLD) extend between the southern latitudes of 23° 30’ and 32° 30’: http://www.dpi.nsw.gov.au/land-and-water/soils/guides/soilpak-series/soilpak.This study was conducted in crop production fields across the cotton-growing belt of eastern, Australia, including regions of New South Wales (NSW) and Queensland (QLD). The soil in this region is mostly characterised as vertisol, the crops are furrow irrigated, and sewing is in elevated rows. Five locations were selected based on a north-south distribution across the region, and that allowed a comparison of fertiliser and crop rotational practices. The selected farm sites were located in Theodore, QLD (farms B and C), Boggabri (farm Q) and Breeza (farm P3) in northern-central NSW, and Carrathool (farm T) in central-southern NSW. Information about crops and production practices including region, crop rotation, and yield from each farm is given in Table 2.1, and climate information is summarised in Table 2.2.

Table 2.1: General information on the 2014/15 cotton seasons from farms participating in this study

Farm	B	C	Q	P3	T
Region	Theodore Qld	Theodore Qld	Boggabri NSW	Breeza NSW	Carrathool NSW
Soil type	Heavy black cracking clay	Light clay with hard setting properties	Brown/Red	Black Self Mulching cracking clay of basalt origin	Clay to clay loam
Crop rotation	Cotton (No rotation)	Cotton (wheat)	Cotton (mung bean)	Cotton (wheat)	Cotton (wheat)
Fertilisation	Urea at 500 kg/ha; Rock Phosphate at 300 kg/ha; NTS Stabilized Boron at 25kg/ha	**Seasonal compost at 10 t/ha) pre-planting;** Urea at 350 kg/ha; Previous year: Muriate of Potash (41% K; 18% S) at 150kg/ha	Cotton Sustain (6.1% N; 12% P; 22.5% K; 2.2% S; 0.55% Zink) @ 80kg per ha; 160 kg Big N (82% ammonia N) per ha	Urea-Ammonium Nitrate solution (170KgN/ha); Phosphate (22kg P/ha)	**Seasonal manure at 5 t/ha pre-planting;** 250kgs/ha cotton starter; 535- 600 kg urea Foliar; 5kg/ha Nutrivant cotton (urea 4.1%, nitrate 4.9%; P 9%; K 37%; Mg 2%; Fe 0.2%; Mn 0.1%; B 0.05%; Cu 0.05%; Mo 0.02%; Zn 0.1%)
Cotton yield range	10-12.5 tone/ha	6.3-11.8 bales/ha	10 bales/ha	8.2-9.9 bale/ha	9.5-11.7 bale/ha

Table 2.2: Climate information for the five locations used in this study (sourced from the Australian Government Bureau of Meteorology (BoM)).

Location	Temperature (°C)	Rainfall (mm)	BoM Temp/humidity	Climate [a]
Theodore, QLD	Mean: 21.15. Mean max: 28.4 Mean min: 13.9 Hottest month: Dec (19.3 min to 33.5 max). Coldest month: July (6.2 min to 21.6 max).	Annual av.: 734 Max: Feb (av. 105.6), Min: Sep (av. 26.3)	Hot, humid summer	BSh Semi-arid, low latitude
Breeza, NSW	Mean: 18.4 Mean max: 24.6 Mean min: 12.2 Hottest month: Jan (18.9 min to 32 max). Coldest month: July (4.8 min to 16.1 max).	Annual av.: 638 Max: Jan (av. 83.7), Min: August (av. 35.6)	Warm summer, cold winter	Cfa Warm oceanic/humid subtropical
Boggabri, NSW	Mean: 18.15. Mean max: 26.1 Mean min: 10.2 Hottest month: Jan (18.3 min to 34.3 max). Coldest month: July (2.1 min to 17.1 max).	Annual av.: 557 Max: Dec (av. 81.5), Min: April (av. 21.6)	Hot dry summer, cold winter	Cfa Warm oceanic /humid subtropical
Carrathool, NSW	Mean: 17. Mean max: 23.9 Hottest month: Jan (17.1 min to 33 max). Coldest month: July (3.5 min to 14.5 max).	Annual av.: 402.2 Max: Oct (av. 38), Min: April (av. 27.6)	Hot dry summer, cold winter	BSk Semi arid, middle latitude

[a] Climate data taken from nearest available recording site (Source: http://www.bom.gov.au/climate/data/stations/) (Koppen-Geiger system).

2.2.2. Soil sampling

Soil samples were collected in 2014, prior to planting in early to mid-spring. Samples were extracted from the soil, from the highest points along elevated rows, using metal corers of 22 mm in diameter and 15 cm deep. Corers were washed and

sterilised with 70% ethanol between samples. To overcome difficulties in extracting soil, which is challenging when dealing with vertisols, a window was cut at one side of each corer. In each sampling round a total of 10 soil samples were collected from a 1 ha plot on each farm. Each sample consisted of ten subsamples, taken along a 100 m section of an elevated row, with a distance of 10 m between subsamples, which were combined to create one sample. The sampled rows were separated from each other by 10 m.

Samples taken along each row (10 samples) were pooled and mixed thoroughly immediately in the farm using a pestle and mortar that were pre-washed with 70% ethanol. Sub-samples for DNA extractions were frozen at -20°C in a field freezer until returned to the laboratory (within 12-48 h), where they were frozen at -80°C. Sub-samples for soil property analyses were collected and stored at 4-16°C.

2.2.3. DNA extraction from soil samples

Soil samples were crushed, ground and homogenised using a mortar and pestle to obtain a homogenous powder. Total DNA was isolated from 0.25 g of each sample using a PowerSoil DNA isolation Kit (MO BIO, Carlsbad, USA), according to the manufacturer's instructions, with the following minor modifications: soil samples were vortexed vigorously at maximum setting (Genie2, Scientific industries, Inc., USA) for 30 minutes, and DNA was eluted in 65 µl of elution buffer, instead of 100 µl. The extracted DNA was stored at -20°C until use.

Genomic DNA quantity and purity were assessed using a Nanodrop ND1000 (Thermo Fisher scientific, USA). Two microliters of each DNA sample were dispensed on the Nanodrop sensors and absorbance was measured at 260/280 nm and 260/230 nm to detect DNA purity, with 260/280 ratios (no protein contamination) of ~1.8, and

260/230 ratios (unwanted organic contamination) between 2 and 2.2 consider to be acceptable. These DNA extraction methods have previously been tested on soil samples spiked with known concentrations of *B. rouxiae* to ensure that DNA can be recovered from different soil types with equivalent efficiency, and that no PCR inhibitors which would impact on the downsteam assays are present in the purified DNA (Dr. M. McMillan, personal communication).

Quality of extracted DNA was also assessed by agarose gel electrophoresis, by loading on a 1.5% agarose gel in TBE buffer (108 g Tris-base, 55 g boric acids, and 9.3 g EDTA per litre, pH 8.2-8.4), and run at 100 V for one hour, against a 1Kb DNA ladder (genomic DNA) or 100bp DNA ladder (PCR products). Gels were stained with GelRed DNA stain (Biotium) and visualized under UV light on a GelDoc system (Biorad).

2.2.4. Cloning of *Berkeleyomyces* spp. ITS region

A segment of the *Berkeleyomyces* spp. internal transcribed spacer (ITS) region was amplified by PCR using the *Berkeleyomyces* spp. specific primers designed by Huang and Kang (2010): Tb1 5' TATTCATTGCTGAGTGGC 3' and Tb2: 5' GGTTTTCCGGCATGTTAT 3'. NCBI BLAST alignments were performed to ensure that these primers are able to detect both *Berkeleyomyces* spp (*B. rouxiae* and *B. basicola).* Each PCR contained 12.5 µl 2X TopTaq Mastermix (Bioline), 1 µl each of forward and reverse primers (final concentration of 0.4 µM), and 1 µl of template DNA, in a final volume of 25 µl. The cycling conditions used are illustrated in Table 2.3.

Table 2.3: PCR cycling conditions for the amplification of the *Berkeleyomyces* spp. ITS region.

Step	Temperature	Time	Number of cycles
Initial denaturation	95°C	5 minutes	35 cycles
Denaturation	95°C	1 minute	
Annealing	54°C	1.5 minutes	
Extension	72°C	1 minute	
Final extension	72°C	5 minutes	

The resulting 330bp PCR fragment was cleaned using a Wizard SV PCR clean up system (Promega) and cloned into a pGEM-T vector using the pGEM-T Easy Vector System (Promega, WI, USA). Cloning was carried out according to the manufacturer's instructions, using the recommended ratio of 3:1 insert: vector DNA. A single white bacterial colony with carrying target DNA (confirmed by colony PCR) was used to inoculate 5 ml of LB broth containing 50 µg/ml ampicillin, and incubated at 37°C for 24 hrs with shaking. Plasmid DNA was extracted using a Qiagen Miniprep kit (Qiagen, Germantown, MD, USA), following the manufacturer's instructions. Plasmid DNA was stored at -20°C until further use.

Plasmids were double-checked for correct inserts using PCR, as described above. The quantity and purity of extracted plasmid was assessed using a Nanodrop ND1000 (Thermo fisher scientific, USA) as described in section 2.2.5. Quality and size of extracted plasmids and/or PCR product was also assessed by electrophoresis using agarose (1.5%) as described in section 2.2.5. A serial dilution (10^1-10^7 gene copies per reaction) of plasmid standard containing the gene of interest was prepared for further studies.

2.2.5. Quantitative PCR

Quantitative PCR analysis was carried out using a CFX96 Touch Real-Time PCR system (Bio-Rad Laboratories, CA, USA). The qPCR reaction mixture comprised 10 µl of SensiFastTN SYBR® No-ROX (Bio-line Reagent, Ltd, USA), 0.8 µl each of forward and reverse primers (final concentration of 400 nM),1 µl of template DNA, and RNase/DNase-free water to a final volume of 20 µl. Each sample was analysed in duplicate against a set of plasmid standards containing 10^1 - 10^7 copies of the *Berkeleyomyces* ITS region (constructed as described in section 2.2.4), and no template (negative) controls were included in each assay. Melting curve and gel electrophoretic analysis were performed to confirm the specificity and the appropriate size of amplified product. The cycling conditions used in this study are illustrated in Table 2.4.

Table 2.4: Cycling conditions for qPCR

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	94°C	5 minutes	
Denaturation	94°C	10 seconds	40 cycles
Annealing	54°C	20 seconds	
Extension	72°C	10 seconds	
Final extension	84°C	20 seconds	
Melting curve: from 55-95°C with 0.5°C steps, 5 seconds for each step.			

Quantitative PCR using the *Berkeleyomyces* spp. ITS region primers resulted in a unique melting curve, with a single band on gel electrophoresis at the expected size of 330bp, as illustrated in Figure 2.1 A and B.

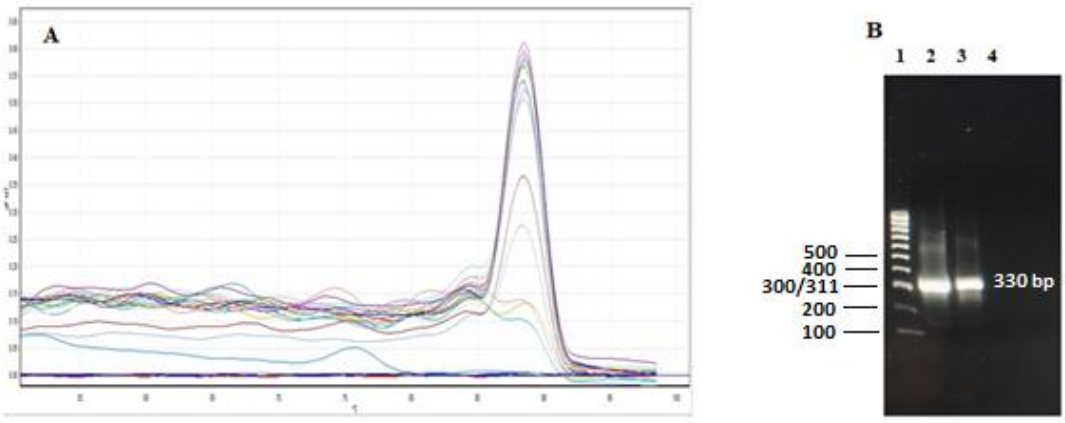

Figure 2.1: (A) Melt curve analysis of qPCR using primer sets of *Berkeleyomyces* spp. (B) Agarose (1.5%) gel electrophoresis of qPCR products from plasmid containing cloned ITS sequence: Lane 1: 100bp DNA ladder; Lane 2, 3: tested plasmids; 4: water control (NTC). Product size: 330bp.

Plasmid standards containing the cloned ITS fragment gave a standard curve with $r^2 > 0.93$ for seven orders of magnitude, ranging from 10^1 to 10^7 copies per reaction, with a high efficiency (0.96%), as illustrated in Figure 2.2.

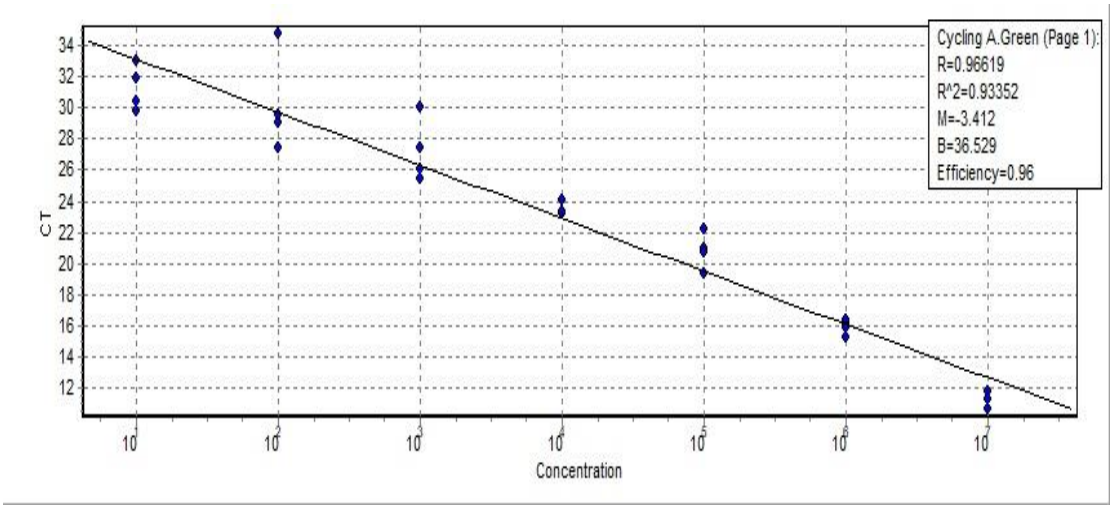

Figure 2.2: Standard curve of qPCR using 10-fold serial dilutions of plasmids containing a region of the *Berkeleyomyces* spp. ITS region

2.2.6. Physical-chemical parameters of cotton soil fields

Soil analysis was performed by A. Coronado and F. Garcia-Orenes, from the University of Miguel Hernandez, Spain. In brief, field-moist soil samples were sieved at 2 mm and stored at room temperature to conduct the physicochemical analysis. Soil organic compound (OC%) was determined as described by Walkley and Black (1934), available K was extracted with 1N ammonium acetate (Knudsen *et al.*, 1982), and Fe, Cu, Zn and Mn were extracted with DTPA (diethylenetriaminepentaacetic acid) (Lindsay and Norvell, 1978) and measured by atomic absorption and emission spectrophotometry by using the absorption of optical radiation (light) by free atoms in the gaseous state. Total nitrogen (N%) was determined by the Kjeldahl method (Bremmer and Mulvaney, 1982). Available phosphorus (AP) was determined by the Burriel-Hernando method (Díez, 1982). Inorganic N (NO_3^- and NH_4^+) was extracted with 2 M KCl (Keeney and Nelson, 1982) and determined by spectrophotometric method.

2.2.7. Data analysis

Gene copy numbers determined by qPCR were analysed using R (version 4) (R core team, 2017). A one-way analysis of variance (ANOVA) was used to test whether there was a significant difference in *Berkeleyomyces* spp. abundance between soil sites, depending on the target sequence abundance data. Differences between farms were compared by Tukey's post-hoc method at the probability level of 0.05. The correlation between the abundance of DNA target sequence with the physiochemical parameters of soils was assessed using Pearson's product correlation (R).

The relationships between the abundance of *Berkeleyomyces* spp. target sequence between farms, as well as between target sequence abundance and soil physiochemical parameters were analysed by principal component analysis (PCA) with Varimax normalized rotation, keeping the number of cases higher than the number of variables (variables ≤N-1) to avoid over parameterisation.

2.3. Results

2.3.1. Abundance of *Berkeleyomyces* spp. in soil

The overall abundance of *Berkeleyomyces* spp. varied by sampling location, although the abundance was not significantly different between farms B, P3 and Q, as illustrated in Figure 2.3. When different fertilisation strategies were considered, in general, soil from cotton farms treated with organic fertilisers showed a higher abundance of *Berkeleyomyces* spp. when compared with soil from farms under mineral fertilisation. This was particularly true of soil from farm T (amended with manure) compared to farm C (amended with compost), where the average copy number of target

sequence was 1.32 x10^8 gene copy no. g^{-1} dry soil for farm T and 6.09 x10^7 gene copy no. g^{-1} dry soil in farm C. Among the farms using mineral fertilisation, farm P3 showed a slightly higher abundance of pathogen, compared with farms B and Q (1.90 x10^7, 8.51 x10^6 and 6.41 x10^6 gene copy no. g^{-1} dry soil, respectively), however this difference was not statistically different.

When taking into account crop rotation used on the farms in this study, farms which rotated cotton with wheat (farms C and T and P3) showed a higher abundance of *Berkeleyomyces* spp. DNA than those rotating with mung bean or growing cotton only. However, the abundance did vary greatly between these farms, and in farm P3 was not significantly higher than that of farm B (cotton monocrop) and farm Q (rotation with mung bean).

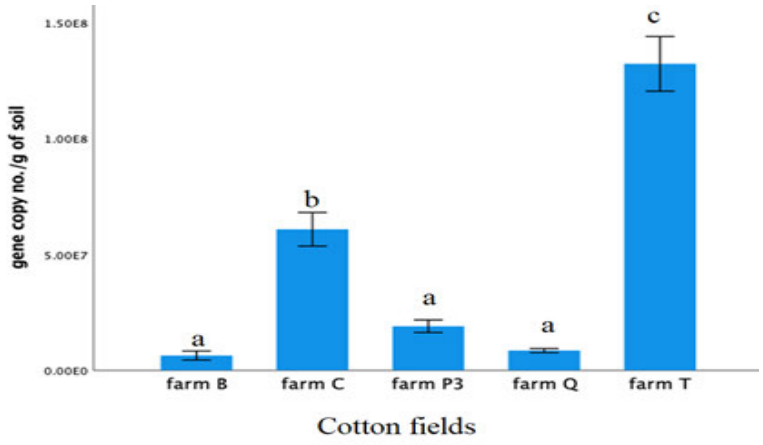

Figure 2.3: Mean copy number (error bars indicate 1 standard deviation) of the *Berkeleyomyces* spp. ITS region per gram of soil for five cotton farms, n = 20 replicates for each farm. Letters above the bars indicate significant differences (one-way ANOVA, P<0.05) between farms. Error bars represent the standard deviation of the means.

2.3.2. Physiochemical parameters of soil

The results of the physiochemical characterization of soils are summarised in Table 2.5. Farms C and T used organic amendments (manure or compost), while farms

B, P3 and Q were under mineral fertilisation. The concentrations of NO_3^- were significantly higher in farms B, P3 and T than in farms C and Q. The percentage of organic compound (OC%) in soil was significantly higher in farm C, while farms T and P3 showed a similar level of OC%, and farms B and Q had the lowest OC%. The organically amended farms C and T contained significantly higher concentrations of Fe and Mn than farms B, Q and P3. There were no significant differences in N%, NH_4^+ concentrations or P% among farms, and K concentrations were similar in all farms with slightly higher concentrations in farm P3. Cu concentration varied across farms, with the highest concentration in farm B. Finally, the Zn showed similar concentrations across all farms, but was slightly higher in farm C.

Table 2.5: Chemical properties of cotton soils.

Farms		N%	NH_4 ppm	NO_3 ppm	OC%	P%	Fe ppm	Cu ppm	Mn ppm	Zn ppm	K%
Organically fertilised farms	**Farm C**	0.094 ^{ns}	92.679 ^{ns}	265.199 ^{a}	3.090^{d}	0.003 ^{ns}	10.320^{b}	1.389 ^{a}	15.143 ^{b}	6.259 ^{c}	381.074 ^{a}
	Farm T	0.118 ^{ns}	91.188 ^{ns}	514.475 ^{c}	2.150^{c}	0.004 ^{ns}	7.788 ^{c}	1.746 ^{ab}	17.040 ^{b}	2.742 ^{ab}	421.520 ^{a}
Mineral fertilised farms	**Farm B**	0.104^{ns}	89.421 ^{ns}	449.200 ^{bc}	1.547^{a}	0.005 ^{ns}	1.085 ^{a}	2.609 ^{c}	3.291 ^{a}	4.023 ^{bc}	542.614 ^{ab}
	Farm P3	0.090 ^{ns}	96.792 ^{ns}	414.685 ^{b}	2.066^{bc}	0.003 ^{ns}	0.467 ^{a}	1.577 ^{ab}	1.463 ^{a}	2.513 ^{ab}	702.201 ^{b}
	Farm Q	0.104 ^{ns}	95.971 ^{ns}	288.757 ^{a}	1.638^{ab}	0.006 ^{ns}	3.271 ^{a}	2.262 ^{bc}	4.690 ^{a}	1.674 ^{ab}	345.159 ^{a}

Mean values of ten soil samples for each farm are presented. Superscript letters indicate significant differences (one-way ANOVA, P, 0.05) between farms: N, available nitrogen; OC, organic compound; P, available phosphorous; K, potassium; Fe, iron; NH_4^- ,ammonium; NO_3^- , nitrate; Mn, manganese; Cu, copper; Zn, zinc.

***ns: non-significant**

2.3.3. Correlation between abundance of *Berkeleyomyces* ITS sequence and soil physiochemical parameters

To understand how soil properties may influence the distribution or abundance of *Berkeleyomyces* spp. a Pearson's product correlation was performed. The results are illustrated in Table 2.6.

Table 2.6: r-values for correlation between physiochemical parameters of cotton fields and *Berkeleyomyces* spp. ITS region copy number. N, available nitrogen; OC, organic compound; P, available phosphorous; Fe, iron; NH_4^-,ammonium; NO_3^- , nitrate; Mn, manganese; Cu, copper; Zn, zinc, gray blocks: non-significant.

Soil physicochemical parameters	Organic fertilised farms		Mineral fertilised farms		
	farm C	farm T	farm B	farm P3	farm Q
N (%)	-0.64	**0.82**	0.52	**-0.83**	-0.46
OC %	**0.85**	-0.52	0.15	**-0.77**	**-0.77**
P%	-0.01	-0.34	**0.74**	-0.20	**0.89**
Fe ppm	**0.75**	**0.87**	**-0.93**	**-0.85**	0.22
Cu ppm	-0.18	0.54	**0.73**	**-0.84**	**0.89**
Mn ppm	**0.77**	**0.92**	-0.62	**-0.88**	0.41
Zn ppm	**0.86**	-0.69	0.11	-0.61	-0.69
NO_3 ppm	**-0.72**	**0.70**	0.67	**-0.85**	**0.92**
NH_4 ppm	0.28	-0.04	-0.38	**0.86**	0.41
K%	-0.01	**-0.64**	**-0.99**	**0.89**	-0.66

In farm C, there was a strong positive correlation between ITS copy number and OC%, and Zn (r = 0.85 and 0.86, respectively) and a moderate positive correlation with Mn and Fe (r = 077, 0.75). Meanwhile there was a moderate negative correlation between ITS copy number with NO_3^- (r = -0.72, respectively). In farm T, a very strong

positive correlation was observed between ITS copy number and the concentration of Mn (r = 0.92), and a strong correlation with N% and Fe (r = 0.82, and 0.87, respectively). Also, a moderate positive correlation was observed between ITS copy number with NO_3^- (r = 0.70). In contrast, there was a weak negative correlation between K% and ITS copy number (r = -0.64). For Farm B, a moderate positive correlation was observed between ITS copy number and P% and Cu (r = 0.74, and 0.73, respectively), and a very strong negative correlation with Fe and K% (r = -0.93 and -0.99, respectively). In farm P3, there was a strong positive correlation of ITS copy number with NH_4^+ and K% (r = 0.86, and 0.89, respectively), and a strong negative correlation with N%, Fe, Cu, Mn, and NO_3^- (r = - 0.83, -0.85, -0.84, -0.88, and -0.85, respectively). For farm Q, a very strong positive correlation was observed between ITS copy number and NO_3 (r = 0.92). Also, a strong positive correlation with P% and Cu (r = 0.89, for each) and a moderate negative correlation with OC% (r = -0.77).

A principal component analysis (PCA) was carried out to understand the relationships between soil parameters and *Berkeleyomyces* spp. abundance across the farm sites. The PCA scores and loading plots are shown in Figure 2.4. The first and second axes explained 41.23% and 29.13% of the cumulative variance respectively, with eigenvalues > 1. PC1 clearly separated the organically amended (farms T and C) from those under mineral fertilisation (farms B, P3 and Q). Farms B (QLD) and Q (NSW) were grouped at distance from farm P3 (Qld) and farm T (NSW) as located apart from farm C (NSW) (Figure 2.4). The second component accounted for 27% variance and separated the manure-treated farm T (NSW) from the compost-treated farm C (Qld) (Figure 2.4). Therefore, each farm was differentiated from each other, giving an indication that each cotton farm, whether under organic or mineral fertilisation, was distinguished from each other, suggesting each farm experiences its

own unique biological, physical and chemical correlation. When the abundance of *Berkeleyomyces* spp. ITS copy number was considered together with environmental factors it was observed that target sequence was accumulated around Mn, Fe and N%, which exhibited a higher abundance in farm T, which was treated with manure amendments (Figure 2.4).

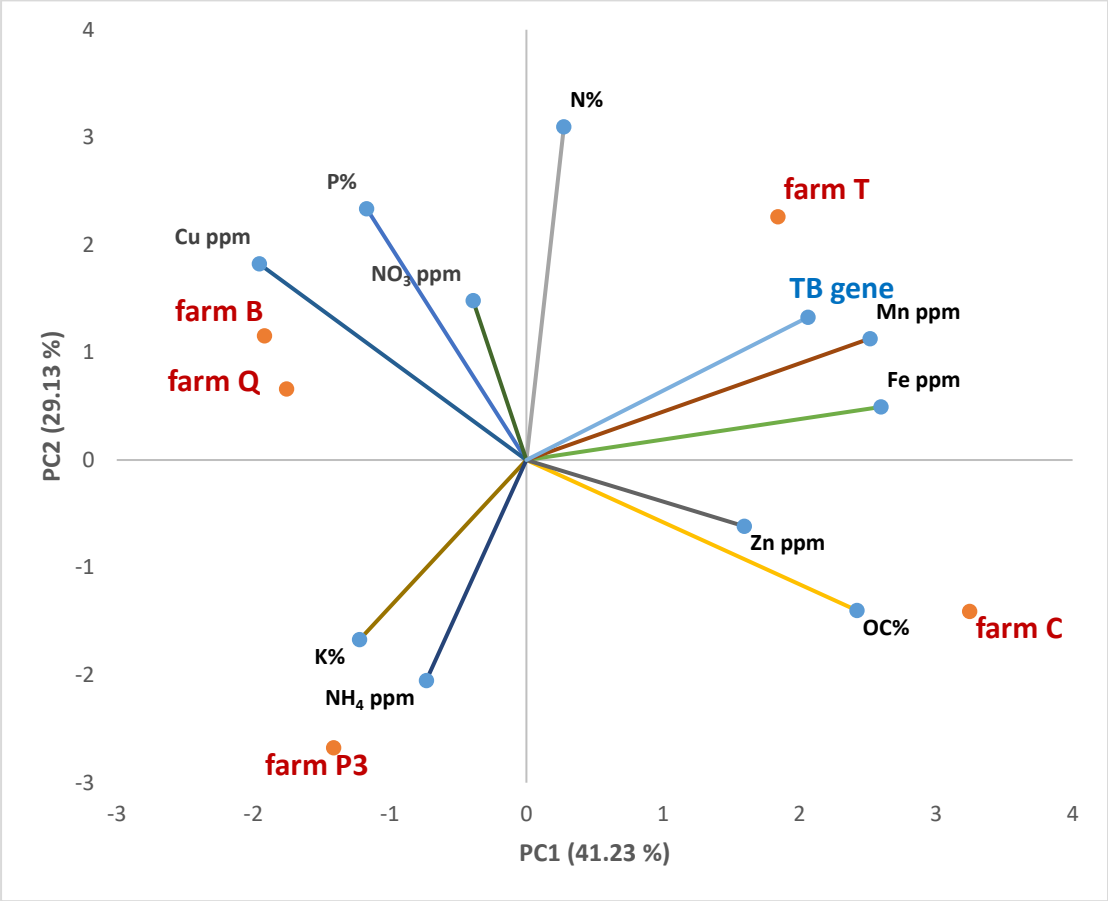

Figure 2.4: Plots from principal component analysis performed on all soil parameters from farms Q, B, T, P3 and C. OC: organic compound; N: available nitrogen; NO_3^-: Nitrate; NH_4^+: Ammonium; Av P: available phosphorus; K: potassium; Fe: iron; Mn: manganese; Cu: copper; Zn: zinc and TB gene: *Berkeleyomyces* spp. ITS region gene.

2.4. Discussion

Detection and quantification of fungal pathogens in soil represents the first step in the study of disease distribution and the monitoring of pathogen dynamics in response to management strategies. Molecular tools, particularly quantitative PCR, are widely considered as efficient and accurate methods to detect and identify fungal plant pathogens (François *et al.*, 2007; Gerhardt *et al.*, 2008; McCartney *et al.*, 2003; Paplomatas, 2004). Recently, the soil-borne fungal pathogens *Berkeleyomyces* spp. were detected using PCR (Thalmann, 2008; Zhao *et al.*, 2009) and qPCR (Huang and Kang, 2010). Consequently, qPCR was used here to assess the abundance of *Berkeleyomyces* spp. in Australian cotton production systems using different fertilisation and crop rotation practices.

Berkeleyomyces spp. were detected in all cotton field soils under study, at varying levels, which is unsurprising given that BRR is considered pandemic in Australia (Nehl *et al.*, 2004). The variable abundance of the BRR pathogen across farms may be attributed to a number of factors. One of these is differences in fertilisation practices. Soils from cotton farms under organic fertilisation showed a higher abundance of *Berkeleyomyces* spp. compared to those under mineral fertilisation. The addition of organic compounds to soils has been proposed to be an effective management option to help suppress disease, by stimulating the growth of beneficial microbes (fungi and bacteria) (Bonanomi *et al.*, 2006; Bonanomi *et al.*, 2017; Bonanomi *et al.*, 2018 Manici *et al.*, 2004; Mazzola *et al.*, 2001; Pérez-Piqueres *et al.*, 2006). The results of the present study are in contrast to these previous findings, with mixed results being observed in relation to pathogen abundance detected in soils and the levels of organic compounds in those soils. In some farms (such as the organically fertilised farm T), there was a significant positive correlation between

pathogen load and soil organic compounds, suggesting that increased organic compounds could increase, rather than decrease, disease. While in other farms (such as the inorganically fertilised farms P3 and Q) a negative correlation between soil organic compounds and pathogen load was observed. These results suggest that it would be incorrect to assume that the addition of organic compounds to soils will suppress pathogen load in all systems. However it should be noted that, in this study, only DNA abundance, and not disease severity, was measured. It is possible that these results only indicate a greater presence of the pathogen in the soil, which may not be directly proportional to disease incidence or severity. However, these results suggest that there are a range of factors that will influence the abundance of *Berkeleyomyces* spp. in soils, and that fertilisation, or use of organic amendments, is only one of these factors.

Berkeleyomyces spp. abundance has previously been shown to be influenced by soil physiochemical factors (Meyer and Shew, 1991a). Higher concentrations of nitrate could enhance the growth of *Berkeleyomyces* spp., due to nitrate levels affecting soil pH. Previous studies have shown that higher (alkaline) pH increases the severity of black rot root disease (Harrison and Shew, 2001; Meyer *et al.*, 1994). In the current study, abundance of the *Berkeleyomyces* spp. ITS sequence did not show any clear positive or negative correlation with nitrate levels across farms. These results are in disagreement with the previous finding of Harrison and Shew (2001), who demonstrated that increased nitrate levels increased the growth of *Berkeleyomyces* spp.

Iron (Fe) availability has been suggested to impact on severity of BRR disease. A previous study found Fe levels to be highly correlated with the appearance of black necrosis caused by *Berkeleyomyces* spp. from infested cotton soils (Delgado *et al.*,

2005). Our results showed that higher soil Fe concentration was strongly correlated with increased abundance of *Berkeleyomyces* spp. ITS region copy number in organically fertilised farms, but not in farms under mineral fertilisation. This has also been observed previously in tobacco fields, where the incidence of BRR disease was reduced in response to reduction in Fe concentration in soil (Ramette *et al.*, 2003). Accumulation of Fe in organic fertilised farms, rather than those using mineral fertilisers, could be attributed to the low availability of microorganisms that produce iron chelating agents, although this was not specifically investigated in this study. The role of microorganisms in producing iron chelating agents has been described in a number of studies (Laurie and Manthey, 1994; Uyanöz *et al.*, 2006; Yang *et al.*, 2004). Buysens *et al.* (1996) suggested that the production of iron chelating agent siderophores by *P. fluorescence* was associated with suppression of *Pythium*-induced damping off in tomatoes. Similar mechanisms could also be involved in the suppression of *Berkeleyomyces* spp. in cotton producing areas, with disease suppressed in areas where these microorganisms are abundant.

Organically fertilised farms exhibited a positive correlation between Mn and *Berkeleyomyces* spp. abundance in comparison with farms using mineral fertiliser. Other finding indicate that Mn is an essential element that handles the production and regulation of enzymes targeting lignin, helping the pathogen to infect the plant (Hofrichter, 2002; Perez and Jeffries, 1992; Posta *et al.*, 1994). Many studies observed that higher Mn availability may promote fungal communities better adapted to decompose lignin (Lindahl and Tunlid, 2015).

Crop rotation is considered as another factor that affects the abundance of *Berkeleyomyces* spp. in cotton fields (Pereg, 2013). The summer/winter wheat rotated cotton farms (Farm C and T) showed the highest abundance of *Berkeleyomyces* spp.

This may be attributed to the capability of wheat, a non-susceptible host for *Berkeleyomyces* spp., to enhance spore germination (Pereg, 2007), which leads to an increased pathogen burden in soil over time. On the other hand, Hulugalle *et al.* (1999) recommend wheat as a rotation crop for cotton under conditions where water is limited, to reduce the severity of BRR. Such conflicting results and recommendations reflect the complexities of interaction between soil, pathogen and plant. It may be that different crop rotations may be effective in different soil types or under different environmental conditions, or could be rotation with wheat may reduce the numbers of one species of *Berkeleyomyces* spp. and have no effect on the other.

The texture of soil also may affect the abundance of *Berkeleyomyces* spp., and its ability to cause widespread infection. Farms T and C in this study are characterised by light to medium clay loam compared with the other farms which had sandy soil to heavy black cracking soil. The higher abundance of *Berkeleyomyces* spp. in medium clay soil, compared with heavy or sandy soil, is in agreement with the previous findings of Nehl *et al.* (2004).

In conclusion, by using quantitative PCR, *Berkeleyomyces* spp. loading in cotton soil was evaluated alongside field fertilisation, rotation and soil physiochemical parameters. The use of organic matter, crop rotation, soil type and soil concentrations of iron and manganese had a relationship with the abundance of *Berkeleyomyces* spp., although this was not always consistent across all farms. While these correlations between pathogen abundance and soil parameters/farm management practices were observed, no one single factor could predict a higher pathogen load in soil. Instead , the abundance of *Berkeleyomyces* spp. in soils is likely to be influenced by the combination of a range of soil and environmental factors, which may be unique to each soil/growing site. The conclusions that can be drawn from this study are also

limited by a lack of information about the correlation by pathogen load in soil and disease severity. Given this, there is a need to further investigate whether there is a direct relationship between the abundance of pathogen identified in soils using techniques such as qPCR, and the incidence and severity of BRR disease.

Chapter 3: Effect of temperature and cotton cultivar on the growth and pathogenicity of *Berkeleyomyces rouxiae* BRIP40192

3.1. Introduction

Black root rot disease in cotton is responsible for significant seedling and yield losses in Australia, however it remains a difficult pathogen to control. One factor that has created challenges in studying this pathogen and developing methods for control is that the relationship between pathogen load and survival in soil and severity of the disease is not well understood (Nehl *et al.*, 2004; Pereg, 2013; Rothrock, 1992). *Berkeyomyces* spp. survive in soil for a long time via the production of a chlamydospores (Nehl *et al.*, 2004; Schippers, 1970), which can then germinate and cause infection under suitable environmental conditions. During the early growing season, Australian cotton farms affected by BRR disease see a reduction in the vigour of the cotton after emergence, and despite compensation there is a subsequent yield penalty (Pereg, 2013). The variance in the pathogenicity and viability of spores in soil has been attributed to the influence of physical factors either on pathogen itself, or on the host plant. Conditions that are unfavorable for plant growth may leave plants more susceptible to fungal attack. Conversely, conditions which are unfavorable for pathogen growth may limit infection (Smith, 1960).

Temperature has been considered as one of the main factors affecting the distribution and severity of BRR disease (David, 1978; Nehl *et al.*, 2004; Pereg, 2013; Reddy and Patrick, 1989; Smith, 1960). Under suitable environmental conditions, including cool temperatures, spores start to germinate and begin invading the tissue of susceptible host plants (Smith, 1960). This interaction between temperature and

infection of susceptible hosts has been demonstrated found an increase of BRR disease of cotton at 20°C compared with 28°C, after ten days of incubation with *Berkeleyomyces* spp. (Mauk and Hine, 1988). A number of studies have shown similar data. Blank *et al.* (1953) reported that cool temperature (26°C) increase the severity of BRR disease in cotton crops. Rothrock (1992) mentioned that severity of BRR disease was increased at soil temperature <24°C. A study conducted by Wheeler *et al.* (2000) demonstrated that effect of *Berkeleyomyces* spp. was most severe at soil temperature 20°C and 24°C, compared with 28°C. The greatest damage is caused by *Berkeleyomyces* spp. when soil temperatures are between 16 and 20°C. This range is favourable to pathogen growth, but not to plant growth (Mauk and Hine, 1988).

One potential strategy for reducing the incidence of BRR disease in cotton is the use of disease-resistant cultivars (Rimbaud *et al.*, 2018). This approach has been used in response to other cotton pathogens. For example, Wang et al. (1999) described variations in resistance/susceptibility to Fusarium wilt, caused by *Fusarium oxysporum,* between different cotton cultivars. However at present no commercial cultivar with BRR disease resistance has been developed. Native Australian cotton diploid *Gossypium* spp. and diploid *G. arboreum* (PI 1415) exhibit a different range of resistance toward BRR disease (Nehl, 1998; Wheeler *et al.*, 1999). *G. arboreum* and *G. herbaceum* variants showed a partial and high resistance to *Berkeleyomyces* spp. respectively (Wheeler and Gannaway, 2007). While these cultivars could potentially be used for crossbreeding to produce a BRR disease-resistant cultivar, this approach has its limitations. Crossbreeding between the diploid cultivar with commercial tetraploid is difficult, and producing a resistant cultivar in this way could lead to a cultivar that, although resistant to this disease, could show susceptibility to other cotton pathogens (Pereg, 2014). While a specific BRR-resistant strain of cotton

does not currently exist, it may be that some common commercial cultivars may differ in their resistance or susceptibility to disease. Understanding these variations could allow producers to take this into account when selecting cultivars, particularly in regions with a history of severe BRR disease.

The main aim of this chapter was to investigate, under laboratory conditions, the correlation between temperature and severity of BRR disease. More specifically, this chapter also aimed to assess disease resistance of different cotton cultivars under cooler and warmer conditions. Furthermore, the relationship between disease severity and pathogen loading in soil was examined. To achieve these aims, five Australian cotton cultivars were grown at different temperatures in the presence of *B. rouxiae* BRIP40192 (a cotton isolate). The percentage of BRR lesions was used as a measurement of disease severity, and qPCR was used to quantify pathogen loading on plant roots and in the rhizospheric soil.

3.2. Methodology

3.2.1. Experimental design

This experiment was designed to investigate the effect of cotton cultivars and growing temperature on the abundance of *B. rouxiae* BRIP40192. Five cotton cultivars were used and grown at three different temperatures (15, 20 and 25°C). The percentage of the root displaying lesions was scored as an estimation of disease severity, root morphology was assessed and shoot water content measured. Quantitative PCR was used to quantify *B. rouxiae* BRIP40192 attached to cotton seedling roots, and in the rhizosphere, by amplification of the ITS region, as described in section 2.2.5.

3.2.2. Soil sampling and preparation

Soil was collected from a commercial cotton farm in Breeza, NSW, Australia (31.25° S, 150.47° E). Breeza has an annual average temperature of 18.4°C and mean annual rainfall of 638 mm. The cropping system was a winter wheat (*Triticum aestivum* L.) and a mid-spring cotton (*Gossypium hirsutum*) rotation. Soil was collected in August 2016. A 15 cm long stainless-steel corer (approximately 2.2 cm diameter) was used to collect soil samples, and was sterilised with 70% ethanol between samplings. Collected soil was removed from the corer, and sieved through 2 mm mesh to remove plant residue, roots and stones. Each 1.5 kg of soil was packed into a sterile container for transport.

A mix of 50% soil (from Breeza, NSW) and 50% sand (from Tingha, NSW) was sterilised to destroy any endogenous *Berkeleyomyces* spores by autoclaving at 121°C for 20 min, three times, each 24 hrs apart. Success of soil sterilisation was assessed by culturing and PCR.

3.2.3. Culture conditions for fungal isolates

The fungal isolate used in this study was wild-type *B. rouxiae* BRIP40192 (isolated from cotton farms, and reclassified according to the new classification), provided by Jan Dean from the Department of Primary Industries, QLD government. Stock cultures of fungal isolates were maintained in glycerol (20%) and stored at -80°C, and sub-cultured every 6 months on 1/2 strength potato dextrose agar (1/2 PDA) containing 1.5% agar. Working cultures were maintained on 1/2 PDA (1.5% agar) and stored at 4°C until needed. Details of all culture media used throughout this thesis are included in Appendix A.

3.2.4. Inoculum preparation

Endoconidia from *B. rouxiae* BRIP40192 were obtained from 5 day old cultures grown on 1/2 PDA (2.2% agar) under a 12 hrs light/12 hrs dark cycle at 25°C, as described by Coumans *et al.* (2009). Spore suspensions were prepared by scraping mycelia from 1/2 PDA (2.2% agar) plates into sterile McCartney bottles containing 7 mL of sterile de-ionized water, and vortexing at high speed for 1 minute. The resulting suspensions were filtered through one layer of dry Miracloth (Calbiochem) (pre-soaked overnight in 70% ethanol and air-dried) to separate chlamydospores and mycelia from endoconidia. The number of endoconidia was estimated using a haemocytometer, and then adjusted with sterile deionized water a concentration of $1x10^7$ endoconidia/ml.

3.2.5. Cotton cultivars

The cotton cultivars used in this study are shown in Table 3.1. The commercial supplier subjected all seeds to standard quality control procedures.

Table 3.1: Cotton (*Gossypium hirsutum*) cultivars used in this study supplied by Cotton Seed Distributors, Australia

Cultivar	Description
Sicot 730	non-GM conventional cultivar
Sicot 71BRF	BRF is short for Bollgard3™ Roundup Ready Flex*
Sicot 74BRF	
Sicot 43BRF	
Sicot 714B3F	B3F for Bollgard3™ Roundup Ready Flex*

***** Bollgard3 ™ Monsanto: cotton cultivars were transgenic and expressed the *Bacillus thurengiensi*s based toxins Cry1ac, Cry2ab and Vip3A protein, while marketed by Monsanto as Roundup ready Flex™: herbicide tolerant trait.

3.2.6. Seed sterilisation

Cotton seeds were sterilised as described by Coumans *et al.* (2009) by soaking in sterile de-ionized water for 5 minutes, then soaking in sterilisation solution (4% bleach, 10% ethanol in sterile water) for 5 minutes. Following surface sterilisation, seeds were rinsed 8 times in sterile de-ionized water. Sterilised cotton seeds were allowed to germinate on yeast mannitol plates at 25°C for 2-3 days in dark.

3.2.7. *In vitro* soil pathogenicity assay of *B. rouxiae* BRIP40192 on cotton

The endoconidial suspension was added to the sterile sand: soil mix to give a final spore concentration of 2.5 x 10^3 endoconidial/g soil. Inoculated soil was placed into sterile 200 ml foam pots and mixed thoroughly. A small indentation was made near the surface of the soil and one germinated, sterilised cotton seed placed in the hole and covered. Samples were then incubated at 15°C, 20°C or 25°C, separately under a 12 hrs light/12 hrs dark cycle for 10 days. Every 2 days, 10 ml sterile distilled water was added. After 10 days, the pot was lain horizontally on a sterile plate. A sterile scalpel blade was used to cut each pot vertically (avoiding cutting the roots) and open it up. The root was tapped gently for 3-5 minutes inside a sterile Falcon tube (50 ml) until all the adherent soil were collected. The soil sample from each root was transferred to a sterile Eppendorf tube (2 ml) and stored at - 20°C. The root was washed by soaking in sterile distilled water to remove the remaining of residual soil, then transferred to a sterile Eppendorf tube (2 ml) and stored at - 20°C. The total length of the tap root, percentage of root affected by lesions, and root morphology (appearance of the tap root and presence of lateral root) were recorded. The wet weight of cotton seedlings (excluding root) was recorded, before drying at 80°C for three days and dry

weight recorded. Nine replicates were carried out for each cotton variety at each temperature, alongside unplanted soil as a negative control.

3.2.8. DNA extraction from soil and roots

DNA was extracted from soil samples using a PowerSoil DNA isolation Kit (MO BIO, Carlsbad, USA), as described previously in section 2.2.5.

Prior to DNA extraction, washed root samples (collected as described in section 3.2.7) were snap frozen in liquid nitrogen, in a pre-chilled pestle and mortar, then crushed to form a fine powder. The frozen powdered tissue was transferred into pre-chilled 2.0 ml Eppendorf tubes and stored at -20°C. Genomic DNA was extracted from 0.25 g of root samples separately using the DNA Power Soil Kit (Mo Bio, Garlsbad, USA), as described for soil samples.

Genomic DNA quantity and purity were assessed using a Nanodrop ND1000 (Thermo fisher scientific, USA), and agarose gel (1.5%) electrophoresis, as described previously in section 2.2.5.

3.2.9. Quantitative PCR

To estimate the number of *B. rouxiae* attached to the roots of cotton seedlings, and in the rhizospheric area, a segment of *B. rouxiae* ITS region was amplified by qPCR using *B. rouxiae* specific primers designed by Huang and Kang (2010): Tb1 5’ TATTCATTGCTGAGTGGC 3’ and Tb2 5’GGTTTTCCGGCATGTTAT 3’ in a total of 20 µl using a CFX96 Touch Real-Timer PCR detection system (Bio-Rad laboratories, CA, USA), as described in section 2.2.5, against a set of plasmid standards containing 10^1 to 10^7 copies of this sequence per reaction.

3.2.10. Data calculation

The percentage of the infected root affected by lesions was calculated using the following equation:

$$\textbf{Percentage of root lesion} = \frac{\textbf{length of infected spot}}{\textbf{length of whole root}} \textbf{ X 100}$$

Shoot water content was calculating using the following equation:

$$\textbf{Water content (WC)} = \frac{(\textbf{Fresh weight of shoot} - \textbf{dry weight of shoot})}{\textbf{Dry weight of shoot}}$$

The number of *B. rouxiae* BRIP40192 attached to cotton roots was estimated in two ways: ITS copy number per gram of root, and ITS copy number per cm of root.

Copy number per gram of root was calculated using the following equation:

Copy number per gram (X) = R x 65^a x 4^b where

R: ITS region copy number per 1 µl DNA, as determined by qPCR
a: as DNA was extracted in a total volume of 65 µl
b: as DNA was extracted from 0.25 g of crushed roots

To find the copy number of the ITS sequence in along the entire root (CR), the following equation was used:

CR = X x CW
Where X = copy number per gram and CW = weight of entire root

Target sequence copy number per gram of root = CR / weight of root

Target sequence copy number per cm of root = CR / total length of root

3.2.11. Statistical analysis

A one-way analysis of variance (ANOVA) was used to test for significant differences in abundance of *B. rouxiae* BRIP40192 between treatments. Differences between cultivars were compared by Tukey's Post-hoc method at probability level of 0.05. Statistical analyses were carried out using the software package R (version 4).

3.3. Results

3.3.1. The effect of *B. rouxiae* BRIP40192 on plant growth and development

The effect of *B. rouxiae* BRIP40192 on plant development of five cotton cultivars was assessed at 15°C, 20°C and 25°C. At 15°C the cotton seeds of all cultivars germinated, but did not grow further, so these samples were excluded from further analysis. This was believed to be due to 15°C representing the lower limits of temperature requirement for cotton development (Bange, 2020). The parameters analysed for the remaining samples were percentage of root affected by lesions, shoot water content and root morphology.

3.3.1.1. Root morphology

The appearance of lesions on the tap and lateral roots differed with temperature. As shown in Figure 3.1 at 20°C, cotton roots exhibited an absence of lateral roots in all cotton cultivars, and large areas of lesion covered the tap roots of those grown in the presence of *B. rouxiae*. At 25°C, all cotton cultivars showed development of some lateral roots, and those grown in the presence of *B. rouxiae* had

significant lesions which were located only on the primary tap root but not on lateral roots (as confirmed by microscopic examination).

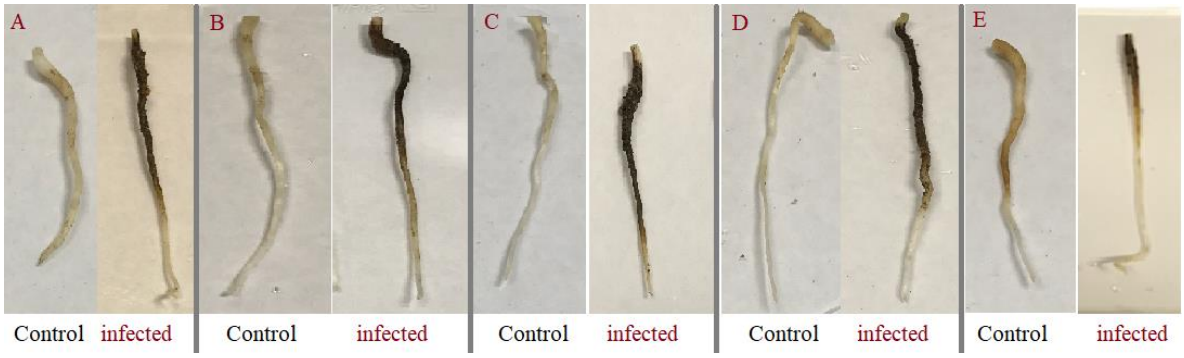

Figure 3.1: Appearance of cotton roots for cultivars grown at 20°C, for control plants and plants grown in the presence of *B. rouxiae* BRIP40192 (infected) (A: Sicot 74BRF, B: Sicot 730, C: Sicot 43 BRF, D: Sicot 71BRF and E: Sicot 714B3F)

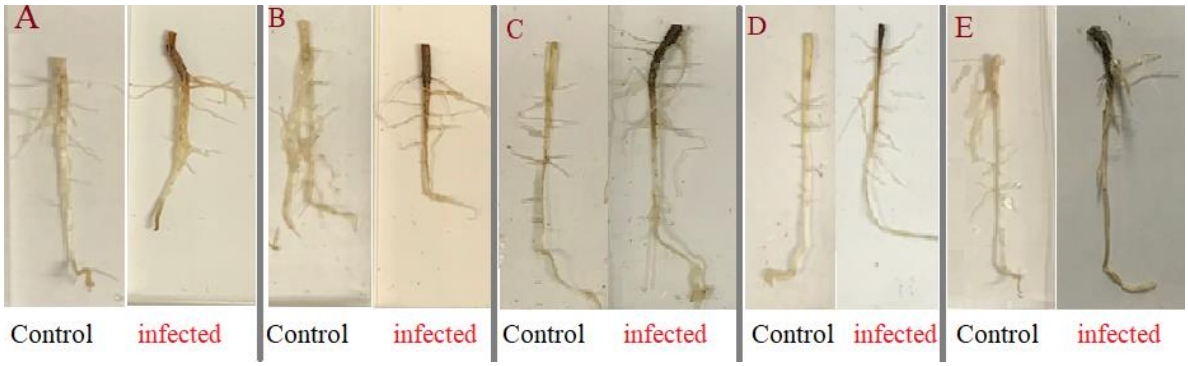

Figure 3.2: Appearance of cotton roots for cultivars grown at 25°C, for control plants and plants grown in the presence of *B. rouxiae* BRIP40192 (infected) (A: Sicot 74BRF, B: Sicot 730, C: Sicot 43BRF, D: Sicot 71BRF and E: Sicot 714B3F)

3.3.1.2. Percentage of lesion

The percentage of roots affected by lesions is used as an estimate of BRR disease severity, as lesions can easily be assessed by the examination of dark coloured spots on cotton roots, and can be confirmed by microscopic examination.

At 25°C there was no significant difference between cotton cultivars ($P<0.05$, Figure 3.3) for the percentage of root affected by lesions. Sicot 71BRF had a slightly higher percentage of the root affected, at 36%, compared with Sicot 730, Sicot 43BRF,

Sicot 74BRF and Sicot 714B3F where the percentage of root affected by lesions was 23%, 31%, 29% and 24%, respectively. Similarly, the percentage of root affected by lesions at 20°C was not significantly different between the cotton cultivars, with Sicot 74BRF having the highest percentage of root affected, at 67%, followed by Sicot 43BRF (63%), Sicot 71BRF and Sicot 714B3F (55% and 50% respectively), while Sicot 730 was the least affected, at 44%.

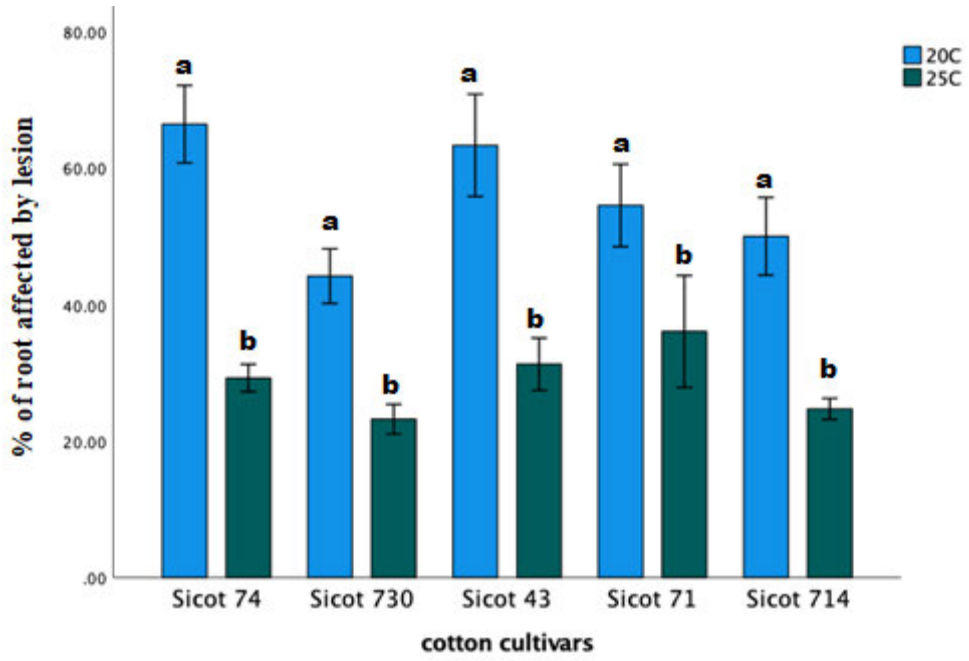

Figure 3.3: Percentage of root affected by lesions from *B. rouxiae* BRIP40192 for cotton cultivars Sicot 71BRF, Sicot 730, Sicot 43BRF, Sicot 74BRF and Sicot 714B3F, at 20°C and 25°C. Each bar represents the mean ± 1 standard deviation for n = 9 for each cotton cultivar. Letters above the bars indicate significant differences (one-way ANOVA, P<0.05) between treatments.

3.3.1.3. Shoot Water content

The results of water content for infected and control plants at 20°C and 25°C is shown in Figure 3.4. At 25°C there were no significant differences between the cotton cultivars, with all cotton cultivars exhibiting similar water content status in both control and infected plants. At 20°C, there was a significant loss of water content when

comparing infected plants with controls for each cotton cultivar. However, no significant difference was observed between the individual cotton cultivars.

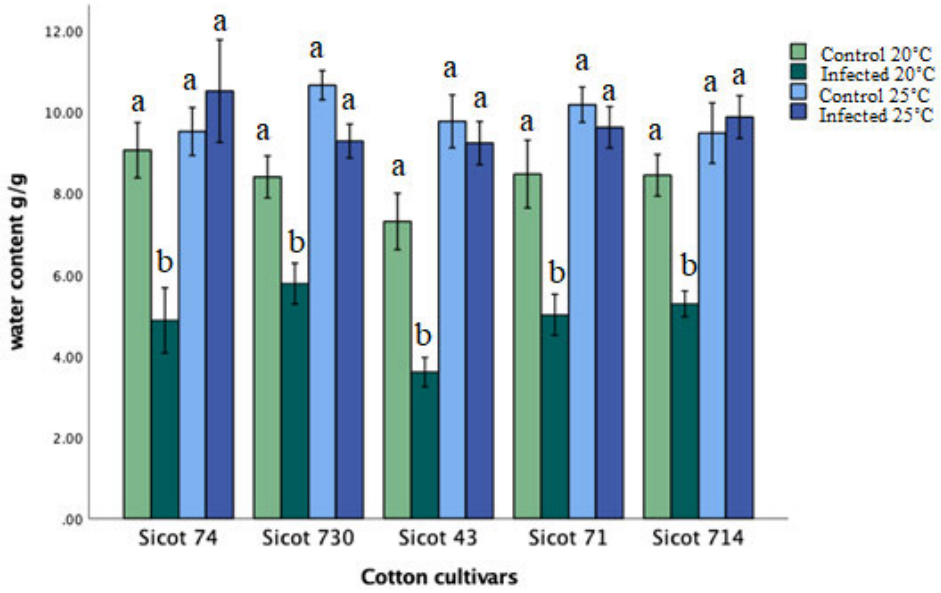

Figure 3.4: Water content (WC) of cotton cultivars (Sicot 71BRF, Sicot 730, Sicot 43BRF, Sicot 74BRF and Sicot 714B3F) infected with *B. rouxiae* BRIP40192 at 20°C and 25°C. Each bar represents the mean ± 1 standard deviation for n = 9 for each cotton cultivar. Letters above the bars indicate significant differences (one-way ANOVA, P<0.05) between treatments.

3.3.2. *B. rouxiae* BRIP40192 ITS copy number in association with cotton roots

The abundance of *B. rouxiae* BRIP40192 associated with the roots of cotton seedlings was assessed using qPCR to detect the ITS region of the pathogen. The copy number of *B. rouxiae* ITS region is reported here as copy number per weight (g) of roots. The copy number per length (cm) of roots was also assessed, but due to the strong correlation recorded between root weight and root length (at 25°C r = 0.938 and at 20°C r =0.984) only the interactions and recoveries per unit root weight are discussed. Results at 25°C showed no significant differences between cultivars, while at 20°C ITS copy number did differ between cultivars. At 20°C, the amount of target

sequence was higher for Sicot 71BRF (2.51 x 10^{12} gene copy number g^{-1} root), compared with Sicot 714B3F and Sicot 43BRF (2.23 x 10^{12} and 1.45 x 10^{12} gene copy number g^{-1} root) (Figure 3.5). Sicot 730 and Sicot 74BRF showed the lowest abundances of target sequence, with a nearly similar trend (7.11 x 10^{11} and 5.48 x 10^{11} gene copy number g^{-1} root, respectively).

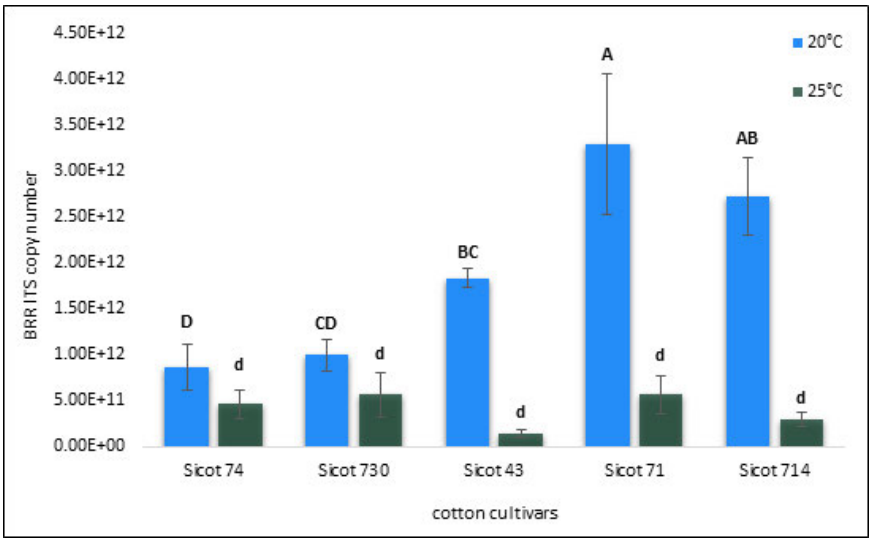

Figure 3.5: Mean abundance of *B. rouxiae* BRIP40192 ITS sequence as assessed by qPCR (n=9), presented as copy number per gram of roots for cotton cultivars Sicot 71BRF, Sicot 730, Sicot 43BRF, Sicot 74BRF and Sicot 714B3F, at 20°C and 25°C. Each bar represents the mean ± 1 standard deviation for n = 9 for each cotton cultivar. Letters above the bars indicate significant differences (one-way ANOVA, P<0.05) between treatments

Overall, the abundance of *B. rouxiae* BRIP40192 detected on plant roots was roughly four times higher at 20°C than at 25°C. Sicot 74BRF and Sicot 730 were the exception to this, with less difference being observed between abundance at 25°C compared to 20°C in contrast to other cultivars.

3.3.3. *B. rouxiae* BRIP40192 ITS copy number in rhizospheric soil

Abundance of *B. rouxiae* BRIP40192 in the rhizospheric soil was also determined by amplification of the ITS region. No target sequence was detected in any

negative control samples (soil not inoculated with endoconidia). The abundance of *B. rouxiae* was higher in soils incubated at 20°C, compared to 25°C, for all cotton cultivars except for Sicot 730, where there was no significant difference in abundance at different temperatures (Figure 3.6). Sicot 730 also had a significantly lower abundance of *B. rouxiae* at 20°C when compared to all other cultivars, a trend that disappeared at the higher temperature of 25°C. At 25°C, Sicot 74BRF and Sicot 43BRF had a lower abundance of *B. rouxiae* ITS sequence than the other three cultivars.

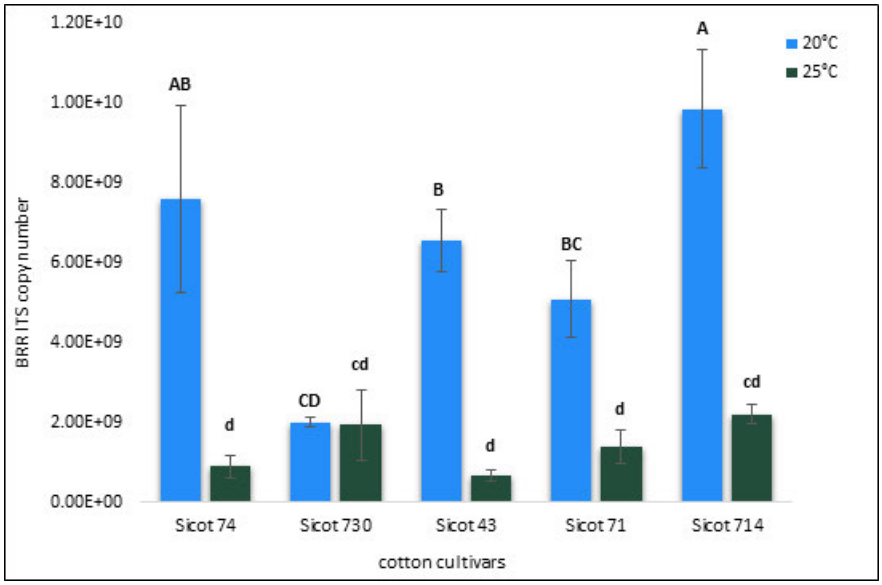

Figure 3.6: Mean abundance of *B. rouxiae* BRIP40192 ITS sequence as assessed by qPCR (n=9), presented as copy number per gram of soil for cotton cultivars Sicot 71BRF, Sicot 730, Sicot 43BRF, Sicot 74BRF and Sicot 714B3F, at 20°C and 25°C. Error bars represent the standard deviation of the means. Each bar represents the mean ± 1 standard deviation for n = 9 for each cotton cultivar. Letters above the bars indicate significant differences (one-way ANOVA, P<0.05) between treatments

For temperature 20°C and 25°C, Sicot 74 BRF there is a positive correlation between ITS copy number in soil with ITS copy number on roots, while Sicot 730 there is no correlation in ITS copy number in soil and root. There is no change in the

pattern for Sicot 43BRF, Sciot 71BRF and 41B3F for both ITS copy number in soil and root.

3.3.4. Correlation among *B. rouxiae* BRIP40192 load in soil, pathogen attached to root, lesions, and water content

The correlation among the results obtained from pathogenicity test of *B. rouxiae* BRIP40192 grown at 20°C and 25°C (pathogen loading in soil, pathogen attached to root, percentage of lesion and shoot water contents) a Pearson's product correlation was performed. The correlation tables for each cultivar are included in Appendix B.

In Sicot 74BRF, a very strong positive significant correlation was observed between copy number of ITS of a fungi attached to root at 20°C and copy number of ITS in rhizosphere at 20°C ($r = 0.96$).

In Sicot 730, a very strong positive significant correlation of copy number of ITS in rhizosphere at 20°C with copy number of ITS of a fungi attached to root at 20°C ($r = 0.92$). The copy number of ITS of a fungi attached to root at 25°C showed a weak positive significant correlation with shoot water content of infected plant at 25°C ($r = 0.67$). In contrast, shoot water content of infected plant at 25°C showed a moderate negative significant correlation with percentage of root lesion at 25°C ($r = -0.75$).

In Sicot 43BRF, a moderate positive significant correlation was observed between shoot water content of control plant at 25°C and copy number of ITS of a fungi attached to root at 25°C ($r = 0.74$). In contrast, a strong negative significant correlation was observed between shoot water content of infected plant at 25°C with percentage of root lesion at 25°C ($r = -0.87$).

In Sicot 71BRF, a very strong positive significant correlation was observed between copy number of ITS in rhizosphere at 25°C and copy number of ITS of a fungi attached to root at 25°C (r = 0.90). A moderate positive significant correlation was between copy number of ITS of a fungi attached to root at 20°C and copy number of ITS in rhizosphere at 20°C (r = 0.75).

In Sicot 714B3F, shoot water content of infected plant at 25°C showed a strong positive significant correlation with shoot water content of control plant at 25°C (r = 0.87). While, the shoot water content of control plant at 25°C showed a weak positive significant correlation with copy number of ITS of a fungi attached to root at 25°C (r = 0.67). The copy number of ITS of a fungi attached to root at 20°C showed a very strong positive correlation with the copy number of ITS in rhizosphere at (r = 0.98). The copy number of ITS in rhizosphere at 25°C showed a strong positive significant correlation with copy number of ITS of a fungi attached to root at 25°C (r = 0.89).

3.4. Discussion

One of the reasons that BRR disease is so difficult to control is that is can remain dormant in soil, in the form of chlamydospores, for long periods of time, and reappear when environmental conditions are favourable. Understanding the factors that control the survival and germination of chlamydospores is important to control this pathogen in the field (Hood and Shew, 1997). Soil temperature has previously been identified as a major factor that can influence severity of BRR disease. The initial findings from this chapter are in agreement with previous research, with a greater abundance of pathogen, and a higher proportion of roots being affected by lesions, at lower growth temperatures. These results are in agreement with previous findings of

Jhorar *et al.* (2004) and Rothrock and Nehl (2000), who found that *B. rouxiae* BRIP40192 infection was most severe when soil was cool. A decrease in shoot water content was also observed at a lower growth temperature (20°C compared with 25°C). This could be attributed to infected roots having a negative impact on growth and development of the whole plant, due to their essential role in absorption of water from the soil. In this study infected plants were observed to develop more lesions and less lateral roots at lower temperatures, and these changes impact on the ability of roots to support plant growth, in agreement with results described by Allen (2001). The results of this study give further support to the idea of delaying planting of cotton crops until soil temperatures increase, to avoid more severe BRR disease and these impacts on plant growth.

Another potential strategy to reduce the incidence or severity of BRR disease is to select cotton cultivars that have greater disease resistance. In this study, five cotton cultivars were grown in the presence of *B. rouxiae*, to determine if there were differences in disease resistance between cultivars, and whether this differed with temperature. Results showed that the numbers of pathogen attached to the cotton roots of Sicot 730, Sicot 43BRF, Sicot 71BRF and Sicot 714B3F was higher at 20°C compared with 25°C. However, there was no significant difference in Sicot 74BRF and number of fungal attached to root at 20°C compared with 25°C. At 25°C, soil around the root for all cultivars except Sicot 730 showed a significant decrease in the number of *B. rouxiae* BRIP40192 compared with 20°C, which clearly indicate that the temperature had an impact on either the proliferation or survival of the pathogen. Cotton cultivars, Sicot 43BRF, Sicot 74BRF and Sicot 714B3F showed a drop in the numbers of *B. rouxiae* BRIP40192 at 25°C compared with 20°C. The differences observed between cultivars in this experiment could potentially be attributed to

differences in the secretions from the roots of each cotton cultivar. Signaling molecules produce by host plants play role an important role in plant- pathogen interactions, and germination of fungal spores is thought to occur in response to root exudate in the rhizosphere (Badri and Vivanco, 2009). Butler *et al.* (2003) mentioned that soil microbial numbers, and their activity, in the rhizosphere is partially under the control of root exudates, implicating plants as having a role in severity of BRR disease. Temperature driven changes in root exudate composition have been described (Badri and Vivanco, 2009; Badri *et al.*, 2009) and these changes could either negatively or positively impact on spore germination and fungal growth (Baker *et al.*, 1997). It has been suggested that temperature effects both quantity and quality of root exudates produced by different plants, supporting the theory that the temperature of plant development, as well as cultivar, could influence the growth of soil-borne pathogens (Hale *et al.*, 1971; O'leary, 1966).

An interesting observation from this *in vitro* study is that there is not necessarily a correlation between the abundance of pathogen detected in soil or in association with roots, and the percentage of plant roots affected by lesions (an indicator of disease severity). Moreover, the percentage of root lesions did not correlate to the shoot water content for all cultivars. At 25°C, the copy number of *B. rouxiae* BRIP40192 ITS sequence in rhizospheric soil and attached to roots did not correlate with shoot water content or percentage of roots affected by lesions. This is in contrast to previous research by Bateman (1963), which mentioned that infection of plants by *Berkeleyomyces* spp. showed a correlation with density of propagules in the rhizosphere. Pathogen density in the cotton field was also found to have a positive relationship with disease severity in a more recent study (Holtz and Weinhold, 1994). One key difference between these earlier studies and the current work is the methods

used to quantify pathogen loads in soils. Part of the reason for this is the reliance on plate culture as a means of assessment of soil load (Nehl *et al.*, 2004; Rothrock, 1992), and that different studies have focused on endoconidia as the infective status of the pathogen (Nehl *et al.*, 2004; Pereg, 2013).

The differences observed in pathogen load in the rhizosphere and attached to roots, and differences in disease severity between different cotton cultivars at different temperatures indicate the central role of the plant in host-pathogen interactions. In this study cotton cultivar Sicot 730 showed the lowest ITS copy number in soil at cooler temperature might be best to grow in Australia to reduce incidence of BRR disease and reduce the pathogen loading in soil. One mechanism that could be responsible for the differences observed between cultivars is the influence of root exudates. However, further study is necessary to determine if signalling molecules produced by cotton roots have a significant effect on pathogen germination and growth, and whether these signalling molecules are pathogen specific or can be detected by a range of microbes in the rhizosphere.

Chapter 4: *Azospirillum brasilense* as a biocontrol of *Berkeleyomyces rouxiae* BRIP40192

4.1. Introduction

Although black root rot is a significant issue for cotton producers, currently there is no permanent solution to this problem. However, a number of strategies have been suggested to assist in the control of this pathogen. These strategies can be classified into three groups: chemical controls, cultural controls and biological controls (Pereg, 2013). Biological control by introducing antagonists represents a promising option to reduce the impact of black root rot disease on cotton production in Australia (Moulynox *et al.*, 2010; Schoina *et al.*, 2011). In general, there are two mechanisms by which biological control agents interact with pathogens: direct and indirect. Direct mechanisms involve interaction directly with the pathogen, for example, through the production of antifungal agents, which affect the pathogen. Indirect mechanisms involve the biocontrol agent influencing pathogens in a non-targeted manner, such as by inducing plants to have pathogen resistance (Diallo *et al.*, 2011; Köhl *et al.*, 2019; Pal and Gardener, 2006; Pearson and Callaway, 2003)

Plant root secretions play a vital role in rhizospheric interactions between plants and microorganisms (Badri and Vivanco, 2009; Badri *et al.*, 2009; Brencic and Winans, 2005; Venturi and Keel, 2016). Spore germination of *Berkeleyomyces* spp., in response to roots secretions, has been documented (Mathre and Ravenscr, 1966; Papavizas, 1968; Patrick *et al.*, 1965). Specific signalling molecules produced by roots promote directional growth of hyphae towards host roots, where they can then cause infection. These signalling molecules secreted by roots probably determine the

intensity of the plant-microbial interaction and influence disease severity. Interruption of the interactions between *Berkeleyomyces* spp. and the cotton host by introducing a suitable biological control agent could be one avenue to either reduce or prevent disease. A number of organisms including *Paenibacillus alvei* strain K-165, *P. fluorescens* CHA0 and *P. aureofaciens* strain 63-28, have been investigated as potential biocontrol against *Berkeleyomyces* spp. (Paulitz *et al.*, 2000; Reddy and Patrick, 1992; Schoina *et al.*, 2011), with mixed results. While these strains show some promise, none are commercially used.

The use of plant growth-promoting bacteria (PGPB) as biological control agents for soil-borne diseases has been reviewed by Bashan and De-Bashan, (2005); Compant *et al.*, (2005) and Hassouna *et al.*, (1998). *Azospirillum* spp. is one of the most widely utilised genera of plant growth-promoting bacteria, due to its ability to enhance growth and yield of several plants of agronomic and ecological importance (Bashan *et al.*, 2004; Bloemberg and Lugtenberg, 2001). *Azospirillum* spp. survive for a long time in the rhizosphere and transfer from root to root under the influence of attractant and repellent signalling molecules (Bashan and Holguin, 1994, 1997; El Zemrany *et al.*, 2006). The ability to adapt to environmental cues that induce morphological transformation and cell aggregation is reported as an important step in the attachment process, and has been related directly with the capability of *Azospirillum* spp. to survive and efficiently colonise the rhizosphere or plant roots (Bashan and Holguin, 1997; Bashan *et al.*, 2004; Fukami *et al.*, 2018).

Many *Azospirillum* species have been suggested to act as biocontrol agents against a range of plant-fungal pathogens. *A. brasilense* Sp245 showed an ability to inhibit the growth of the fungal pathogen *Rhizoctonia*, *in vitro* (Russo *et al.*, 2008). Siderophores production, acetylene reducing activity and antagonistic ability by *A.*

brasilense showed antifungal activity against *Colletotrichum acutatum*, *Alternaria* spp., *Bipolaris* spp. and *Fusarium* spp. pathogens (López-Reyes *et al.*, 2017; Tortora *et al.*, 2011). An auxin-like molecule called phenylacetic acid (PAA) in supernatant extracts from *A. brasilense* Sp7 and *A. brasilense* Sp245 showed inhibitory activity against *Fusarium oxysporum*, *Fusarium matthiolae*, *Neurospora crassa* and *Alternaria bassicicola* (Somers *et al.*, 2005). Resistance to the leaf spot pathogen *Pseudomonas syringae*, induced by *A. brasilense*, has also been recorded in *Arabidopsis thaliana* (Solano *et al.*, 2008). In addition, *A. brasilense* SBR, mixed with rhizobacteria strains *Azotobacter Chroococcun* ZCR, and *Klebsiella pneumoniae* KPR, exhibited biological control properties against sunflower root-rot disease complex caused by three fungal pathogens (*Fusarium solani, Macrophomina phaseolina* and *Rhizoctonia solani*), which was attributed to the production of antifungal compounds and IAA (El_Komy *et al.*, 2020). Rhizobacterial community analysis of BRR disease suppressive soil indicated a greater presence of *Azospirillum* spp. in comparison with conducive soil (Kyselková *et al.*, 2009). This body of evidence suggests that *Azospirillum* spp. could potentially be a good candidate as a biocontrol against BRR disease. However, there is a need to understand how interactions between *Azospirillum* spp. and fungal pathogens may take place. A recent study proposed that beneficial bacteria, such as *Azospirillum* spp. can attach to the hyphae of pathogenic to move towards plant roots, where they can promote plant growth/suppress plant disease (Palmieri *et al.*, 2020). This process by which bacteria attach to and move/migrate along the fungal hyphae is termed the "fungal highway" (Simon *et al.*, 2015; Toljander *et al.*, 2006; Warmink *et al.*, 2011). Bianciotto *et al.* (2001) reported that extracellular polysaccharide production by *A.brasilense* is involved in its attachment to arbuscular mycorrhizal fungi. Whether *Azospirillum* can

attach to, or move along, hyphae of *Berkeleyoymces* spp. is yet unknown, and investigating these processes could give insight as to whether, and how, *Azospirillum* spp. could work as a biological control against this pathogen.

Understanding the interactions between *Berkeleyomyces* spp., the plant host, and candidate biocontrol agents will assist in the identification of a suitable microbial biological agents that could be employed as a part of a disease control strategy. The main goal for this chapter was to investigate the potential of *A. brasilense* to act as a biocontrol to manage infection with *B. rouxiae* BRIP40192 (cotton isolate). A first goal was to investigate the directional growth capability of *B. rouxiae* BRIP40192 towards cotton seedlings, in response to signalling molecules produce by cotton seedlings. Furthermore, the ability of *A. brasilense* strains to block this interaction between *B. rouxiae* BRIP40192 and cotton seedling was examined. Finally, the role of oxygen as an environmental signal that triggers cellular transformation, clumping and aggregation in the *A. brasilense*, which are important processes in plant growth promotion and potentially biological control was investigated.

4.2. Methodology

4.2.1. Culture condition and inoculum production for fungal isolates

The source and culture conditions for the *B. rouxiae* BRIP40192 isolate used in this study are as previously described in section 3.2.3. The preparation of endoconidial suspension to use as an inoculum is as described in section 3.2.4.

4.2.2. Culture conditions for bacterial isolates

The *A. brasilense* strains used in this study are listed in the Table 4.1. *A. brasilense* strains were grown on minimal lactate medium (Appendix A) (Galimand *et al.*, 1989) or nitrogen- free malate medium (Appendix A) (Sriskandarajah *et al.*, 1993), supplemented with 20 mM of ammonium chloride and 40 μg/ml Congo red. Bacteria were cultured for 18 hrs at 28°C.

Table 4.1: *A.brasilense* strains used in this study

Bacterial strain	Genotype or phenotype[a]	Reference
Azospirillum brasilense		
- Sp7	Wild-type	(Tarrand *et al.*, 1978)
- Sp245	Wild-type	(Baldani *et al.*, 1983)
- Sp72002	Tn5 induced mutant of Sp7, CR^-, $Floc^-$	(Pereg-Gerk *et al.*, 1998)

[a] CR, Congo red binding; Floc, flocculation

4.2.3. Cotton cultivars and sterilisation

Cotton cultivars used in this study are the same as those described previously in section 3.2.5. Sterilisation of cotton seeds prior to use was carried out as described in section 3.2.6.

4.2.4. Directional growth of *B. rouxiae*

The directional growth of *B. rouxiae* BRIP40192 isolates towards germinating cotton seeds (Sicot 730, Sicot 71BRF, Sicot 74BRF, Sicot 43BRF and Sicot 714B3F) was tested on 1.2% Noble agar in round plates (90 x 14 mm). 20 ml of Noble agar was

poured in each plate. A volume of 70 µl of endoconidial suspension (containing 3.5 x 10^5 endoconidia/ml) of *B. rouxiae* BRIP40192 isolate was absorbed onto a strip of sterile filter paper (6.5 x 1 cm) (Whatman no.1 chromatography paper). Strips were placed directly onto the Noble agar plate, 2 cm away from four sterilised seeds. Seeds were spread out evenly in a straight line along the plate. This experimental set up is illustrated in Figure 4.1. Matching controls were set up, using a strip of filter paper inoculated with 70 µl of sterilised de-ionized water in place of the sterile seeds.

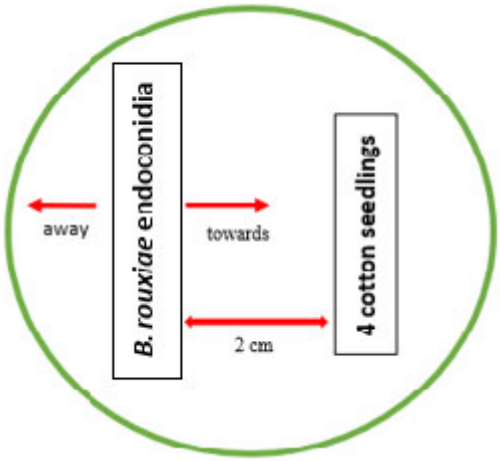

Figure 4.1: Schematic showing the experimental design to detect the directional growth of hyphae towards cotton cultivars (Sicot 730, Sicot 43BRF, Sicot 71BRF, Sicot 74BRF and Sicot 714B3F).

All plates were incubated for 7 days at 26°C under a 12 h light / 12 h dark cycle. When the roots of seedlings reached approximately 2 cm they were replaced with fresh sterilised seeds. Seven days after inoculation, mycelial growth was examined under the stereomicroscope (Nikon Clipse 50i, Japan) to confirm the presence of *B. rouxiae* BRIP40192 chlamydospores or mycelia. Growth directionality results were assessed by measuring the distance of the mycelial growth from the side of the strip towards the seedlings, and subtracting the distance of the mycelial growth away from the seedlings. The experiment was performed with five replicates for each cotton cultivar

with three time repeated for each experiment. Statistical analysis using R package (version four) was carried out to find the average, standard deviation and perform a one-way ANOVA to test for differences between cotton cultivars, at $p<0.05$.

4.2.5. Testing *B. rouxiae* BRIP40192 growth inhibition by *A. brasilense* in the presence cotton:

The effect of *A. brasilense* (Sp7, Sp245 and Sp72002) on the germination of fungal spores and growth of hyphae toward cotton seeds was tested on 100 mm square plates containing 1.2% Noble agar (12 g/L Difco Noble agar in MilliQ water). An aliquot of 70 µl of bacterial culture ($1x10^6$ cell/ml) was applied to one strip of sterile filter paper (Whatman No.1 chromatography paper, 65 mm x 10 mm), and 70 µl of sterile de-ionized water was applied as a control on a second strip of sterile filter paper. The two strips were placed 15 mm on either side of an identical strip containing a volume of 70 µl of endoconidial suspension ($3.5x10^5$ spores/ml*)* of *B. rouxiae* BRIP40192. Four surface sterilised cotton seeds (Sicot 74BRF) were allowed to germinate on yeast mannitol plates at 26°C for 2 days, until roots were between 5-10 mm long. Two germinated seeds were placed 5 mm away from each of the control and treatment strips, thus 20 mm from the edge of the central strip containing fungal spores. This experimental set up is shown in Figure 4.2. Seeds were removed and replaced on days two and four with fresh germinating seeds. After seven days, the growth of fungal hyphae towards the germinating seeds on each side was measured from the edge of the central strip to the front of the growing hyphae. All plates were incubated for 7 days at 26°C in a 12 h light / 12 h dark cycle. Seven days after inoculation, mycelial growth on all 1.2% Noble agar plates was examined under the stereomicroscope (Nikon Clipse 50i, Japan) to confirm the presence of *B. rouxiae*

BRIP40192 chlamydospores or mycelia. The experiment was performed five times with three replicates for each cotton seed cultivars.

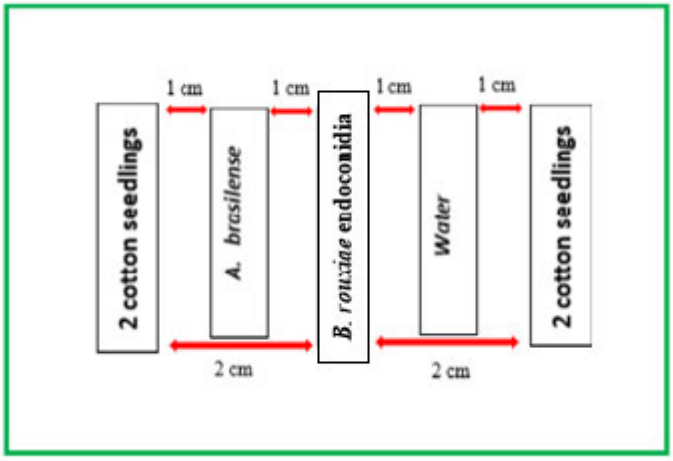

Figure 4.2: Schematic showing the experimental design to detect the inhibition of *B. rouxiae* BRIP40192 growth towards cotton seeds by *A. brasilense* strains (Sp7, Sp72002 and Sp7245).

4.2.6. Antagonism test

Dual cultures were used to examine the antagonistic effect of *A. brasilense* strains on mycelial growth of *B. rouxiae* BRIP40192. A 2 mm sterilised paper disc was dipped into a log phase ($1x10^6$ cell/ml) bacterial suspension of *A. brasilense* grown on LB broth and placed in the middle of a Petri dish containing ½ PDA. A 5 mm mycelial plug of *B. rouxiae* BRIP40192 was collected from the margin of a seven day old culture, and placed 2 cm away from the *A. brasilense* paper disc. Three replicates were used for each *A. brasilense* strain, and a set of three plates containing only pathogen and no bacteria were used as a control. All plates were incubated in an inverted position at 26°C in a dark for 7 days. The radius of fungal growth was measured for both experimental and control plates each day. The formula used to assess the percentage of fungal growth inhibition was (r1-r2/r1) x100, where r1 is the

radial growth of the fungus on the control plate, and r2 is the radial growth of the fungus in the presence of bacteria (Trivedi *et al.*, 2008).

4.2.7. Interaction of *A. brasilence* with *B. rouxiae* BRIP40192 hyphae

4.2.7.1. Attachment of A. brasilense strains to B. rouxiae BRIP40192 hyphae in suspension culture

Attachment assays were carried out as described by Bianciotto *et al.* (1996) with some modification. *A. brasilense* Sp7 and Sp245 wild-type strains and a mutant strain (Sp72002) were used to study their ability to physically interact with fungal hypha. 100 µl of an overnight culture at final concentration of 1 x 10^6 cell/ml was used to inoculate a set of 10 conical flasks (50 ml) containing 10 ml ½ PDB (pH was adjusted to 7) for each strain. 100 µl of 3.5 x 10^5 spore/ml of *B. rouxiae* BRIP40192 was added to each flask. A set of 10 control flasks were set up for each bacterial strain, using micro glass beads in place of the fungal suspension. All flasks were incubated at 26°C with shaking at 180 rpm. After 48 hrs of incubation, the fungal material, and the glass beads from the controls, were used to prepare slides by directly applying a drop of fungal material in the middle of a slide, which was covered with a cover slip and examined by light microscope (Nikon Clipse 50i, Japan).

4.2.7.2. Slide agar culture technique

A slide agar culture technique was designed to study the ability of bacteria to move along the fungal hyphae. A sterile Petri dish lid (55 mm x 14 mm) was placed upside down inside a large sterile Petri dish (90 mm x 14 mm). A microscope slide

(25.4 mm x 76.2 mm, thickness 1 mm-1.2 mm) was laid on the small Petri dish lid. A thin layer of water agar (0.3%-0.6%) was poured onto the slide. Two 20 mm x 10 mm strips of sterile filter paper (Whatman no.1 chromatography paper) were used to apply inoculums of *A. brasilense* and *B. rouxiae* BRIP40192. The first strip of filter paper was dipped into a log phase culture broth of *A. brasilense* (1 x 10^6 cell/ml) and placed directly on the slide. 20 µl of endoconidia suspension (3.5 x 10^5 endoconidia/ml) of *B. rouxiae* BRIP40192 was absorbed on the second strip of filter paper, and placed 100 mm away from the first one. This experimental set up is illustrated in Figure 4.3. Ten replicates were carried out for each strain (*A. brasilense* Sp7, Sp245 and Sp72002), plus two controls for each strain (bacteria alone). The Petri dishes were incubated for 2-3 days at 26°C, and the slides examined directly using a light microscope (Nikon Clipse 50i, Japan).

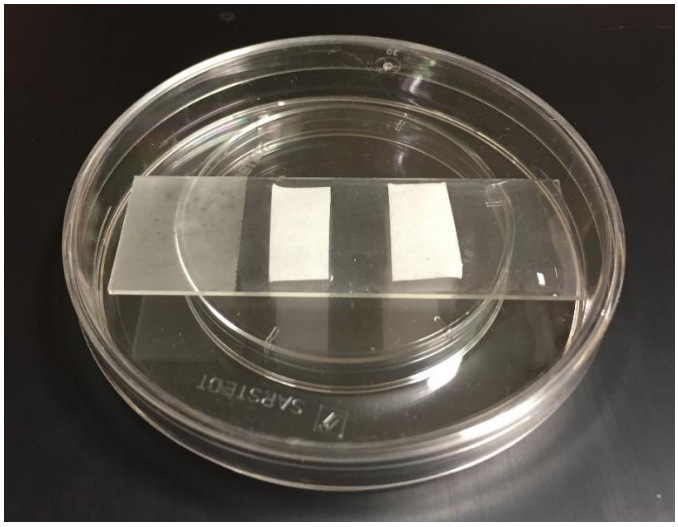

Figure 4.3: Photography of the slide agar culture technique assembly.

4.2.7.3. Fungal Highway test

4.2.7.3.1. Sample collection

The fungal highway assays were performed following a modified procedure of Leben (1984). 1/2 PDA (pH was adjust at 7) was poured on both sides of a sterile two-compartment Petri dishes (92 x 16 mm, Sarstedt, Germany) to a depth of 7-8 mm. A mycelial plug of 5 mm diameter was collected from the margins of 7 day old culture of *B. rouxiae* BRIP40192 and placed into one compartment of the Petri dish, 2 cm away from the divider. An aliquot of 70 µl of bacterial culture (*A. brasilense* Sp7, Sp245 (wild-type) and Sp72002 (mutant), at a concentration of 1 x 10^6 cell/ml) was applied along the Petri dish, equidistant between the mycelial plug and dish divider. For controls, 70 µl of sterile dH_2O was applied in place of the bacterial suspension. All plates were incubated in an inverted position at 26°C in the dark. After 10 days, hyphae were scraped from the un-inoculated compartment of the dish and stored at –20°C until DNA extraction was carried out. Ten plates were used for each strain with 10 controls. Figure 4.4 illustrate a schematic of fungal highway assay.

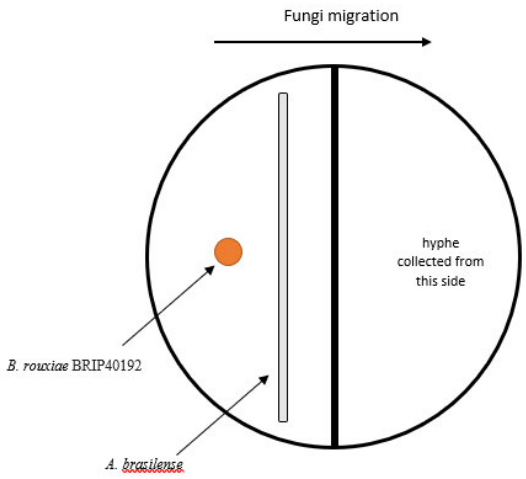

Figure 4.4: Schematic of the 'fungal highway' experimental procedure used to test the ability of *A. brasilense* strains to move along *B. rouxiae* BRIP40192 hyphae.

4.2.7.3.2. DNA extraction and PCR

Genomic DNA was extracted from the scraped hyphae of each plate using a DNA PowerSoil kit (Mo Bio, Carlsbad, USA), following the manufacturer's manual with some modifications: hyphae were vortexed vigorously using dial 9/10 maximal power on vortex-Genie2 (Scientific Industries, Inc., USA) for 30 minutes, and DNA was eluted in 65 µl of elution buffer. The extracted DNA was stored at -20°C until use.

Primers were designed to amplify the *FlcA* gene of *A. brasilense* Sp7 and Sp245, based on the available in the NCBI database under accession numbers Y12363.1 and HE577327.1, respectively. Primer3 software (http://frodo.wi.mit.edu/) was used to design the following primers: FlacA-1-Forward: 5' AGC GTC AAG GTG ATG ATG G 3' and FlacA-1-Reverse: 5' GAC TTG AAG ATC GCC GTG A 3'. Polymerase chain reaction (PCR) was carried out using the Mastercycler nexus PCR cycler (Eppendorf, Germany). Each reaction contained 12.5 µl of 2 X TopTaq Mastermix (Bio-line), 1 µl (0.4 µM) each of forward and reverse primers, and 1 µl of template DNA, in a final volume of 25 µl using nuclease-free water. Samples were run in triplicate and a no template control (NTC) was included with each run. Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 51°C for 1.5 minute, and elongation at 72°C for 1 minute, and a final elongation step at 72°C for 5 minutes. The PCR product was observed by gel electrophoresis as describe in section 2.2.3.

4.2.8. Flocculation of *A. brasilense* under controlled oxygen levels

A single colony of *A. brasilense* strains (SP7, and Sp245), grown on solid nutrient agar medium, was used to inoculate 10 ml of nutrient broth in a conical flask (50 ml), and incubated in a shaking incubator (200 rpm) overnight at 28°C. The overnight culture was used to inoculate fresh nutrient broth to an absorbance of 0.1 at 600 nm (SpectraMax®M2e, USA) and incubated in a shaking incubator (200rpm) at 28°C. The optical density was monitored until the culture reached a log-phase absorbance of 0.5-0.7(A_{600}). The inoculums were harvested by centrifugation at 4200 rpm for 10 minutes at room temperature, and the bacterial pellet resuspended in 10 ml of minimal medium to wash. Cells were again collected by centrifugation at 4000 rpm for 10 minutes. Cells were then resuspended in 5 ml minimal medium, and used to inoculate culture flasks under various concentrations of oxygen.

Culture flasks with different concentrations of oxygen were prepared as follows. First, anaerobic conditions were created by sealing sterile 50 ml flasks with 20.5 mm sterile Suba Seal red rubber septa (Sigma-Aldrich). 10 ml of Minimal medium (Appendix A) supplemented with 0.5 mM KNO_3 and 8 mM fructose was added using a sterile syringe. A sterile reusable needle luer lock (Gauge 23 X 100 mm stainless steel, Livingstone) was inserted through the septum, and used to flush nitrogen through the flask for 1 minute (a second sterile needle was inserted to remove oxygen from the flask), an oxygen analyser was used to monitor oxygen level. This set up is illustrated in Figure 4.5. Different concentrations of oxygen (5%, 15%, 25%, aerobic) were created in each flask by injecting oxygen through a sterile needle until the desired concentration was reached. Additionally, two control were used, aerobic (positive control) and anaerobic (negative control). Each flask was inoculated with the

previously prepared bacterial suspension to an absorbance of 0.3-0.4 at 600 nm, using a sterile syringe. Flasks were incubated in a shaking incubator (200 rpm) at 28°C until a standard bacterium (aerobic) reached full flocculation, as determined by visual examination (after 12 hr). Nine replicates were used for each treatment.

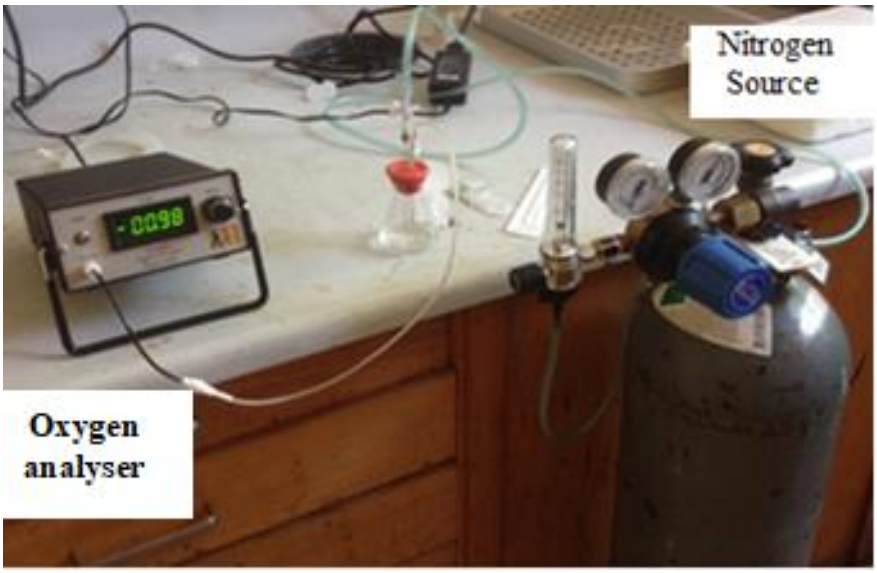

Figure 4.5: Preparation of culture flasks containing different oxygen concentrations. Nitrogen was used to flush oxygen from the flasks to create anaerobic conditions. An oxygen analyser (Volumetrics Inc./USA) was used to monitor oxygen levels.

Following floc formation, turbidity of the supernatant was measured using a spectrophotometer at 600 nm (SpectraMax®M2^{e,} USA). Each culture was then filtered through Whatman filter paper (no. 1), dried at 80°C for 2 hours, and weighed to determine total floc weight. A one-way ANOVA using Duncan' test was carried out to detect significant differences between oxygen levels. Statistical analysis was carried out using R (version 4).

4.3. Results

4.3.1. Directional growth of *B. rouxiae* towards cotton seedlings

Germinating seeds of five cotton cultivars were used to observe differences in directional growth of *B. rouxiae* BRIP40192 hyphae towards cotton seedlings. Hyphal growth and development of chlamydospores was induced after 4-5 days of incubation, for all cotton cultivars examined. No differences in the rate of seed germination or growth was observed between the different cultivars. No hyphal growth or chlamydospore formation was observed in any of the controls. Figure 4.6 illustrates the directional hyphal growth towards cotton seedlings. No growth was observed on the side of the plate away from the seedlings. From visual assessment, and examination under the stereomicroscope, the hyphal density was greater using cultivars Sicot 71BRF and Sicot 74BRF compared to other cultivars.

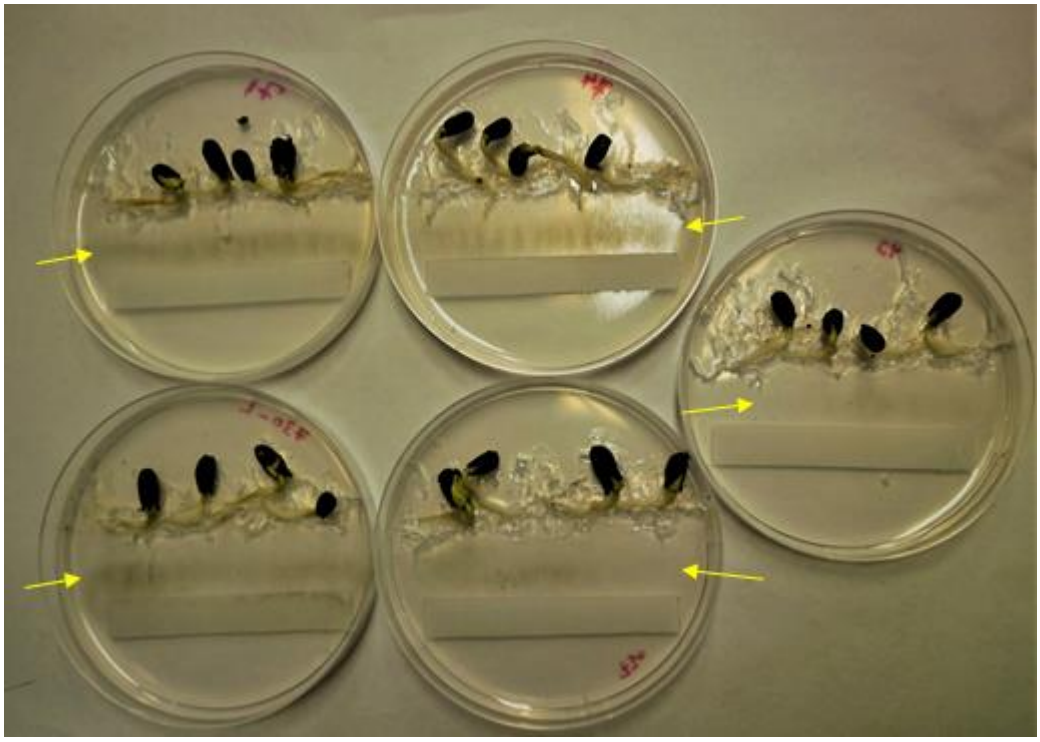

Figure 4.6: Directional growth of *B. rouxiae* BRIP40192 hyphae towards cotton cultivars seedlings growing on 1.2% Noble agar for 7 days at 26°C. A, B, C, D and E letters represent Sicot 74BRF, Sicot 71BRF, Sicot 730, Sicot 43BRF and Sicot 714B3F. Yellow arrows show the growth of hyphae.

When fungal directional growth was quantified, differences were observed between cotton cultivars. As illustrated in Figure 4.7, *B. rouxiae* BRIP40192 exhibited the strongest growth towards Sicot 74BRF and Sicot 71BRF (1.278 cm and 1.222 cm, respectively), compared with Sicot 730 (0.922 cm). The least fungal directional growth was observed towards both Sicot 43BRF and Sicot 714B3F (0.789 cm, and 0.711 cm, respectively).

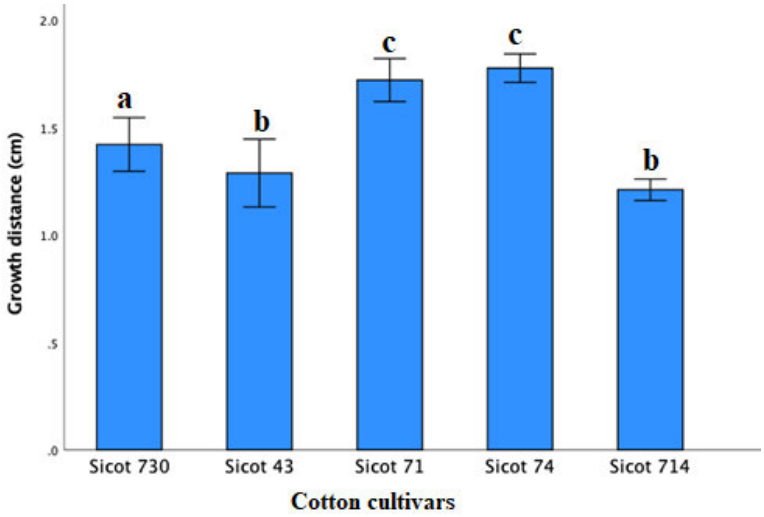

Figure 4.7: Quantification of directional growth of *B. rouxiae* BRIP40192 isolate towards germinating seedlings of five cotton cultivars (Sicot 730, Sicot 43BRF, Sicot 71BRF, Sicot 74BRF and Sicot 714B3F). Bar plots represent means (mean ± SE, n=5). Different letters above the error bars indicate significant differences at p≤ 0.05 using Duncan's test.

4.3.2. Growth inhibition of *B. rouxiae* BRIP40192 by *A. brasilense* in the presence of cotton

The ability of *A. brasilense* to inhibit the directional growth of *B. rouxiae* towards cotton seedlings was tested using methods described in section 4.2.5. All *A. brasilense* strains used (Sp7, Sp245, and Sp720002) blocked the growth of *B. rouxiae* BRIP40192 hyphae towards cotton seedlings, with no hyphal growth being observed on the half of the plate containing bacteria. As an example, this is illustrated in Figure 4.8. In contrast, when a paper strip containing water was used as a control, *B. rouxiae* BRIP40192 growth towards cotton seedlings was observed. To confirm the presence of *B. rouxiae* BRIP40192 chlamydospores or mycelia, all plates were examined under stereomicroscope.

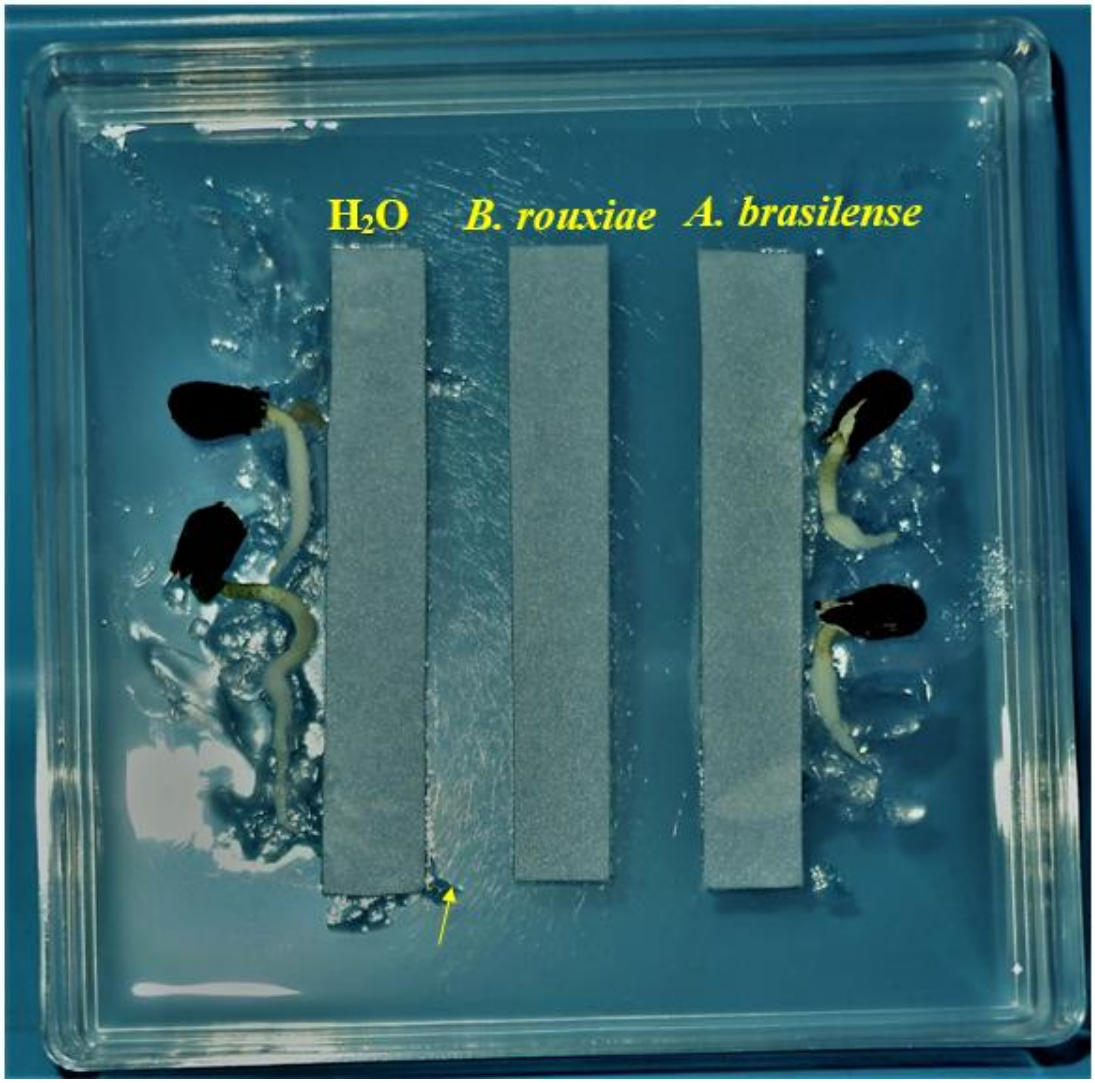

Figure 4.8: Growth inhibition of *B. rouxiae* BRIP40192 hyphae (middle strip) by *A. brasilense* Sp7 (Right strip) with the presence of Sicot 730 cultivar seedlings on 1.2% Noble agar for 7 days at 26°C. Water (left strip) was used as a control. Yellow arrows indicate regions of hyphal growth

4.3.3. Antagonism test

Dual culture assays were used to detect antagonism between *A. brasilense* and *B. rouxiae* BRIP40192. Figure 4.9 illustrates the results of these dual culture assays. No significant differences were observed in the radial growth of *B. rouxiae* when grown in the presence of any of the three *A. brasilense* strains (Sp7, Sp245 or Sp72002) when compared to controls. These results show that there is no antagonistic interaction between these organisms.

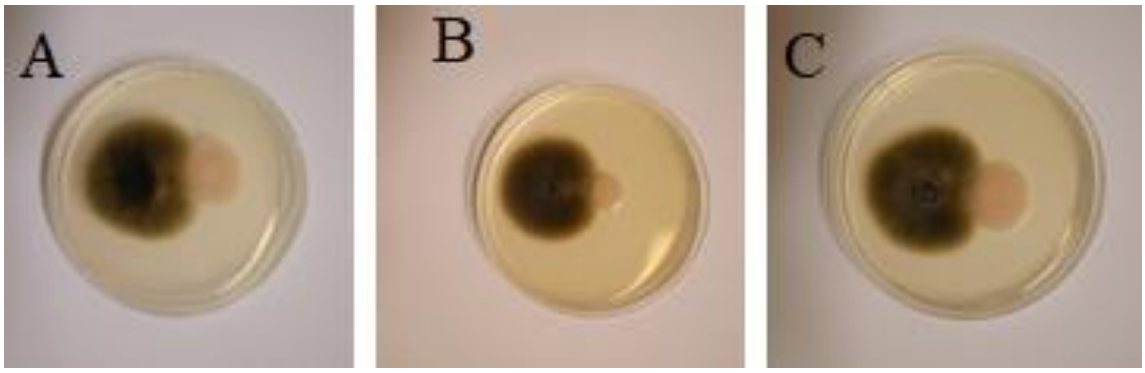

Figure 4.9: Dual culture test for antagonism between *A. brasilense* strains (A: Sp7, B: Sp72002, C: Sp245) and *B. rouxiae* BRIP40192.

4.3.4. Interaction of *A. brasilense* with *B. rouxiae* BRIP40192 hyphae

Three different techniques were used to study the interaction between *A.brasilense* and *B. rouxiae* BRIP40192 hyphae. When grown together in a suspension culture, *A. brasilense* strains demonstrated the ability to attach to *B. rouxiae* BRIP40192 hyphae as shown in Figure 4.10. However, shaking for 5 seconds, at low speed, was enough to detach the bacteria cells from the hyphae, indicating that this hyphal attachment is weak.

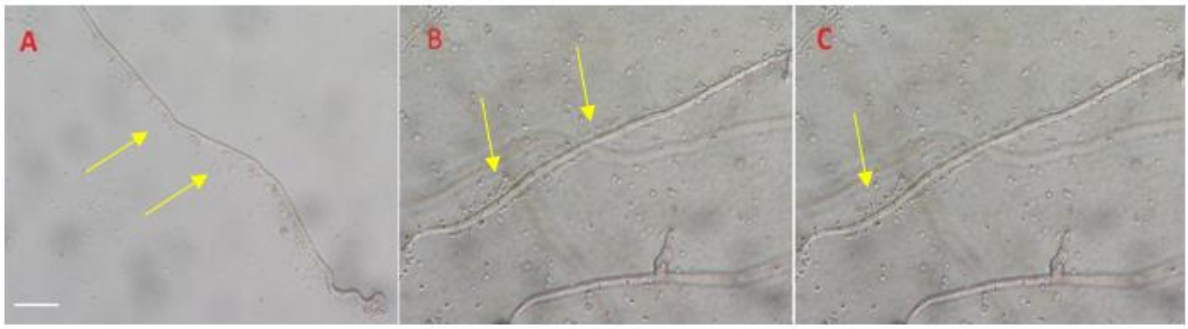

Figure 4.10: *A. brasilense* attachment to *B. rouxiae* BRIP40192 hyphae in suspension. A: *A. brasilense* Sp7; B: *A. brasilense* Sp72002 and C: *A. brasilense* Sp245. Yellow arrows show *A. brasilense attached along B. rouxiae* hyphae. Bar= 10 µm.

Experiments carried out using a slide agar culture technique (section 4.2.7.2) also indicated that *A. brasilense* can interact with *B. rouxiae* hyphae. In these experiments *A. brasilense* strains *(*Sp7, Sp72002 and Sp245) were all observed to attach to, and move along, the *B. rouxiae* BRIP40192 hyphae (this attachment is shown in Figure 4.11).

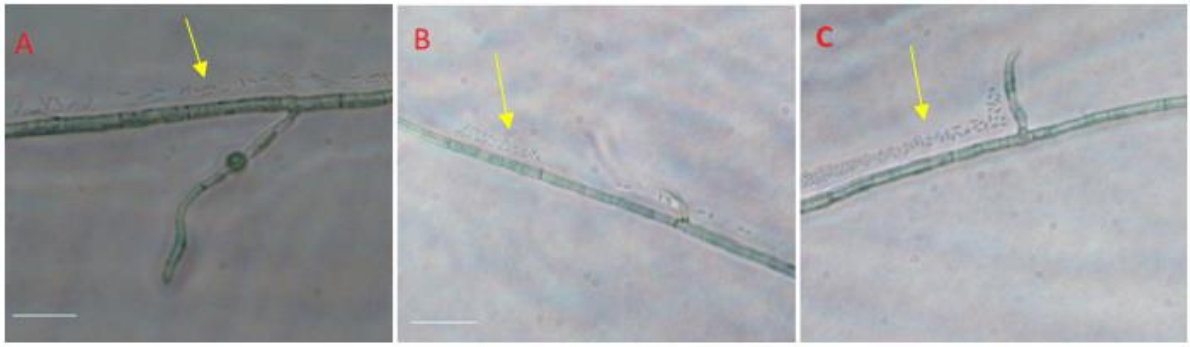

Figure 4.11: *A. brasilense* strains move along the *B. rouxiae* BRIP40192 hyphae. *A. brasilense* Sp7; B: *A. brasilense* Sp72002 and C: *A. brasilense* Sp245. Yellow arrows show *A. brasilense* moving along *B. rouxiae* hyphae. Bar= 10 µm

To confirm the ability of *A. brasilense* strains *(*Sp7, Sp72002 and Sp245) to attach to, and move along, *B. rouxiae* BRIP40192 hyphae, a "fungal highway" test was carried out as described in section 4.2.7.3. Genomic DNA extracted from hyphae which crossed the divider of a compartmentalised plate was used as a template to

detect the *A. brasilense flcA* gene, which was used as an indicator of the presence of bacteria. For the three strains tested, *flcA* DNA was only detected for *A. brasilense* Sp245 (results of the PCR are shown in Figure 4.12). No DNA was detected from *A. brasilense* Sp7 or Sp72002, suggesting that only Sp245 had the ability to move along the *B. rouxiae* hyphae for a significant distance.

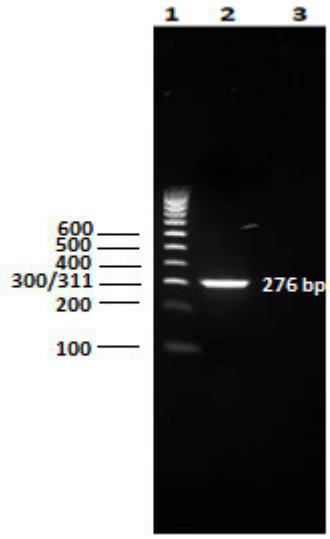

Figure 4.12: Gel electrophoresis of PCR product from FlcA-1 primer set in the study (1.5% agarose gel). Lane 1: 100bp DNA ladder; lane 2: *FlcA*- gene product (the expected size 276bp) and lane 3: NTC.

4.3.5. Aggregation of *A. brasilense* in response to oxygen

Two parameters were used to assess the ability of *A. brasilense* strains Sp7 and Sp245 to flocculate under different concentrations of oxygen: the dry weight of flocs, and the optical density of the culture suspension. As presented in Figure 4.12, the dry weight of both Sp7 and Sp245 flocs was significantly different (p-value > 0.05) under different concentrations of oxygen. Under aerobic conditions, both strains were able to flocculate fully, with a dry weight of 0.007 g and 0.006 g of flocs being collected for Sp7 and Sp245 respectively. Under decreasing oxygen concentrations (from 25%,

15% then to 5%) the rate of flocculation for both strains decreased, as indicated by the decrease in floc weight, with only 0.001 g of flocs being collected for both strains under 5% oxygen (Figure 4.13). Measurements of turbidity of the suspension cultures also illustrate this change in flocculation with changing oxygen concentration. The absorbance of both Sp7 and Sp245 cultures was lowest under aerobic conditions (0.049 and 0.054, respectively), when most of the cells had flocculated. The failure of cells to flocculate with decreasing oxygen concentration was illustrated by the significant increases in turbidity at 25, 15 and 5% oxygen (Figure 4.13).

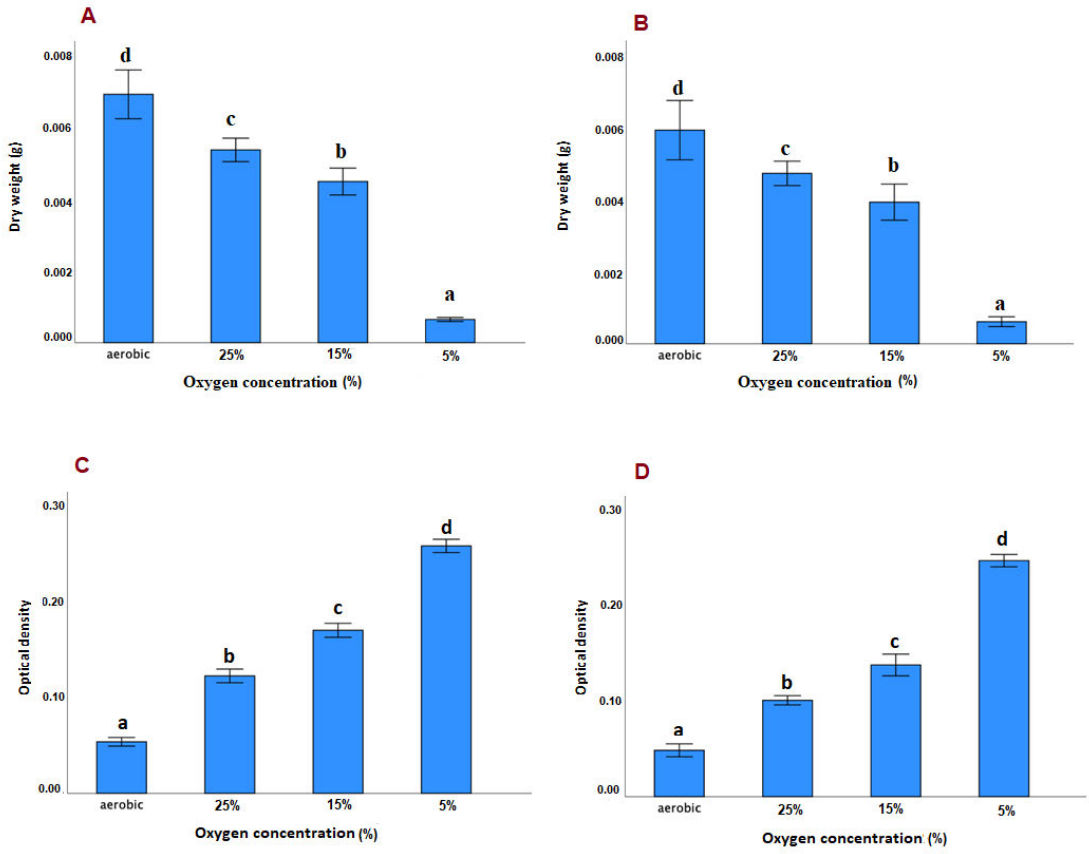

Figure 4.13: Dry weight (in grams) of flocs collected for *A. brasilense* Sp7 (A) and *A. brasilense* Sp245 (B). Turbidity of culture suspension at optical density (600 nm) for *A. brasilense* Sp7 (C) and *A. brasilense* Sp245 (D). Under aerobic conditions and under different concentrations of oxygen (25%, 15% and 5%). The bar plots represent mean ± SE (n= 9). Different letters above the bars indicate significant differences at P≤ 0.05 using Duncan's test.

4.4. Discussion

In order for *Berkeleyomyces* spp. to infect susceptible host plants, under suitable environmental conditions, spores start to germinate, and the germ tube elongates to reach plant roots and cause infection (Hood and Shew, 1997). The role of root exudate in stimulating hyphal elongation of mycorrhizal fungi has been described previously (Bécard and Piché, 1989; Elias and Safir, 1987; Nagata *et al.*, 2016). In the current study, root exudates produced by cotton seedlings stimulated pathogen germination and growth for all cotton cultivars tested. The influence of root exudates on the hyphal morphology among the five-cotton cultivar clearly appears in hyphal density, length, and the degree of branching (Figure 4.4). As density of *B. rouxiae* BRIP40192 hyphae increases over time, chlamydospore formation is triggered as a result of depletion in nutrient availability. An *in vitro* study by Hood and Shew (1997) explained that the primary factors inducing chlamydospore production in *Berkeleyomyces* spp. was nutrient availability. Knox *et al.* (2014) revealed that different cotton cultivars have a significant influence on the microbial diversity in the rhizosphere region, through the production of different root exudates. This difference in root exudates could explain the differences in the growth of *B. rouxiae* in association with the different cotton cultivars used in this study. However, it should be noted that the quantity of exudates produced by each cultivar in this study was not measured. While there was no observable difference in the rates of germination and growth of the different cultivars, it cannot be ruled out that some cultivars may have produced more or less exudates than others, which in turn could have influenced fungal growth. Quantification of root exudates from different cultivars would be an interesting avenue for future study. Given the significant role played by root exudates

in fungal germination growth, a potential strategy to control this pathogen could be to block access to the plant signalling molecules, which may help prevent germination and growth towards cotton roots.

Appling *A. brasilense* strains between cotton seedling and pathogen showed that *A. brasilense* was able to block the growth towards cotton seedling, but it was not directly antagonistic. Four hypotheses could explain this suppression of hyphal growth, despite the presence of susceptible host root seedlings. First, *A. brasilense* could stimulate the cotton root to produce an active toxic compound that inhibits *B. rouxiae* BRIP40192 growth directly. Secondly, *A. brasilense* could produce a chemical compound that causes a physiological change in cotton roots that blocks the production of specific signalling molecules that trigger spore germinations and hyphae directional growth. Thirdly, cotton roots could induce *A. brasilense* to produce an active antifungal compound that inhibits fungal growth, or finally, *A. brasilense* may utilise compounds produced by cotton roots, which play a role as signalling molecules or as nutritional support for fungal growth, and essentially deplete these resources, leaving the fungi unable to grow. While the mechanism is as yet unknown and each of these possibilities needs further investigation, the results of this study suggest that *A. brasilense* strains can act as a biological barrier to prevent *B. rouxiae* BRIP40192 from interacting with cotton roots and causing infection, and thus it is of significant interest as a biological control.

While *A. brasilense* was able to block the growth of *B. rouxiae* towards cotton seedlings, the two organisms showed no antagonistic activity in dual assays. Therefore, to test what sort of interactions may be taking place instead, three tests were performed to examine direct interactions between bacterial cells and fugal hyphae. In liquid culture *A. brasilense* strains tended to make a weak attachment with fungal

hyphal. A slide agar technique, designed in this work, showed that *A. brasilense* was able to move along channels created by hyphae during growth in semisolid agar medium. However, among the three *A. brasilense* strains, only one out of 10 replicates of *A. brasilense* Sp245 showed a positive result in the fungal highway experiment, indicating that this bacteria does not have the ability to travel for long distances along fungal hyphae, at least not in *in vitro* culture. Bianciotto *et al.* (2001) studied the attachment of *A. brasilense* wild-types and *A. brasilense* EPS partial mutants to the arbuscular mycorrhizal structure. *A. brasilense* wild-types established a compact bacterial biofilm on both hyphal structure and spores, while a few cells of *A. brasilense* EPS mutants observed anchoring on the fungal structure surfaces. These results appear to be in agreement with the present study, where *A. brasilense* was observed to attach to fungal hyphae, even if unable to move along the hyphae for long distances.

A. brasilense is well known as a plant growth promoting bacteria, which has a positive effect on plant growth and crop production under suitable environmental conditions (Oda and Vanderleyden, 2000; Sadasivan and Neyra, 1985). Oxygen represents one of the environmental factors that has a significant impact on *Azospirillum* behaviour in soil (Zhulin *et al.*, 1996). High oxygen concentration is considered as a metabolic stress to *A. brasilense*, by blocking/impeding its oxidative metabolism (Bible, 2012; Bible *et al.*, 2008; Blaha and Schrank, 2003). Under aerotaxic conditions, *A. brasilense* undergoes a vegetative transformation into an alternative form that allows for long-term survival, (Qi *et al.*, 2013; Russell *et al.*, 2013). The effect of oxygen stress was investigated in this study to assess how it impacts on *A. brasilense* growth. Under reduced oxygen conditions, the bacteria the flocculation decreased. These observations reveal that oxygen plays a vital role in *A. brasilense* flocculation, which in turn will reduce impact on its ability to act as a

biocontrol. To interact with and move along fungal hyphae, and to influence plant growth, *A. brasilense* needs to be in an active, motile state, rather than in a dormant, cyst like form. Oxygen concentrations in the rhizosphere will impact on this, with low oxygen resulting in increased flocculation, and therefore potentially less biocontrol activity.

The results of this chapter demonstrate that different cotton cultivars vary in their release of extracellular signalling molecules, which impacts on the germination and growth of *B. rouxiae* BRIP40192. They also demonstrated that *A. brasilense* strains could block the growth of *B. rouxiae in vitro.* While growth was suppressed, there was no direct antagonism between these organisms; instead, this effect was possibly due to *A. brasilense* accessing the same signalling molecules, released from cotton roots, as the pathogen. However, it is important to note that a positive interaction between a candidate biocontrol agent and the pathogen *in vitro* does not mean that it will be an effective biological control *in vivo*. Further investigation of these interactions in conditions mimicking the soil environment is required. Here we have investigated the impact of one such environmental factor, oxygen concentration, and showed that it will impact on the ability of *A. brasilense* to grow and remain active in the soil environment, which should be taken into consideration when considering this bacteria as a biological control.

Chapter 5: The role of border cells in microbial-plant interactions

5.1. Introduction

Root microbiomes, influenced by plant root secretions, have an impact on plant growth and productivity. The biochemical interaction between plant roots and soil microbes is mediated by the chemical compounds released by plant roots (root exudates) combined with the metabolic activity of the soil microbial community (Nardi *et al.*, 2000). These biochemical interactions can either repel or attract members of the microbial community that surround the root (Huang *et al.*, 2014). In addition to root exudates, thousands of active cells are released daily by each single root tip (Hawes *et al.*, 2016; Hawes and Pueppke, 1986).

Cells sloughed from the root cap of growing plants are known as border cells, and are considered as one of the driving forces behind plant-soil-microbial interactions in the rhizosphere. Border cells are specialized root cells that detach from the root cap meristem while embedded in mucilage, surrounding the root surface. These cells contain essential components such as mitochondria, Golgi stacks and Golgi-derived vesicles (Doornbos *et al.*, 2012; Hawes *et al.*, 2012; Stubbs *et al.*, 2004), and can remain viable in rhizosphere for one week or more (Vermeer and McCully, 1982). The number of border cells that are released from root caps varies across plant species. For example, the number of border cells produced by *Gossypium hirsutum* (Malvaceae) cultivars ranges from 6000 to 16000 cells per root tip of a germinated seeds (Knox *et al.*, 2020; Knox *et al.*, 2007), while *Pisum sativum* (Fabaceae), *Cucumis melo* (Cucurbitaceae) and *Capsicum annum* (Solanaceae) produce approximately 4500,

2500 and 100 border cells per root tip, respectively (Groot *et al.*, 2004; Hamamoto *et al.*, 2006; Hawes *et al.*, 2002).

Consequently, border cells have the potential to interact with microflora in the rhizosphere (Hawes *et al.*, 1998), and hence shape plant growth, due to their involvement in root-microorganism interactions (Brigham *et al.*, 1995; Morgan *et al.*, 2005; Wen *et al.*, 2007). Border cells attract beneficial soil microorganisms that support plant growth through the production of growth promoting hormones and nutrient supplements (Chaparro *et al.*, 2012). On the other hand, border cells can also inhibit plant diseases by entrapping pathogenic bacteria, fungi and nematodes, using the mucilage surrounding roots (Doornbos *et al.*, 2012). Border cells of cotton show a specific response to *Pythium dissotocum* (Goldberg *et al.*, 1989), while pea roots showed an enhanced ability to produce border cells and mucilage to cover the root tips when exposed to the pathogen *Nectria haematococca* (Gunawardena *et al.*, 2005; Wen *et al.*, 2007), and they also inhibit the development of *Aphanomyces euteiches* (Cannesan *et al.*, 2011). Moreover maize root cap cells can elicit a defensive response to *Colletotrichum graminicola* (Sherwood, 1987).

Pea *(Pisum sativum)* border cells release chemicals that effect expression of the virE gene, important in *A. fumefaciens* pathogenesis and expression of nod genes from *R. leguminosarum* bv *viciae* and *R. melilofi* (beneficial bacteria) (Zhu *et al.*, 1997). Hawes *et al.* (2012) reported that extracellular root tip DNA might also support plant pathogen defence by trapped pathogen. Extracellular root tip DNA released by root tips was found to be different between six cotton cultivars (Knox *et al.*, 2020). The ability of border cells and extracellular DNA to trap lead (Pb) to a nontoxic level have also been studied (Huskey *et al.*, 2018; Huskey *et al.*, 2019).

Understanding the interactions between pathogens and host, and with other microorganisms in the rhizosphere, may lead to new methods to reduce the incidence of BRR disease (Pereg, 2014). *Azospirillum brasilense* was found to be suppress the growth of *Berkeleyomyces rouxiae* toward cotton seedling (in this study, chapter 4), despite not being directly antagonistic to the pathogen. The primary goal of this study was to understand and further investigate the interaction taking place between *A. brasilense* and *B. rouxiae* BRIP4019 and cotton border cells. To determine if these interactions are specific to cotton border cells, interactions of fungi and bacteria with wheat border cells was also considered. Other bacterial and fungal isolates from cotton farms, which had shown an ability to suppress *Berkeleyomyces* spp., were also tested to determine if there was any interaction between them and cotton and wheat border cells, with a view to improving our understanding of potential biocontrol options for *Berkeleyomyces* spp.

5.2. Methodology

5.2.1. Culture conditions for bacterial isolates

The bacterial strains used in this study are listed in Table 5.1. Complete medium was nutrient broth (NB, Difco) for all bacteria except, *A. brasilense* strains, which were grown on minimal medium containing 10 mM lactate (Galimand *et al.*, 1989) or nitrogen-free malate medium (Sriskandarajah *et al.*, 1993), supplemented with 20 mM of ammonium chloride and 40 μg/ml Congo red. The preparation of different culture media is described in Appendix A. In general, bacteria were cultured for 18 hrs at 28°C.

Table 5.1: Bacterial isolates used in this chapter

Bacterial strain	Genotype or phenotype[a]	Reference
Azospirillum basilense - Sp7 - Sp245 - Sp72002	 Wild-type Wild-type Tn5 induced mutant of Sp7, CR^-, $Floc^{-*}$	 (Baldani *et al.*, 1983; Tarrand *et al.*, 1978) (Pereg-Gerk *et al.*, 1998)
Actinobacteridae bacterium - 1TB	 Wild-type	Lily Pereg lab, University of New England, Australia These isolates isolated from cotton farms (2.2.1) showed *Berkeleyomycess* spp. suppressiveness (personal communication, results unpublished)
Bacillus aryabhattai - 8SC - $CNT3^h$	 Wild-type Wild-type	
Bacillus cereus - CNT15	 Wild-type	
Bacillus subtilis - XNT4	 Wild-type	
Agrobacterium tumefaciens - 9P3B	 Wild-type	
Pseudomonas aeruginosa - 6BF - 11CG	Wild-type	
Pseudomonas orryzihabitans - 3P3H	Wild-type	
Enterobacter cloacae - 6BG	 Wild-type	
Streptomyces luteogriseus - $CNT26^h$	Wild-type	

[a] CR, Congo red binding; Floc, flocculation

5.2.2. Culture conditions for fungal isolates

The fungal isolates used in this study are listed in Table 5.2. Stock cultures of fungal isolates were maintained in glycerol (20%) and stored at -80°C and subcultured every 6 months on 1/2 strength potato dextrose agar (1/2 PDA) containing 1.5% agar. Fungi were cultured for 5 days at 25°C with working cultures maintained at 4°C until needed.

Table 5.2: Fungal Isolates used in this chapter

Fungal Stains	Genotype or phenotype	Reference
Berkeleyomyces rouxiae BRIP40192 (isolated from Cotton).	Wild-type	Jan Dean, Dept. of Primary Industries, OLD govt.
Trichoderma - *atrovirdie* DB-T7 - *viride* SH2 - *harzianum* BK35 - *gamsii* UASWS1428	Wild-type	Lily Pereg lab., University of New England, Australia These isolates isolated from cotton farms (2.2.1) showed *Berkeleyomycess* spp. suppressiveness (personal communication, results unpublished)
Penicillium decumbens - 16A-3	Wild-type	
Penicillium citrinum - 14A-1	Wild-type	
Aspergillus terreus - ATE1	Wild-type	
Clonostachys rosea - 30T	Wild-type	
Fennellia flavipes - isolate16C-1	Wild-type	
Thysanophora longispora - 913B	Wild-type	
Fusarium solani - 48X3-P0-P7-2	Wild-type	
Talaromyces pinophilus - 16F-10	Wild-type	

5.2.3. Inoculum preparation

Reproduction structures, including spores, conidia, macroconidia and endoconidia were harvested from fungal isolates (Table 5.2), as described in section 3.2.4.

5.2.4. Seed cultivars and sterilisation

Cotton cultivars used in this study are described in section 3.2.5 and seed sterilisation in section 3.2.6.

Durum wheat (*Triticum durum,* cultivar Wollaroi) seeds were obtained from University of New England. Sterilisation was carried out as described by Zeman *et al.* (1992): wheat seeds were sterilised by soaking in Tween 20 detergent (1 ml/L of sterile distilled water) for 1 minute, under vacuum. Seeds were then washed with sterile distilled water four times. Seeds were then soaked in 0.5% $HgCl_2$ for 1.25 minute under vacuum, before rinsing five times with sterile distilled water, using a vacuum/air cycle of 30 seconds for each wash. Sterilised seeds were allowed to germinate on yeast mannitol plates at 25°C for 2 days in dark.

5.2.5. Preparation of border cells

Border cells were prepared following methods described by Knox *et al.* (2007). 2-3 days following germination, cotton and wheat seedlings were recovered and the terminal 15-20 mm of radicles was immersed in 1 ml of sterile deionised water in a 1.5 ml microtube (for cotton seedlings), or in 100 µl of sterilised deionised water in a 0.5 ml tube (for wheat seedlings). The root tips were allowed to imbibe for 5 minutes then agitated with 10 repeated flushes of either 100 or 20 µl volumes, for cotton and wheat, respectively. The seedling's radicle was removed, and the resulting suspension examined microscopically to confirm successful collection of border cells. Cotton and wheat border cells illustrated in Figure 5.1.

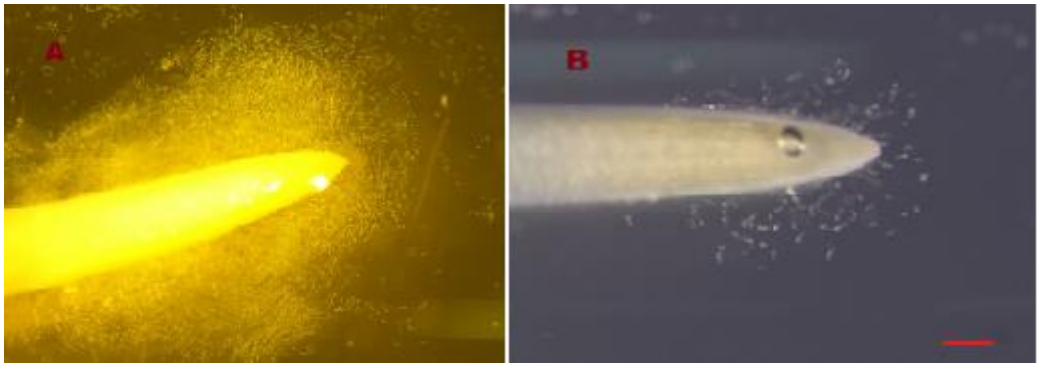

Figure 5.1: Border cells have dispersed away from the root tip after immersion in deionized distilled water for 30 sec. (A) cotton border cell Sicot 730 and (B) wheat border cell. Bar marker=3 mm (Image: this study).

5.2.6. Bacterial Binding assays:

Bacterial binding assays were carried out as described (Curlango-Rivera *et al.*, 2013; Hawes and Pueppke, 1987) with some modifications. A 100 µl suspension of border cells (cotton or wheat) was adjusted to a density of 2.5 X 10^2 cells/ml and placed into 0.5 µl microtube. Log phase bacterial cultures (150 µl of OD_{600} 0.5) were added to the border cells, mixed and incubated at 20°C. Attachment of bacteria to border cells were detected in intervals by direct microscope (Nikon Clipse 50i, Japan) examination. After 2 hrs of incubation at room temperature, each border cell/bacteria suspension was stirred, and attachment examined microscopically.

From each cotton seedlings border cell suspension were prepared in triplicate (100 µl of 2.5-3 X 10^2), followed by preparing slides from each treatment in order to count at least 550 border cells from each seedling to calculate the percentage of the bacterial attachment to these border cells, via count the border cells had at least a few bacteria attached divided by the total border cells. Wheat border cells (250-300 cell from each seedling) were similarly prepared and at least 550 border cells counted to calculate the percentage of bacterial attachment to these border cells. Attachment was recorded as positive when at least a few bacterial cells were attached the border cell surface. This

experiment was repeated three times, with 15 cotton seeds and a 15 wheat seeds used each time.

5.2.7. Timing of interactions between bacteria and border cells

To determine the specific time that bacterial isolates start to attach to cotton (Sicot 730) and wheat border cells, a 100 µl samples of border cells, containing 2.5-3 X 10^2 cells/ ml, were placed into sterile one concave slides. 100 µl of bacterial suspension containing 1 X 10^6 cfu/ml was added and covered with a sterile cover slip and monitored directly under a microscope (Nikon Clipse 50i, Japan). The time it took for the bacteria to start to attach to border cells was noted. For each bacterial isolate, the reaction was repeated using border cells collected from 10 seedlings of cotton and wheat.

5.2.8. Stability of bacterial attachment to border cells

To determine the stability of the attachment of *A. brasilense* strains to border cells, the bacterial/border cell suspension was incubated for 1, 2, 3, 4, 5 and 6 hours at 20°C and then agitated in a 1.5 ml micro-tube by vortexing at high speed for 60 seconds. For each period, a slide of the culture was prepared and examined under the light microscope (Nikon Clipse 50i, Japan) to determine the stability of the attachment by comparing the number of bacteria adhering to the border cell post incubation with those initially attached.

5.2.9. Role of extracellular DNA in *A. brasilense* strains attachment to border cells

To determine if extracellular DNA was involved in the attachment of *A. brasilense* strains to border cells, DNase I was added the bacteria/border cell suspensions at a concentration of 1 unit per 10 µl of suspension. Attachment of bacteria to border cells was observed by microscopic examination at 1, 2, 3, 4, 5 and 6 hours of incubation at approximately 20°C. Microscope slide prepare and examined under the light microscope (Nikon Clipse 50i, Japan) to determine the stability of the attachment.

5.2.10. Fungal spore interaction with border cells:

Fungal binding assays were carried out as described for bacterial binding assays, but using a suspension of endoconidia in place of bacterial suspensions. Briefly, 100 µl suspension of border cells was adjusted to 2.5 X 10^2 cells/ml were placed into 0.5 µl microfuge tube. Spore suspension (100µl, 1 X 10^5) were added to the border cells in each microfuge tube. After 12 hrs of incubation at approximately 20°C, the suspension of each microfuge tube was stirred with a micropipette and attachment of spores to border cells detected by microscopic examination. This experiment was repeated three times, with 15 cotton seeds and a 15 wheat seeds used each time.

5.3. Results

5.3.1. Bacterial Binding assay

Out of the ten bacterial strains tested (in addition to *A. brasilense*), four demonstrated the ability to attach to cotton (Sicot 730) border cells. The percentage of border cells had bacteria attached was calculated after incubation for 2 hrs. Results

(summarised in Table 5.3) showed that all *A. brasilense* strains and *Bacillus subtilis* were able to completely attach to border cells. Other bacteria exhibiting attachment were *Pseudomonas orryzihabitans* (16%), *Actinobacteridae bacterium* (15%) and *Agrobacterium tumefaciens* (17%) Typical results from these binding assays are illustrated in Figure 5.2). In contrast, *Bacillus aryabhattai*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* strain SMVIT, *Streptomyces luteogriseus* and *Enterobacter cloacae* all failed to attach.

Table 5.3: Binding assay of soil bacterial attachment to cotton (Sicot 730) border cells at 20˚C after 2 hours.

Bacterial strains	**Percentage of* attachment**	**Time for bacteria to start attaching**
***Azospirillum brasilense* Sp7**	100%	1 minute
***Azospirillum brasilense* Sp72002**	100%	1 minute
***Azospirillum brasilense* Sp245**	100%	Seconds
Bacillus subtilis	100%	5 minutes
Bacillus aryabhattai	-	-
Bacillus cereus	-	-
Pseudomonas aeruginosa	-	-
Pseudomonas orryzihabitans	16.4%	5 minutes
***Pseudomonas aeruginosa* strain SMVIT**	-	-
Actinobacteridae bacterium	14.7%	5 minutes
Streptomyces luteogriseus	-	-
Agrobacterium tumefaciens	17%	5 minutes
Enterobacter cloacae	-	-

*Percentage of cells with bacteria attached.

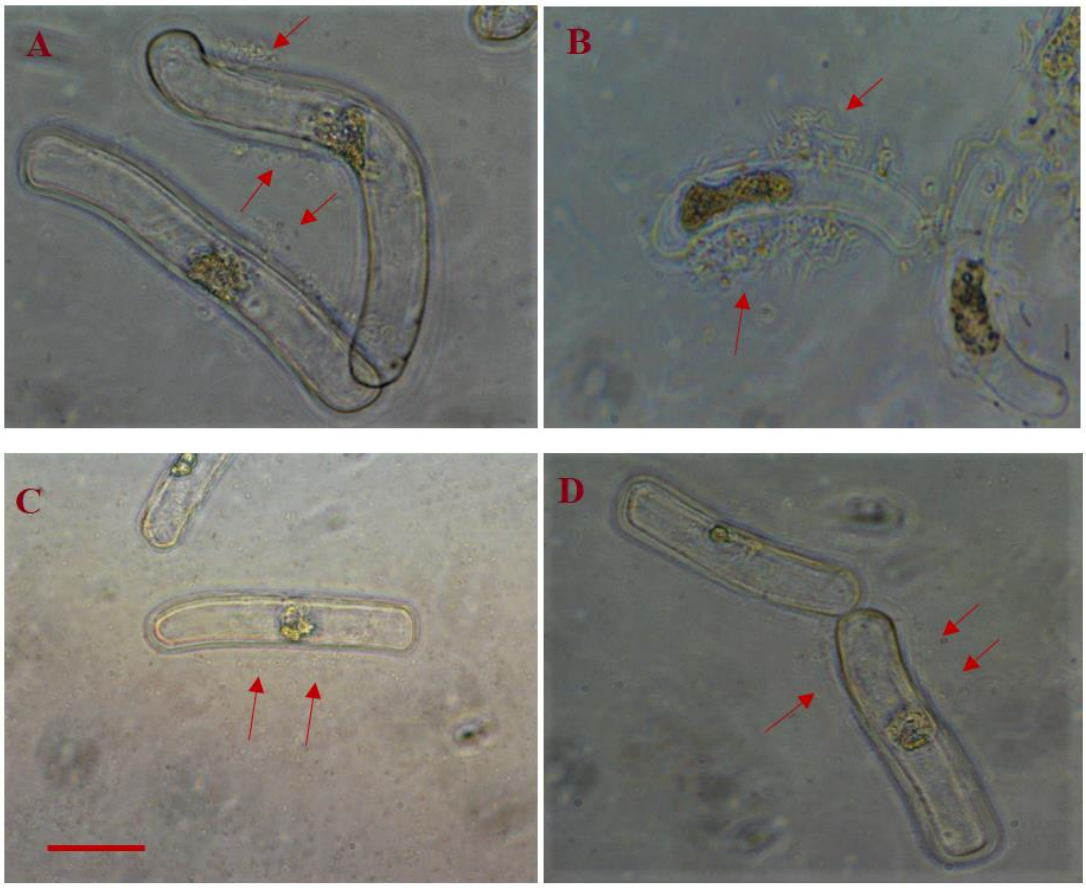

Figure 5.2: Typical results of bacterial binding assays using border cells of cotton (Sicot 730) after 2 hrs incubation at 20°C. *Actinobacteridae bacterium* (A), *Bacillus subtilis* (B), *Pseudomonas orryzihabitans* (C) and *Agrobacterium tumefaciens* (D). Red arrows show bacteria attached to border cells. Bar marker = 10 µm

Out of the additional ten bacterial strains tested only *Bacillus subtilis* and *Pseudomonas orryzihabitans* had the ability to attach to wheat border cells. The percentage of attachment after two hours incubation is shown in Table 5.4, with 100% of wheat border cells attached with *Bacillus subtilis* and 81% of wheat border cells attached with *Pseudomonas orryihabitans*. Typical results of these binding assays are shown in Figure 5.3. There was no attachment detected for *Bacillus aryabhattai, Bacillus cereus, Pseudomonas aeruginosa, Pseudomonas aeruginosa* strain SMVIT, *Actinobacteridae bacterium, Streptomyces luteogriseus, Agrobacterium tumefaciens* and *Enterobacter cloacae*.

Table 5.4: Binding assay of bacterial attachment to wheat border cell

Bacterial strains	Percentage of* attachment	Time for bacteria to start attaching
***Azospirillum brasilense* Sp7**	100%	1 minute
***Azospirillum brasilense* Sp72002**	100%	1 minute
***Azospirillum brasilense* Sp245**	100%	1 minute
Bacillus subtilis	100%	5 minutes
Bacillus aryabhattai	-	-
Bacillus cereus	-	-
Pseudomonas aeruginosa	-	-
Pseudomonas orryzihabitans	81.25%	5 minutes
***Pseudomonas aeruginosa* strain SMVIT**	-	-
Actinobacteridae bacterium	-	-
Streptomyces luteogriseus	-	-
Agrobacterium tumefaciens	-	-
Enterobacter cloacae	-	-

*Percentage of cells with bacteria attached.

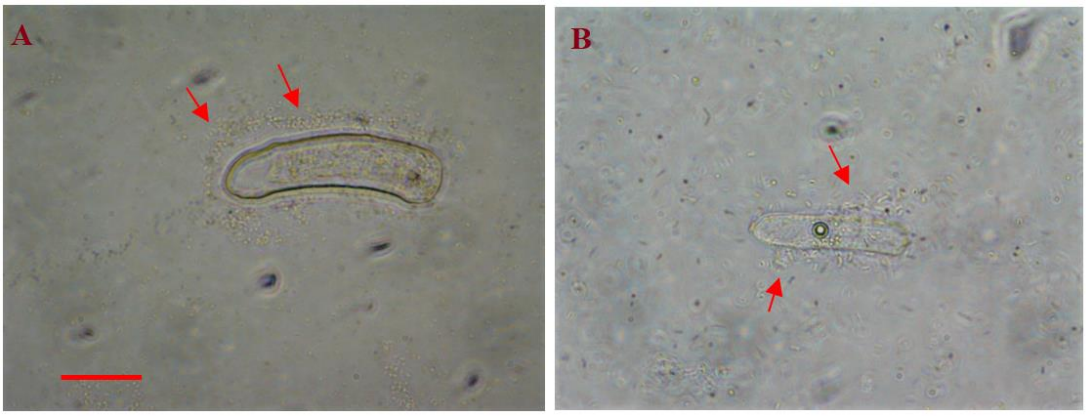

Figure 5.3: Assay of soil bacterial binding to wheat border cells, *Pseudomonas orryzihabitans* (A), *Bacillus subtilis* (B). After 1 hrs of incubation at 20°C. Red arrows show bacteria attached to border cells. Bar marker=10 µm

5.3.2. Attachment of *A. brasilense* to border cells

All *A. brasilense* strains (Sp7, Sp245 and Sp72002) showed the ability to attach to both cotton (Sicot 730) and wheat border cells (Figures 5.4, part A and part B). *A. brasilense* strains showed motility towards border cells after one minute in culture. The number of bacteria attached to border cells was observed to quickly

increase over time, until they formed a biofilm layer covering the border cell surface. After 2 hours of incubation all *A. brasilense* in the suspension had attached to border cells, completing surrounding these cells.

Border cells of different cotton cultivars (Sicot 71BRF, Sicot 74BRF, Sicot 43BRF and Sicot 714B3F) were also tested and the results showed a similar pattern of interaction with *A. brasilense* strains (Sp7, Sp245 and mutant Sp72002) as Sicot 730.

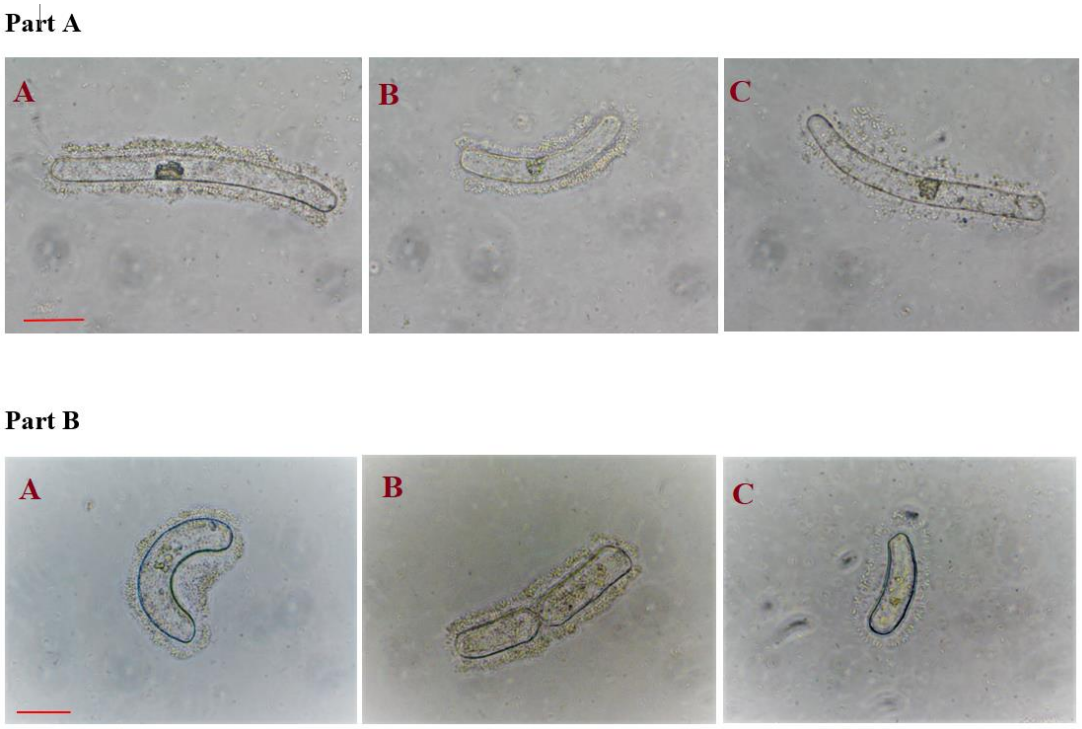

Figure 5.4: Part A: Assay of binding *Azospirillum brasilense* strains to cotton (sicot 730) border cells, strain Sp7 (A), strains Sp72002 (B) and strain Sp245 (C). After 1 hrs of incubation at 20°C. Bar marker=10 µm. Part B: Assay of binding *Azospirillum brasilense* strains to wheat border cells, strain Sp7 (A), strains Sp72002 (B) and strain Sp245 (C). After 1 hrs of incubation at 20°C Bar marker=10 µm

To test the strength of bacterial attachment, cultures were disrupted by vortexing. Vortexing the cell suspensions at high speed did not detach *Azospirillum* from the border cells of either cotton or wheat. Treatment with DNaseI did not prevent the attachment of *A. brasilense* to cotton or wheat border cells, liberate the trapped *A.*

brasilense from the border cells. *Bacillus subtilis* XNT4, which also demonstrates the ability to attach to border cells, was used as a control; When DNaseI was added, *B. subtilis* XNT4 was prevented from attaching to border cells.

5.3.3. Fungal isolates interaction with border cells

The ability of fungal isolates to attach to cotton and wheat border cells after 12 hrs were illustrated in Table 5.5. For cotton border cells, five out of fifteen fungal strains tested were able to directly attach to border cells. These fungi were *P. decumbens* strain 16A-3, *P. citrinum* strain 14A-1, *F. flavipes* isolate16C-1, *T. longispora* strain 913B and *V. dahliae* VCG2A. Attachment of each of these strains to cotton border cells is shown in Figure 5.5. For wheat border cells, three out of fifteen fungi showed the ability to attach to border cells. These fungi were *C. rosea* strain 30T, *F. flavipes* isolate16C-1 and *T. longispora* strain 913B as illustrated in Figure 5.6.

Table 5.5: Interaction of cotton (Sicot 730) and wheat border cells and fungi after 12 hrs of incubation at 20°C, (+) fungi attached to border cells, (-) fungi not attached to border cells

Fungal strains	Border cells	
	Cotton border cells	Wheat border cells
B. rouxiae BRIP40192	-	-
Penicillium decumbens strain 16A-3	+	-
Penicillium citrinum strain 14A-1	+	-
Aspergillus terreus strain ATE1	-	-
Clonostachys rosea strain 30T	-	+
Fennellia flavipes isolate16C-1	+	+
Thysanophora longispora strain 913B	+	+
Fusarium solani strain 48X3-P0-P7-2	-	-
Talaromyces pinophilus strain 16F-10	-	-
T. atrovirdie DB-T7	-	-
T. viride SH2	-	-
T. harzianum BK35	-	-
T. gamsii UASWS1428	-	-
F. oxysporum sp.	-	-
V. dahliae VCG2A	+	-

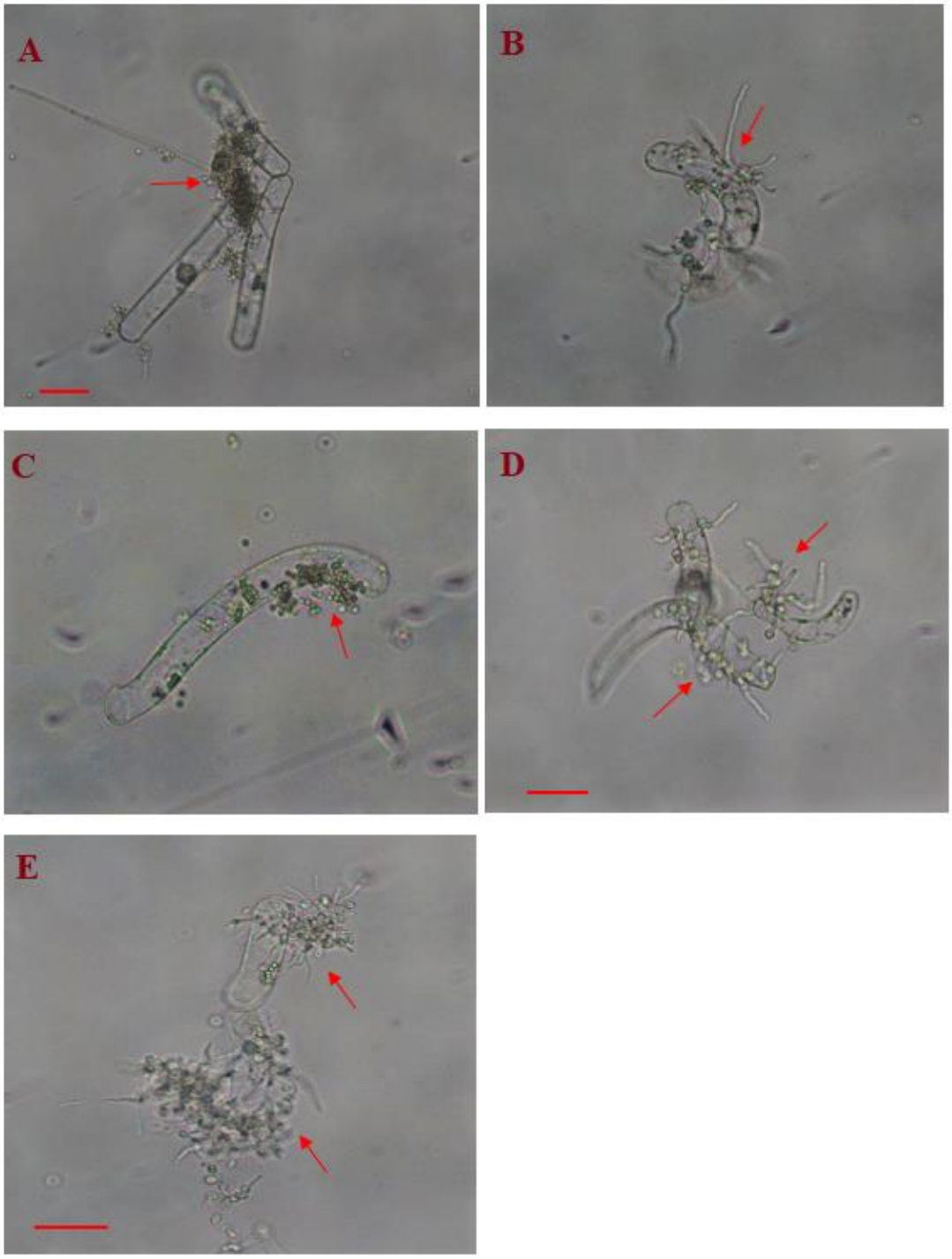

Figure 5.5: Assay of soil fungal binding to cotton (Sicot 730) border cells, *Penicillium decumbens* strain 16A-3 (A), *Thysanophora longispora* strain 913B (B), *Fennellia flavipes* isolate16C-1 (C), *Penicillium citrinum* strain 14A-1 (D) and *V. dahliae* VCG2A (E). After 12 hrs of incubation at 20°C. Red arrows show fungi attached to border cells. Bar marker=10 µm

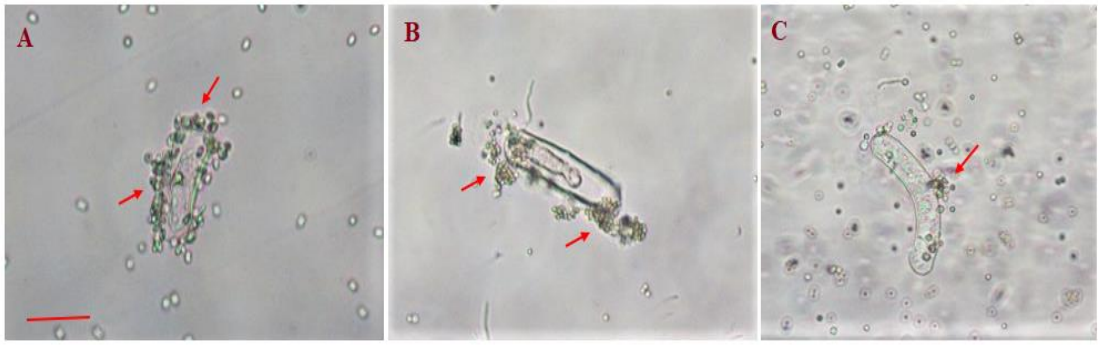

Figure 5.6: Assay of soil fungal binding to wheat border cells *Clonostachys rosea* strain 30T (A), *Fennellia flavipes* isolate16C-1 (B and *Thysanophora longispora* strain 913B (C). After 12 hrs of incubation at 20°C. Red arrows show fungi attached to border cells. Bar marker=10 µm

5.3.4. *B. rouxiae* BRIP40192 interaction with border cells

No clear interaction was observed between *B. rouxiae* BRIP40192 and either cotton (Sicot 730) or wheat border cells in the binding assay. However, when the reaction was examined over a period of 12 hrs, and then after 24 hrs, it was noted that in a few instances, some *B. rouxiae* endoconidia germinated, and hyphal extensions started to grow toward cotton border cells. Where hyphal and border cell contact was made, it was noted that there was no evidence of any penetration, instead hyphae were observed to grow around or on the surface of border cells, as illustrated in Figure 5.7.

Border cells of different cotton cultivars (Sicot 71BRF, Sicot 74BRF, Sicot 43BRF and Sicot 714B3F) were also tested and showed no interaction with *B. rouxiae* BRIP40192.

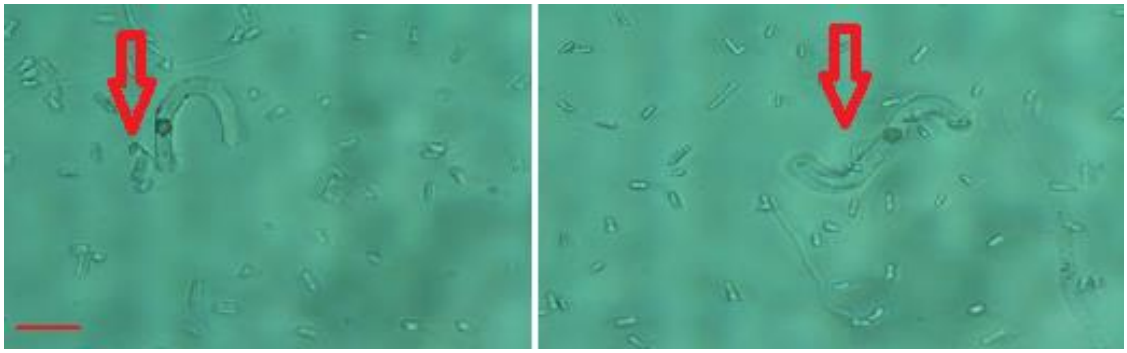

Figure 5.7: Assay of binding of *B. rouxiae* BRIP40192 to cotton border cells. After 12 hrs of incubation at 20°C Bar marker=10 µm. Arrows showed that some endospores start to grow towards the border cells but do not penetrate

5.4. Discussion

It is clear that border cells have the potential to influence the interactions between plants and microbial communities in the rhizosphere (Brigham *et al.*, 1998; Hawes *et al.*, 2000). Being able to manipulate these interactions, through a more detailed understanding, could provide a basis for the control and management of some plant pathogens. This chapter investigated the interactions between cotton and wheat border cells with *A. brasilense* strains, identified as a potential biological control agent, *and B. rouxiae*. BRIP40192, an important soil-borne pathogen that impacts on Australian cotton production.

The presence of different flagellation in *Azosprillum* species, especially *A. brasilense*, make this bacteria highly motile (Bashan and Levanony, 1990; Brigham *et al.*, 1998; Hall and Krieg, 1983; Hawes *et al.*, 2000; Krieg and Döbereiner, 1984). *Azosprillum* strains have previously demonstrated positive chemotaxis in response to root exudates (Bashan and Levanony, 1987, 1990; Heinrich and Hess, 1985), which helps to determine the orientation of *Azosprillum* migration toward roots and further root colonisation (Bashan, 1986; Bashan and Holguin, 1994; Broek and Vanderleyden, 1995; Cocking, 2003). The findings in this study provide further support that root

exudates, and specifically those of border cells, play a role in attracting *A. brasilense* strains towards cotton and wheat, and could potentially influence the ability of these bacteria to promote plant growth and/or supress pathogen interactions with roots.

Wild-type strains of *A. brasilense* Sp7 (a root surface coloniser) and *A. brasilense* Sp245 (a root surface and tissue coloniser). Schloter and Hartmann (1998) showed a strong interaction with both cotton and wheat border cells. It is possible that border cell secretions and/or surface materials may activate biofilm formation and exopolysaccharide production by *A. brasilense* strains, allowing them to attach to the border cell surface. The role of the sugar arabinose in exopolysaccharide (EPS) and capsular polysaccharide (CPS) composition in *A. brasilense* has previously been described as playing an important role in cell aggregation (Burdman *et al.*, 2000). Root exudates have been proposed to increase the percentage of arabinose in EPS of *A. brasilense* Cd, which is then associated with cell flocculation, attachment and colonisation processes (Fischer *et al.*, 2003). The expression of outer membrane proteins of *Azospirillum* spp. has also been found to be influenced by root exudates (Van Bastelaere *et al.*, 1993). In the current study, it was observed that *A. brasilense* Sp72002 (a *flcA*$^{-}$ mutant) showed the same attachment to cotton and wheat border cells as the wild-type *A. brasilense*. Previously the *flcA* gene has been described as being the regulatory gene responsible for *A. brasilense* flocculation and attachment to plant roots (Pereg-Gerk *et al.*, 1998). The observation that the *flcA* mutant is able to attach to border cells suggests that attachment is not controlled by this gene, but instead either by the specialized structure of border cells surface, in comparison to other root cells, or to the specific signalling molecules produces by border cells. Border cells produce more than 100 extracellular proteins (Wen *et al.*, 2017; Wen *et al.*, 2007) which may trigger genes, other than *flcA,* and control attachment to border cells (Burdman *et al.*,

2000; Michiels *et al.*, 1991). Attachment of *A. brasilense* strains which previously showed the ability to block/inhibit *B. rouxiae.* BRIP40192 growth (Chapter 4) to both cotton and wheat border cells give an indication that border cells might support the interaction between beneficial microbes and cotton and wheat roots. They may create a form of biological barrier around the roots, and in this way directly influence other organisms in the root microbiome.

Root border cells have also been described as playing a role in trapping of pathogens and preventing them from reaching plant roots (Hawes *et al.*, 2016; Hawes *et al.*, 2012). Border cells of cotton (Sicot 730) and wheat showed no clear interaction with *B. rouxiae* BRIP40192. In spite of this, examination of the reaction with time under constant condition revealed the occasional observation of spore germination and fungal growth towards cotton border cells. These results may be either a normal growth response of *B. rouxiae* hyphae in the suspension or a response to a chemical or DNA based signal from the cotton border cells, which stimulated fungal hyphae growth toward these cells.

This study also tested a number of bacterial and fungal strains that had previously been isolated from the soil of cotton farms showing reduced incidence of BRR disease (disease suppressive soils). The ability of these strains to interact with cotton and wheat border cells was mixed. Some of these strains from disease suppressive soils were able to interact with border cells, and these could be potential candidates for further investigation, alongside the bacteria *A. brasilense*. The interaction of some of these soil isolates with cotton and wheat border cells reflects the importance of border cells in plant microbial interaction, via either trapping of pathogens, or by influencing the microbial diversity in the rhizosphere. However, it should be noted that a number of serious cotton pathogens, including *Fusarium* and

Verticillium species, did not show any attachment or interaction with cotton or wheat border cells. The mechanisms by which plant and pathogen interact are highly complex, and clearly many pathogens can interact with plants in ways that do not involve border cells. The lack of interaction between *B. rouxiae* (and these other fungal pathogens) and cotton/wheat border cells suggests that plants may use other mechanisms, rather than border cell trapping, to reduce disease.

Chapter 6: General discussion and conclusion

6.1. General discussion

Black root rot disease is a wide spread, soil-borne disease, which impacts Australian cotton production, causing significant loss of yield (Nehl *et al.*, 2004). Currently there are no effective methods used to stop the spread or incidence of the disease (Pereg, 2014) . This thesis is part of larger project that aims to identify methods that could be used to prevent or minimise the impact of BRR disease on the Australian cotton industry. Ideally, an effective, environmentally sustainable control method could be identified to allow producers to control pathogen growth in soil and prevent loss of yield due to infection. To do so, this thesis first aimed to investigate the presence of *Berkeleyomyces* spp. in soils from Australian cotton farms, and understand the relationship between soil properties, farm management practices and pathogen abundance (Chapter 2). It then aimed to increase understanding of how soil temperature and different cotton cultivars may influence the abundance of this pathogen in soils, and the severity of black root rot disease in cotton seedlings (Chapter 3). Given the drive to identify an effective control method for this pathogen, this thesis then aimed to assess the potential for the plant growth promoting bacteria *Azospirillum brasilense* to act as a biological control agent against black root rot disease (Chapter 4). Having identified that *A. brasilense* showed some ability to block the growth of *B. rouxiae* towards cotton seedlings, the final chapter of this thesis then aimed to further investigate this process, and to determine if the border cells of plants may play a role in the interactions between cotton, *A. brasilense*, and *Berkeleyomyces* spp. (Chapter 5).

Detection of the BRR pathogen in soils before and after planting is an essential step in disease control and helping to minimise disease severity. Rapid detection, before the appearance of symptoms, is important to enable management plans to be activated to stop or reduce the spread of disease. Molecular methods, which are highly specific and efficient, are now being used widely to detect plant pathogens (Atkins and Clark, 2004; McCartney *et al.*, 2003). These tools can also be used to track pathogen loads in soils over time, and could be useful in determining the effectiveness of any pathogen control methods. Throughout this thesis PCR, or qPCR, was used to detect and quantify *Berkeleyomyces* spp. in soils. While this technique is rapid and efficient, it should be mentioned that this technique cannot distinguish between different fungal forms (spores or vegetative cells), or even between live and dead fungal material. However, this technique does overcome many of the limitations of traditional culture-based techniques, and can be applied in analysing and tracking pathogen in soils from the same farm across seasons and years, to feed in to a BRR disease management plan.

Cultural methods are one of the management strategies often followed by cotton growers in an attempt to minimise the incidence of BRR disease (Abawi and Widmer, 2000; Nehl *et al.*, 2004). One cultural method suggested is the use of organic material. However, in contrast to previous studies, in this study farms amended with organic fertiliser (manure and compost) showed a higher abundance of the BRR pathogen compared with farms amended with mineral fertilisers. The variability in pathogen numbers could be attributed to the ability of organic fertiliser to support the growth of fungal pathogen by supplying essential nutrients. While organic fertilisation has been suggested to help control plant soil-borne pathogen, (Bonanomi *et al.*, 2010), many other farming practices and soil parameters should also be considered. Results

presented in this thesis suggest that factors such as trace element concentrations in soil may also play a role in influencing the loading of pathogen in soil. Rotation of cotton with other crop species is also often recommended to producers as a method to help control incidence of BRR disease. However, many questions still remain to be answered about the role of crop rotation in disease control. While wheat is often recommended as a rotation crop, the results of this thesis did not support the notion that rotation with wheat reduced BRR disease. Instead, these findings suggest that no one factor (crop rotation, fertilisation strategy or other management practise) alone is responsible for dictating pathogen loads in soils rather a complex interplay of numerous factors will influence the abundance and severity of disease.

Two such factors that was investigated further in this thesis were temperature and the influence of cotton cultivar. The findings described here regarding temperature support previous studies that have found that lower temperatures support increased pathogen growth. However, the more interesting and novel results of this thesis concern the differences observed between cotton cultivars. Of the cultivars tested in this work, Sicot730 showed the greatest resistance to infection with *B. rouxiae*, as indicated by the lowest proportion of roots being affected by lesions, at both 20 and 25°C, and may therefore be a good choice for either early or late planting. These findings highlight the importance of cultivar type on the proliferation and induction of pathogenicity. Host-microbe interactions are controlled in part by signalling molecules produced by plant roots. The effect of temperature on the quantity and quality of production of root exudates has been reviewed previously (Hale *et al.*, 1971; O'leary, 1966). That pathogen abundance in soil or attached to roots decreased with temperature in some cultivars, and increased in others suggests that signalling

molecules produced by cotton roots either increase in quantity, or change in composition, at lower versus higher growth temperatures.

A novel aspect of this thesis was the investigation of the ability of wild-type strains (*A. brasilense* Sp7 *and A. brasilense* Sp245) and mutant strain *A. brasilense* Sp72002 (*FlcA* mutant) to block the growth of the pathogen *B. rouxiae* BRIP40192 towards cotton seedlings. *Azospirillum* spp. is known globally as a plant growth promoting bacteria (Bashan *et al.*, 2004; Bloemberg and Lugtenberg, 2001). Its ability to suppress plant pathogens had been studied (Bashan and De-Bashan, 2010; Somers *et al.*, 2005), and make this bacteria a good potential candidate to use as a biological control agent. Previous studies from the Pereg laboratory found that treating cotton seeds and seedlings with *A. brasilense* reduced the incidence of lesions caused by *Berkeleyomyces* spp. (Moulynox *et al.*, 2010). In this thesis these interactions were studied further, and it was determined that, while *A. brasilense* can attach to fugal hyphae and prevent fungal growth, there was no direct antagonistic interactions between the bacteria and *B. rouxiae*. Rather, it appears that *A. brasilense* may instead act as a biological barrier that prevents the signalling molecules produced by cotton seedlings triggering the germination of fungal spores and directional growth of hyphae. While these results are promising, it is important to note that these preliminary laboratory studies may not reflect the ability of this bacterium to suppress pathogen growth in field soils. To be an effective biological control agent *A. brasilense* must be able to survive and remain active under the changing environmental conditions experienced in the rhizosphere. One of these environmental factors is oxygen concentration, which may fluctuate in the rhizosphere. *A. brasilense* is characterised by its ability to undergo morphological transformation under stressful conditions, which can include low oxygen concentrations. This thesis described differences in

flocculation under low oxygen of wild type and a mutant strain of *A. brasilense (*Sp72002). While the mutant strain shows impaired flocculation, it also showed the same ability to block fungal growth towards cotton seedlings as the wild type strains. Given that rhizospheric conditions, such as oxygen concentration, can fluctuate significantly, one strategy to consider in the use of biological control is to inoculate soils with a mix of *A. brasilense* strains, to ensure that there is at least one that will survive and remain effective under the different conditions that may be encountered in different soils.

It was hypothesised that the interactions between *A. brasilense, B. rouxiae* BRIP40192 and cotton seedlings may be controlled in part by border cells produced by roots. The studies described here showed that *A. brasilense* strains were able to strongly interact with both cotton and wheat border cells, while *B. rouxiae* BRIP40192 did not interact with border cells from either cotton or wheat. Other bacterial and fungal strains, isolated from soils described as being disease suppressive, showed a diverse ability to interact with cotton or wheat border cells. *A. brasilense* is highly motile (Bashan and Levanony, 1990; Brigham *et al.*, 1998; Hall and Krieg, 1983; Hawes *et al.*, 2000; Krieg and Döbereiner, 1984) and produces a range of extracellular protein that could be responsible for this attachment to border cells, likely in response to signalling molecules being produced by those cells. The diversity of the ability of tested soil microbes to interact with border cells indicates that these cells probably influence the growth of some, but not all, rhizospheric microbes. An interesting finding here was that there was no direct interaction between *B. rouxiae* BRIP40192 and border cells. However, this pathogen clearly showed directional growth towards cotton seedlings in previous experiments. Together, these findings indicate that the

signalling molecules inducing germination and directional growth in the presence of cotton are produced by other root cells, and not by the border cells.

The results described here highlight opportunities for future research in a number of areas. Firstly, further analysis of the associations between pathogen abundance and disease severity and the physical and chemical parameters of soil, crop rotation and fertilisation strategy is needed in order to better understand the contribution of each of these factors to BRR disease. Monitoring of the same farms across the growing season and over a period of years will help to establish how these farming practices may affect pathogen load in the soil in the long term. Longer term monitoring would also assist in more definitively answering the question as to whether pathogen load in soil, as detected using molecular techniques, correlates with disease severity, as there is currently conflicting reports about this relationship. A second avenue for further investigation is the differences between cotton cultivars in their ability to resist infection by *Berkelyomyces* spp. As observed in this thesis, some cultivars may be more or less susceptible to infection at different temperatures. Further understanding the mechanisms behind this would allow producers to make more informed decisions when selecting cultivars in regions where BRR disease is endemic. This thesis also describes some preliminary studies into the potential use of *A. brasilense* as biocontrol. While these initial results are promising, a significant amount of work, including field studies, needs to be carried out to see if these same effects can be observed *in vivo*. At the same time, wide-scale testing of other soil microbes, particularly those isolated from soils that show natural disease suppression, could also identify other putative biological controls. Understanding, at molecular level, the factors that control the interaction between cotton, bacteria such as *A. brasilense,* and

Berkeleyomyces spp. can be used to develop and deliver an effective biological control agent.

6.2. Conclusion

The rapid spread of black root rot disease in Australian cotton fields, and the increasing cost to cotton producers trying to control it, is one of the main challenges faced by this industry. Early and rapid detection of the causative pathogen, *Berkeleyomyces* spp., represents the first step in being able to implement control measures. Soil samples from cotton farms have diverse physiochemical properties, different crop rotation history, and different history of fertilisation, all of which may influence abundance of the pathogen. Understanding how these factors influence pathogen growth and disease severity could allow producers to make more informed farm management decisions and potentially decrease yield loss. The bacterium *A. brasilense* shows potential as a biological control agent against at least one pathogenic strain, *B. rouxiae* BRIP40192, *in vitro*. Further study is now required to see if the same interactions can also take place in the field. Finally, using molecular and cellular tools to better understand *Berkeleyomyces* spp. interactions with host plants and other soil microbes will support attempts to clarify the biology of this economically important pathogen. This knowledge can, in turn, minimise its impact on the Australian cotton industry.

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Appendix A

Luria Broth (LB)
Bacto trypton 10 g/L
NaCL 5 g/L
Yeast extract 5 g/L

Nutrient Broth
Beef extract 3g/L
Peptone 5 g/L

Phosphate solution pH 6.8-7 (for minimal medium)
K_2HPO_4 16.7 g/L
KH_2PO4 8.7 g/L

$MgSO_4$/NaCl solution (for minimal lactate)
$MgSO_4$ 29 g/L
NaCL 48g/L

Trace element solution (for minimal medium)
In 1 Litre:
$MnSO_4\,H_2O$ 250 mg
$ZnSO_4\,7H_2O$ 70 mg
$CoSO_4\,7H_2O$ 14 mg
$CuSO_4\,5H_2O$ 12.5 mg
H_3BO_3 3mg

Minimal medium
Add phosphate solution, 100 ml; $MgSO_4$/NaCl solution, 10 ml; complete with DW to 1 litre. Autoclave at 121°C for 20 min.

Minimal Lactate medium
Prepare minimal medium supplemented with 6.3 ml/L of sodium lactate.
Pefore use add 10 ml of $CaCl_2$ solution (7 g /L), 10 ml of trace element solution, 1 ml of $FeCl_3\,6H_2O$ solution (10 g/L) and 1 ml of $Na_2MoO_4\,2H_2O$ solution (0.8 g/L).
Each of the solution were autoclaved separately.

Nitrogen free medium
In to 800 ml of DW add:
Malic acid 5 g
$K_2HPO_4\,3H_2O$ 0.5 g
$MgSO_4\,7H_2O$ 0.2 g
NaCl 0.1 g
$CaCl_2$ 0.02 g
Fe-EDTA 4 ml (1.64% aqueous, w/v);
2 ml of trace element solution (supplemented with 0.2 g/L $Na_2MoO_4\,2H_2O$).
Adjust pH to 6.8 with KOH and make up to 1 litre with DW.

Sterilised by autoclave at 121°C for 20 min.

Yeast mannitol agar (YMA)
Yeast extract 1 g/L
Mannitol 10 g/L
K_2HPO_4 0.5 g/L
$MgSO_4\,7H_2O$ 0.2 g/L
Add 15 g/L of agar and sterilise by autoclave at 121°C for 20 min.

Appendix B

Correlation among *B. rouxiae* BRIP40192 load in soil, pathogen attached to root, lesions, and water content

Sicot 74 BRF	Shoot water contents of control palnt at 25°C	Copy number of ITS of a fungi attached to root at 25°C	Copy number of ITS in Rhizosphere at 25°C	Percentage of root lesion at 20°C	Percentage of root lesion at 25°C	Copy number of ITS of a fungi attached to root at 20°C	Copy number of ITS in Rhizosphere at 20°C	Shoot water contents of control plant at 20°C	Shoot water contents of infected plant at 20°C	Shoot water contents of infected plant at 25°C
Shoot water contents of control plant at 25°C	**1.00**									
Copy number of ITS of a fungi attached to root 25°C	0.65	**1.00**								
Copy number of ITS in Rhizosphere at 25°C	0.39	0.57	**1.00**							
Percentage of root lesion at 20°C	0.40	0.54	0.66	**1.00**						
Percentage of root lesion at 25°C	0.14	-0.12	0.25	0.41	**1.00**					
Copy number of ITS of a fungi attached to root 20°C	-0.27	0.46	0.21	-0.11	-0.32	**1.00**				
Copy number of ITS in Rhizosphere at 20°C	-0.10	0.62	0.15	-0.01	-0.33	**0.96**	**1.00**			
Shoot water contents of control plant 20°C	0.13	0.17	-0.03	-0.22	0.30	0.41	0.42	**1.00**		
Water contents infected shoot at 20°C	-0.23	-0.22	-0.22	0.20	0.06	-0.12	-0.10	-0.39	**1.00**	
Shoot water contents of infected Plant at 25°C	-0.26	-0.24	-0.15	-0.10	-0.41	-0.17	-0.20	-0.12	-0.37	**1.00**

Sicot 730	Percentage of root lesion at 20°C	Shoot water content of control plant at 20°C	Shoot water content of infected shoot at 20°C	Shoot water content of control plant 25°C	Copy number of ITS in the rhizosphere at 20°C	Copy number of ITS of a fungi attached to root 20°C	Copy number of ITS in the rhizosphere at 25°C	Copy number of ITS of a fungi attached to root 25°C	Shoot water content of infected plant at 25°C	Percentage of root lesion at 25°C
Percentage of root lesion at 20°C	**1.00**									
Shoot water content of control plant at 20°C	-0.53	**1.00**								
Shoot water content of infected plant at 20°C	0.24	-0.54	**1.00**							
Water content of control at 25°C	0.04	-0.19	-0.26	**1.00**						
Copy number of ITS in the rhizosphere at 20°C	0.34	0.43	-0.05	-0.53	**1.00**					
Copy number of ITS of a fungi attached to root 20°C	0.17	0.57	0.06	-0.41	**0.92**	**1.00**				
Copy number of ITS in the rhizosphere at 25°C	0.32	0.49	-0.10	-0.34	**0.96**	**0.93**	**1.00**			
Copy number of ITS of a fungi attached to root 25°C	-0.12	0.32	0.22	-0.56	0.66	**0.70**	0.66	**1.00**		
Shoot water content of infected Plant at 25°C	-0.36	0.39	-0.21	-0.17	0.43	0.38	0.45	**0.67**	**1.00**	
Percentage of root lesion at 25°C	-0.13	-0.09	0.12	-0.03	-0.46	-0.33	-0.52	-0.33	**-0.75**	**1.00**

Sicot 43 BRF	Shoot water content of control plant 20°C	Shoot water content of control plant 25°C	Copy number of ITS of a fungi attached to root 25°C	Copy number of ITS of a fungi attached to root 20°C	Copy number of ITS in the rhizosphere at 20°C	Copy number of ITS in the rhizosphere at 25°C	Percentage of root lesion at 20°C	Shoot water content of infected plant 25°C	Percentage of root lesion at 25°C	Shoot water content of infected plant 20°C
Shoot water content of control plant 20°C	**1.00**									
Shoot water content of control plant 25°C	**0.72**	**1.00**								
Copy number of ITS of a fungi attached to root 25°C	0.46	**0.74**	**1.00**							
Copy number of ITS of a fungi attached to root 20°C	0.60	**0.73**	0.59	**1.00**						
Copy number of ITS in the rhizosphere at 20°C	-0.43	-0.64	-0.25	-0.63	**1.00**					
Copy number of ITS in the rhizosphere at 25°C	0.05	0.17	-0.30	0.18	**-0.83**	**1.00**				
Percentage of root lesion at 20°C	-0.10	0.24	0.24	-0.07	0.34	-0.44	**1.00**			
Shoot water content of infected plant 25°C	-0.12	-0.22	-0.25	-0.06	-0.17	0.31	**-0.75**	**1.00**		
Percentage of root lesion at 25°C	0.09	-0.07	-0.08	-0.19	0.21	-0.16	0.70	**-0.87**	**1.00**	
Shoot water content of infected plant 20°C	-0.17	-0.10	-0.25	-0.10	-0.08	0.20	-0.50	0.52	-0.49	**1.00**

Sicot 71 BRF	Shoot water content of infected plant 25°C	Shoot water content of infected plant 20°C	Copy number of ITS of a fungi attached to root. 20°C	Copy number of ITS in the rhizosphere at 20°C	Copy number of ITS in the rhizosphere at 25°C	Copy number of ITS of a fungi attached to root 25°C	Percentage of root lesion at 20°C	Shoot water content of control plant 20°C	Percentage of root lesion at 25°C	Shoot water content of control plant 25°C
Shoot water content of infected plant 25°C	**1.00**									
Shoot water content of infected plant 20°C	-0.45	**1.00**								
Copy number of ITS of a fungi attached to root. 20°C	-0.22	-0.12	**1.00**							
Copy number of ITS in the rhizosphere at 20°C	0.06	0.33	**0.75**	**1.00**						
Copy number of ITS in the rhizosphere at 25°C	-0.04	-0.28	**-0.69**	**-0.95**	**1.00**					
Copy number of ITS of a fungi attached to root. 25°C	-0.25	-0.15	0.52	**-0.86**	**0.90**	**1.00**				
Percentage of root lesion at 20°C	-0.13	0.29	0.18	-0.36	0.57	0.59	**1.00**			
Shoot water content of control plant 20°C	-0.40	-0.12	0.57	-0.15	-0.03	-0.02	-0.57	**1.00**		
Percentage of root lesion at 25°C	-0.10	-0.31	-0.04	0.10	-0.12	-0.12	-0.20	0.26	**1.00**	
Shoot water content of control plant 25°C	-0.33	-0.23	-0.24	0.04	-0.14	0.14	-0.08	0.05	0.28	**1.00**

Sicot 714 B3F	Shoot water content of infected plant 25°C	Shoot water content of control plant 25°C	Copy number of ITS of a fungi attached to root 20°C	Copy number of ITS in the rhizosphere at 20°C	Copy number of ITS in the rhizosphere at 25°C	Copy number of ITS of a fungi attached to root. 25°C	Shoot water content of control plant 20°C	Percentage of root lesion at 20°C	Shoot water content of infected plant 20°C	Percentage of root lesion at 25°C
Shoot water content of infected plant 25°C	**1.00**									
Shoot water content of control plant 25°C	**0.87**	**1.00**								
Copy number of ITS of a fungi attached to root 20°C	0.44	**0.67**	**1.00**							
Copy number of ITS in the rhizosphere at 20°C	0.48	**0.72**	**0.98**	**1.00**						
Copy number of ITS in the rhizosphere at 25°C	0.31	0.61	**0.95**	**0.98**	**1.00**					
Copy number of ITS of a fungi attached to root 25°C	0.37	**0.67**	**0.82**	**0.88**	**0.89**	**1.00**				
Shoot water content of control plant 20°C	-0.16	-0.37	-0.31	-0.49	-0.57	**-0.71**	**1.00**			
Percentage of root lesion at 20°C	0.06	-0.05	0.17	0.03	-0.02	-0.15	0.53	**1.00**		
Shoot water content of infected plant 20°C	-0.02	-0.15	-0.37	-0.35	-0.41	-0.21	0.09	-0.50	**1.00**	
Percentage of root lesion at 25°C	-0.12	0.21	0.01	0.00	0.00	0.19	-0.09	-0.44	0.41	**1.00**