GENOMICS OF QUANTITATIVE RESISTANCE TO BROWN RUST (*Puccinia melanocephala*) IN A SUGARCANE BREEDING POPULATION

By

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ABSTRACT

The Sugarcane Industry contributes approximately 400 000 jobs and ZAR 8 billion annually to South Africa's economy. Due to climate change and the subsequent threat posed by disease, these figures have been on the decline. Brown rust, a contributor to this decline is caused by the basidiomycete *Puccinia melanocephala* Syd. and P. Syd., which previously resulted in 50% yield losses in susceptible varieties. This highlighted the need for improved screening and breeding techniques which will result in the replacement of susceptible varieties.

The objectives of this study were to:

a) Adopt and optimise a glasshouse whorl inoculation screening technique applicable for mass screening of large populations.

b) Develop a rapid and cost effective rust resistance screening technique using detached leaves.

c) Utilise two flanking marker sets (R12H16 and 9O20-F4-PCR primers) for the rust resistance *Bru1* gene in a diagnostic polymerase chain reaction (PCR) to identify rust resistant genotypes lacking *Bru1* and possessing either quantitative resistance or an alternative major qualitative resistance gene.

d) Correlate rust phenotypic data to AFLP marker data for the Linkage Disequilibrium (LD2) mapping population.

e) Utilise suppression subtractive hybridization (SSH) profiling on rust challenged genotypes to discover differentially expressed genes between susceptible and resistant (susceptible *Bru1* negatives taken away from resistant *Bru1* negatives); and resistant genotypes (resistant *Bru1* positives taken away from resistant *Bru1* negatives). 4

Results from the glasshouse whorl inoculation trials showed the technique could be reliably used to screen large populations, as two independently conducted pot trials showed highly correlated rust ratings. A visually assessed detached leaf assay (DLA) was developed using selected genotypes. Chlorophyll fluorescence and SPAD readings were used in the DLA to determine the leaf photochemical efficiency (PI_{ABS}) with relation to chlorophyll content,

resulting in reduced assessment time of at least two days. PCR diagnostics revealed 31% of LD2 did not possess either flanking marker, 8% had one or the other marker, and 61% had both markers. The overall rust phenotypic ratings (rating scale of 0-10) and *Bru1* status of the genotypes was used to group the population, with the *Bru1* negative genotypes containing all three rating categories (resistant 0-3.5; intermediate 3.51-6.5; susceptible 6.51-10); while the *Bru1* positive genotypes were all resistant. The phenotypic data was correlated to AFLP data using the Pearson product-moment correlation coefficient and stepwise multiple linear regression employed to build marker based models to use for predicting non-*Bru1* mediated resistance. SSH analysis was then subsequently conducted on genotypes selected on the basis of *Bru1* status and AFLP correlation data. Two subtraction cDNA libraries were constructed and the cDNA inserted into electro-competent *Escherichia coli* cells. PCR on transformed cells revealed cDNA inserts ranging from 200- 1300bp. BLAST analysis of the cDNA sequences indicated the presence of high proportions of disease and drought stress related sequences in the libraries. Analysis of the sequences in both libraries showed that the resistant *Bru1* negative genotypes contained oxidative stress related sequences which were however absent in the *Bru1* positive resistant genotypes. The library comparing the *Bru1* negative resistant genotypes against the *Bru1* negative intermediate and susceptible genotypes showed a higher proportion of differentially expressed sequences coding for putative disease resistance proteins, highlighting their presence in the resistant genotypes. Both subtraction libraries also contained high proportions of a leucine rich repeat protein coding cDNA which contained a conserved domain homologous to that of a disease resistance protein conferring resistance to *Pseudomonas syringae* in *Arabidopsis thaliana*. The outcomes of this study will subsequently enable an improved understanding of sugarcane-rust resistance mechanisms and improved breeding and screening techniques for sugarcane by identifying SSH and AFLP markers linked to rust resistance QTLs or alternative *R* genes.

FACULTY OF SCIENCE AND AGRICULTURE

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I, **Terence Tariro Mhora**, declare that

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Co-supervisor

Signed: _____ Name: R. S. Rutherford Date: _____

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Chapter 1

Introduction and Rationale

Sugarcane is a large perennial grass belonging to the tribe *Andropogoneae*, family *Graminae* and is of the genus *Saccharum* (Asnaghi *et al*., 2004). The sugarcane industry in South Africa is responsible directly and/or indirectly for providing up to 429 000 jobs and eight billion rand a year to the national economy (www.sasa.org.za). These large contributions are almost entirely resulting from sugar production. More recently, sugarcane has been more widely used as a source of ethanol, an environmentally friendly additive or substitute to petroleum based fuel (Geller, 1985). Ethanol has reduced fuel imports by Brazil by up to 20%, proving the benefits that sugarcane has besides that of sugar production (Xavier, 2007). These developments underline the potential beneficial impacts sugarcane could have on the South African economy. Sugarcane is however prone to disease which can result in devastating losses, such as the rust outbreak experienced in the late 1970's, when losses of up to 50% were experienced in Cuba due to the rust causing fungal pathogen *Puccinia melanocephala* (Purdy *et al*., 1983). The South African sugarcane industry was also affected by *P. melanocephala* during that period, with an outbreak occurring in the 1974/75 season in which the variety N55/805 was affected (Bailey, 1979). More recent losses in the South African industry have occurred, with estimates of 26% reduction in yield on the rust susceptible variety N29 (McFarlane *et al*., 2006).

A widely recognized solution to this problem is the use of resistant varieties (Bischoff and Gravois, 2004; Purdy *et al*., 1983). R570, a rust resistant sugarcane variety predominantly grown in Mauritius and the Reunion Islands was found to contain a qualitative rust resistance gene named *Bru1* (Le Cunff *et al*., 2008). Flanking markers for the *Bru1* gene have been used to show the presence of this gene in some of the sugarcane varieties in the South African industry. The use of a major resistance gene as the sole protective measure against disease is however risky, as major genes are prone to break down due to the high genetic plasticity of pathogen populations (Hu *et al*., 1996). This is in contrast with quantitative resistance which offers partial resistance and is also controlled by multiple loci (Hoarau *et al*., 2001). Quantitative trait loci (QTL) do not normally follow simple Mendelian inheritance, making their selection difficult. Studies have shown that the stacking of resistance QTLs can achieve similar or even more effective disease resistance when compared to that conferred by major genes (Parlevliet and Van Ommeren, 1988). The breeding and selection cycle used to

successfully develop a commercial sugarcane variety is approximately 12-15 years, which makes it important to find more efficient and rapid means of identifying and detecting quantitative resistance loci (QRLs). It is with this background that the aims of this research were formulated. The aim was to determine the genetic basis of rust resistance within a sugarcane linkage disequilibrium mapping population, and this determined the following objectives:

1 To develop a rapid method for rust resistance screening.

A detached leaf assay (DLA) was developed and optmised for screening purposes based on literature sources (Braithwaite, 2005; Jackson *et al*., 2008; Twizeyimana *et al*., 2007; Zhao *et al*., 2011). Varieties with known rust responses (**N12**- Rust resistant; **N29**- Rust susceptible; **N39**- Rust tolerant) were used to develop the DLA. Visual analysis and chlorophyll associated parameters were used to determine the extent of rust infection and assign rust resistance ratings.

2 To screen population LD2 for resistance to rust.

A whorl inoculation technique (Sood *et al*., 2009) was adopted and used to screen 80 genotypes from the South African Sugarcane Research Institute's (SASRI) linkage disequilibrium (LD2) mapping population. Rust responses which included lesion severity and sporulation were recorded for each replicate and used to calculate and assign rust ratings to each genotype. The DLA was used to screen selected genotypes from LD2 so as to verify this method's ability to give comparable results to those obtained from whorl inoculation.

3 To relate resistance ratings to presence or absence of markers for the *Bru1* major resistance gene and also to AFLP markers.

Bru1 PCR marker analysis was conducted on each LD2 genotype to determine whether it contained *Bru1* or not. Two flanking markers were used to determine the presence or absence of *Bru1* in the population. The *Bru1* marker analysis data and rust resistance ratings obtained from whorl inoculation where correlated to previously acquired AFLP marker data (Butterfield, 2007). Stepwise multiple linear regression was used to obtain the best markers for rust resistance using both lesion ratings and overall rust ratings obtained from whorl inoculation for those genotypes lacking *Bru1*.

4 To identify candidate genes involved in the rust resistance response.

Suppression subtractive hybridization was used to compare the expression profiles among rust challenged resistant *Bru1* negative; resistant *Bru1* positive; and susceptible *Bru1* negative sugarcane genotypes from LD2. cDNA sequences obtained from this process were cloned, sequenced and analysed using BLAST algorithms.

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Chapter 2

Literature Review

2.1 Background

Sugarcane is a tropical perennial grass in the genus *Saccharum*, which is thought to have originated in New Guinea and spread to South East Asia and Western India around 327 B.C. (Baucum *et al.,* 2009; Grivet *et al.*, 1994; James, 2004; Kampen, 2002). Sugarcane cultivation, now widespread in most tropical and sub-tropical regions is globally located between the latitude 36.7° north and 31.0° south of the equator (James, 2004).

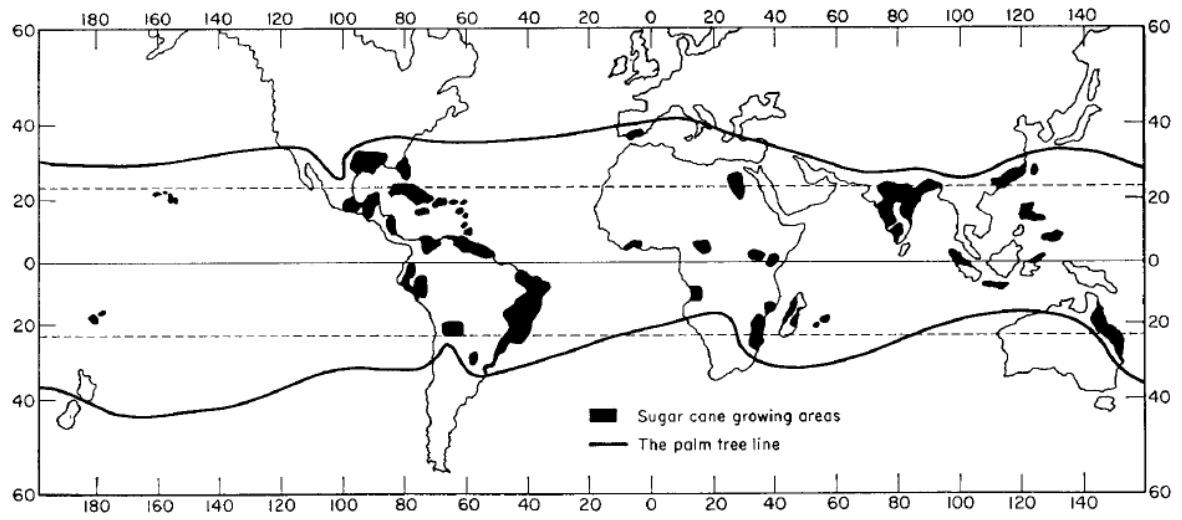

Fig 2.1: World distribution of sugarcane cultivation and the Palm Tree Line (James, 2004).

The South African sugar industry is located predominantly in Kwa-Zulu Natal and extends from Northern Pondoland in the Eastern Cape to the Mpumalanga Lowveld. In South Africa, sugarcane was first cultivated as a commercial crop on the North Coast of Kwa-Zulu Natal between 1847 and 1851, with the first public sale of sugar from these plantations being made in Durban in 1855 (Lewis, 1990; Van Antwerpen *et al*., 2005). From having only 4 953 hectares of sugarcane in 1860, there were 392 000 ha of land under cane in 2010, with 2.2 million tons of sugar being produced in that year (Anon, 2010a; Lewis, 1990). The average area under sugarcane in the past 15 years has been approximately 430 000ha, with an average of 2.5 million tons of sugar being produced (Anon, 2010a).

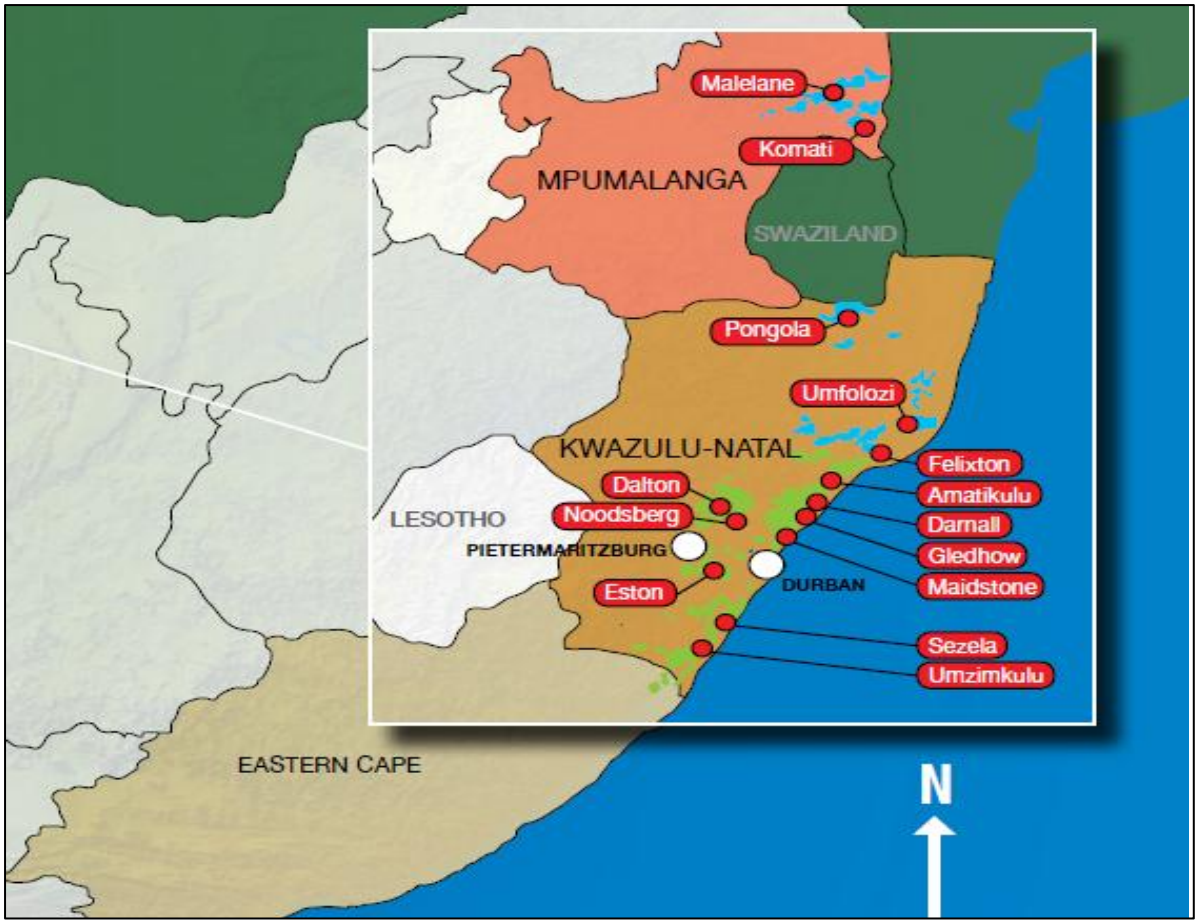

Fig 2.2: South African sugarcane growing region (Anon, 2010a).

Sugarcane has in recent years found use as more than just a source of sugar, as it is now extensively used in ethanol production. Ethanol is a biofuel used in countries such as Brazil, where it is used as an additive to oil based fuels and in some instances, as a substitute (Martines-Filho *et al.*, 2006; Rudorff *et al.*, 2010). This makes sugarcane an important crop with both a high economic and industrial potential, as biofuels are seen as solutions to mitigating greenhouse emissions as well as an alternative fuel source to dwindling oil reserves (Rudorff *et al.*, 2010).

2.1.2 Economic importance

Sugarcane is a renewable, natural agricultural resource which mainly provides sugar, and more recently ethanol, fibre and numerous other by-products with ecological sustainability (Bolling and Suarez, 2001). Out of the world's total white crystal sugar production, approximately 79% comes from sugarcane, with the remainder coming from sugar beet (Grivet and Arruda, 2001; Anon, www.illovo.co.za, 2012). Molasses, the main by-product of sugar production, is the raw material for

alcohol production and thus for alcohol-based industries. Excess bagasse is used as raw material in the paper industry and also in the co-generation of power in most sugar mills (Chen and Negulescu, 2002). Sugarcane has been successfully used in a number of countries in the production of ethanol for use in blending with petrol. This has aided these countries to save millions in foreign exchange, as they effectively save up to 20% on petroleum fuel imports, and inject this directly into their economies by manufacturing ethanol themselves (Geller, 1985; Rudorff *et al.*, 2010; Xavier, 2007).

Worldwide, sugarcane is cultivated in 127 countries and occupies an area of 32 million ha (Cordeiro *et al.*, 2007). Of these countries, Brazil has the highest area under sugarcane (6.96 million ha), while South Africa has an average 430 000 ha (Anon, 2010a; Zuurbier and van de Vooren, 2008). These figures have seen South Africa consistently ranked in the top 15 sugar cane producers in the World, behind leaders Brazil and India (Anon, 2010b; Grivet and Arruda, 2001).

The sugarcane industry in South Africa is responsible for both direct and indirect employment of approximately 429 000 people and has an approximate 35 300 registered sugarcane growers annually producing an average 20 million tons of sugarcane (Anon, 2010a). The South African economy is reliant on the sugar industry contributing a significant share of its export earnings (Cordeiro *et al.*, 2007). This is shown by figures that show that the sugarcane industry directly generates an average of R8 billion per year to the South African economy (Anon, 2010a).

2.1.3 Taxonomy and Morphology of Sugarcane

Botanically, sugarcane (*Saccharum* spp. hybrids) belongs to the *Andropogoneae* tribe of the monocotyledonous family *Gramineae* (Asnaghi *et al.*, 2004; Grivet and Arudda, 2001). Modern sugarcane cultivars (*Saccharum* spp. hybrids) have been derived from crosses amongst the wild species *S. spontaneum* and *S. robustum* with domesticated *S. officinarum* (James, 2004; Baucum *et al.*, 2009). Sugarcane comprises a large number of varieties, each a single clone producing stalks with variations in colour, wax cover, internode shape, length and diameter (Bremmer, 1961a; Cordeiro *et al.*, 2007). The internodes are regularly spaced and each possesses a single nodal bud capable of asexual propagation. N29 is a commercial variety in the South African sugarcane industry which was initially deemed to be rust tolerant, but became susceptible after introduction into the cooler climate of the Midlands (McFarlane *et al*., 2006). N29 is characterised by high sucrose content, is a fairly erect variety with a medium width blade, a hairy purple sheath and pinkish wax coated internodes (N29 Information sheet). The N12 variety however is rust resistant and has slight differences when its characteristics are compared to those of N29. N12 is erect, also has a purple

waxy sheath and has narrower leaves which are much paler in colour than N29. The variety N12 has green to yellow internodes and often lacks hair on its sheath (N12 Information sheet).

2.2 The Genome of Sugarcane

The genome of cultivated sugarcane is a complex, highly polyploid and aneuploid interspecific hybrid with between 2n=100 and 2n=130 chromosomes (Grivet and Arruda, 2001; Raboin *et al.*, 2006). Most of these chromosomes originate from the sugar-producing species *S. officinarum* (2n=80), with 15 to 25% of these being derived from the wild species *S. spontaneum* (2n=40 to 128) (Asnaghi *et al.*, 2004). A small percentage of the genome (5- 10%) has also been contributed by interspecific recombination with other *Saccharum* species, namely *S. barberi*, *S. robustum*, and *S. sinense* together with other related grass genera, namely *Miscanthus*, *Narenga* and *Eriunthus* (Cuadrado *et al*., 2004; Grivet and Arruda, 2001; Hoarau *et al*., 2001; Le Cunff *et al*., 2008; Ming *et al.*, 2006). Genomic *in situ* hybridization has demonstrated that recombination is also possible between homoeologous chromosomes in modern cultivars (Fig 2.3) (Grivet and Arruda, 2001).

Breeding of modern sugarcane cultivars was initiated in the early 19th century by breeders in Java, who produced interspecific hybrids between *S. officinarum* and *S. spontaneum*, and backcrossed them twice with *S. officinarum* as the recurrent parent (Cordeiro *et al.*, 2007; Grivet *et al*., 1996). *S. officinarum* was used as the female parent and *S. spontaneum* as the pollen donor, resulting in progeny with a 2n+n chromosome number (Butterfield, 2007). This was a result of either fusion of two megaspore nuclei after the second mitotic division or endo-duplication that takes place in the nucleus of the chalazal megaspore of *S. officinarum* (Grivet *et al*., 1996; Bremer, 1961b). Both these occurrences resulted in the formation of 40 bivalents. The chromosome number after the second backcross became normal.

Considering that introgression has considerably increased the genome complexity, the meiosis of modern sugarcane cultivars appears to be fairly regular, mainly involving prevalent bivalent pairing, rare univalent and multivalents (Raboin *et al*., 2006; Grivet *et al*., 1996). This almost normal trend is due to the changes that occur within the genome of newly hybridized organisms to ensure the genomes are coordinated and act in harmony (Cuadrado *et al*., 2004).

Synteny clusters between sugarcane and some other members of the *Andropogoneae* tribe such as maize and sorghum have been revealed through the use of DNA probes. Sugarcane linkage groups have shown syntenic relationships to the repeated regions in maize and sorghum, indicating close relationships with respect to chromosomes among the genomes of these crops (Dufour *et al*., 1997).

Syntenic positions in the *Andropogoneae* genomes have been useful in the detection of numerous quantitative trait loci (QTLs) (Raboin *et al.*, 2006).

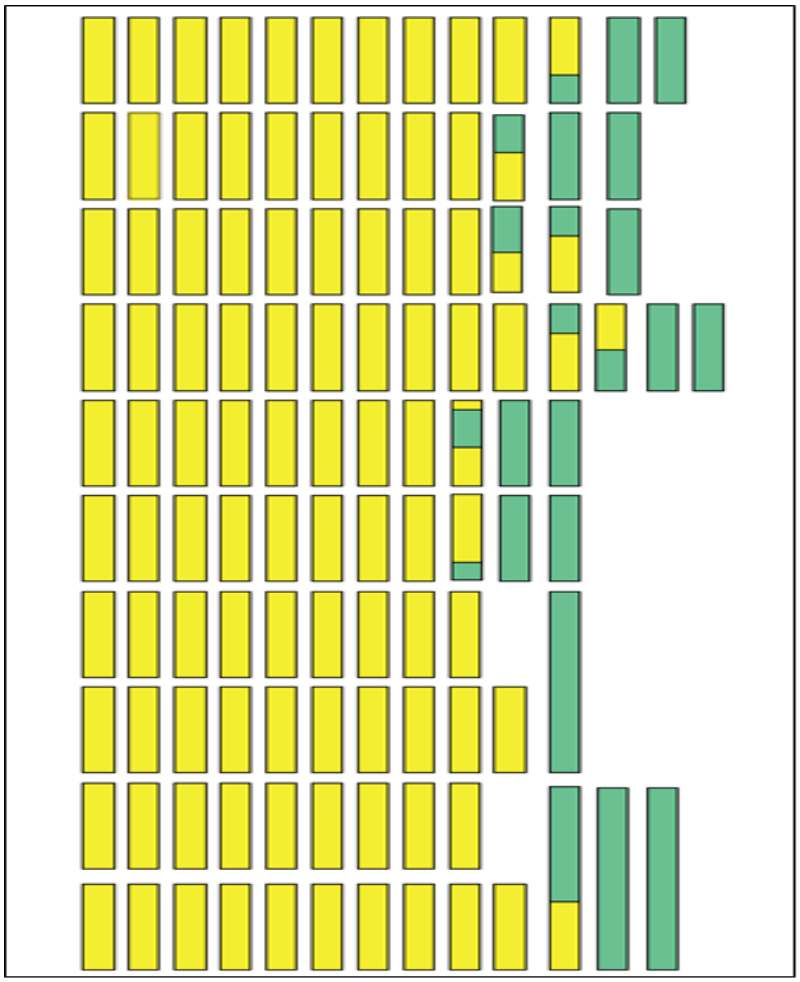

Fig 2.3: Schematic of the genome of a current sugarcane cultivar. Each yellow bar represents *S. officinarum* and each green bar *S. spontaneum*. Chromosomes aligned within the same row are homologous (Grivet and Arruda, 2001).

Though heterologous probes derived from these syntenic regions with other members of the *Andropogoneae* tribe have not enabled detection of any major genes, they have assisted in the improvement of existing genetic maps (Grivet and Arruda, 2001). Genetic maps based on molecular markers have proved more useful for understanding genome structure and for the isolation of important genes (Maureira and Osborne, 2004). Breakthroughs in the genetic mapping of sugarcane were reported in 1992 for *S. spontaneum* and in 1996 for *S. officinarum* (Asnaghi *et al*., 2000). This technology quickly spread to the modern cultivars and Q165 and R570 were mapped soon after, using RFLP and/ or RAPD markers (Asnaghi *et al.*, 2000; Raboin *et al.*, 2006). Subsequent use of AFLP and bulked segregant analysis in the development of a more detailed genetic map resulted in the identification of a major rust resistance gene *Bru1*, which was identified in the sugarcane cultivar R570 (Asnaghi *et al*., 2000; Asnaghi *et al.*, 2004).

Despite the complexity of the sugarcane genome, *Bru1* has a moderate to high heritability. *Bru1* is a durable gene that has maintained its integrity, is stable and has not broken down in over 20 years that the R570 cultivar has been cultivated (Asnaghi *et al.*, 2004). This gene was also found to be a monogenic and dominant allele as it gave a three to one segregation ratio of resistance in the progeny resulting from selfed R570 (Asnaghi *et al.*, 2000).

2.3 Disease in Sugarcane

Sugarcane is commonly grown over large contiguous areas, is a ratoon crop and is also propagated vegetatively which subsequently makes it prone to disease build-up (Bailey, 2004; Bremer, 1961a). Diseases in sugarcane are caused by a variety of organisms which include fungi, bacteria and viruses. The diseases resulting from these organisms have been compiled and described, the first manuscript being published in 1938 by Stevenson and Rands, with the latest one being compiled this past decade. The complilation of the latest manuscript was mainly coordinated by the International Society of Sugar Cane Technologists (ISSCT) (Bailey, 2004). Brown rust of sugarcane is an example of such a disease which is found in this manuscript. This disease has resulted in moderate to severe losses in sugarcane yield in India, Australia and South Africa, amongst other sugarcane producing countries (Purdy *et al*., 1983; Raid and Comstock, 2000; Taylor *et al*., 1986).

2.4 Brown Rust of Sugarcane

Brown rust of sugarcane is caused by *Puccinia melanocephela* H. & P. Sydow, a basidiomycete fungus (Hoy and Hollier, 2009; Purdy *et al*., 1983; Raid and Comstock, 2000). *P. melanocephala* is an obligate biotrophic parasite which incites new infections only on living host tissue. Changes in varietal susceptibility to rust have been observed over the years, suggesting the existence of several brown rust variants (Comstock and Milligan, 2007; Jackson *et al.*, 2008; Raid and Comstock, 2000; Sood *et al*., 2009).

This disease, formerly known as common rust, has had considerable economic impact on global sugarcane industries (Asnaghi *et al.,* 2004; Raid and Comstock, 2000). The causative fungal pathogen is now widespread and found almost everywhere where sugarcane is grown (Dixon, 2010; Hoy and Hollier, 2009; Raid and Comstock, 2000).

2.4.1 History of Brown rust

Brown rust was first recorded in South Africa in 1941, then in Madagascar in 1962, in Mauritius in 1964 and in the Réunion Islands in 1965 (Hull *et al*., 2008; Nagarajan and Singh, 1990). *P. melanocephala* was then reported in the America's in 1978, where it was thought to have reached the Dominican Republic via trans-oceanic high altitude air currents from Cameroon in Africa (Purdy *et al.*, 1983; Raid and Comstock, 2000). From the Dominican Republic, brown rust spread to the other Caribbean countries, northern South America, Central America and to the United States, where a severe epidemic resulting in losses of up to 50% was experienced on susceptible varieties which made up over half their sugarcane crop. In the same year (1978), the epidemic spread to Australia, which had never observed *P. melanocephala* in its crop before (Purdy *et al*., 1983). *P. melanocephala* is now widespread and found almost everywhere sugarcane is grown, with some recent reports confirming its continuing spread (Dixon, 2010; Kelly *et al*., 2009).

2.4.2 History of Brown Rust in South Africa

P. melanocephala was first described in 1907 by H. and P. Sydow on bamboo and was only considered a disease of economic importance in India in 1949 (Purdy, 1985; Ryan and Egan, 1979). The origin of the fungus in South Africa is unknown, though its presence in the local industry was reported as early as 1941 on the variety Co301 (Bailey, 1979a). This period was the beginning of an epiphytotic which extended into the 1950s, with an estimate made that enough wind-borne rust spores were produced to infect an area the size of the whole of South Africa (Hull *et al*., 2008). There was a further outbreak of rust in the 1974/75 season in which variety N55/805 was affected and losses of 23% were recorded (Bailey, 1979b; McFarlane *et al*., 2006). Brown rust has since been found in Mozambique, Zimbabwe, Zambia, Malawi, Tanzania, Kenya and Uganda since this initial report and is likely to be present in virtually all sugarcane growing countries in future (Hull *et al.*, 2008). There was a resurgence of brown rust after 2000 in South Africa, which was prevalent particularly in the varieties N29 and N33 and resulted in yield losses of 10- 26% in yield loss trials for the N29 variety (McFarlane *et al*., 2006; Ramouthar, 2009).

2.4.3 Economic Importance

Rust is an economically important disease which results in losses due to severe reductions in yield because of the loss of effective leaf area (Bailey, 2004). Losses are also a result of the combined

effects of reductions in the numbers of millable stalks, stalk diameter, canes per stool and stalk biomass (Purdy *et al*., 1983; Raid and Comstock, 2000). Brown rust was initially regarded as an economically unimportant disease in the America's until the 1978-1982 seasons, when heavy losses were incurred due to an outbreak which occurred in the South and North American continents (Purdy *et al*., 1983; Taylor *et al.*, 1986). The disease affected yield in susceptible varieties and losses as high as 50% were registered in Mexico (1981-82) on the susceptible cultivar B4362 (Asnaghi *et al*., 2004; Purdy *et al*., 1983). The epidemic also resulted in indirect losses being incurred, as varieties were frequently withdrawn from commercial production because of susceptibility to rust (Kelly *et al*., 2009). Cuba, which had 40% of its crop being a susceptible variety, reduced the hectarage of this variety to 28% the following season so as to curb losses (Purdy *et al*., 1983).

In South Africa, the 1979 epidemic resulted in significant losses in the variety N55/805, with an estimated 100 000 ton loss of cane annually (Bailey, 1979b). In recent years, a resurgence in the incidence of brown rust resulted in the previously rust tolerant variety N29 becoming susceptible, resulting in an estimated 26% loss (McFarlane *et al*., 2006).

2.4.4 Taxonomy and Morphology of *Puccinia melanocephala*

Puccinia melanocephala belongs to the Phylum Basidiomycota, class Pucciniomycetes, Order Pucciniales, family Pucciniaceae and the genus *Puccinia* (Agrios, 2005; Ramouthar, 2009). *Puccinia melanocephala* and a close relative, *P. kuehnii*, are regarded as pathogens of economic importance with regards to sugarcane (Purdy *et al.*, 1983; Raid and Comstock, 2000). *P. melanocephala* is distinct by its appearance primarily on the abaxial side of leaves, with cinnamon-brown, linear (up to 4mm) uredinia (Fig 2.4). The urediniospores are ellipsoidal (25-39 X 17-28µm) and cinnamon to dark brown in colour (Dixon, 2010). The echinulate spores have a darker cell wall and lack a thickened apical wall when compared to those of *P. kuehnii* (Purdy, 1985; Purdy *et al*., 1983). The urediniospores have 4-5 equatorial germ pores. The paraphyses are capitate, golden, club shaped structures with 1-2.8µm thick wall and a 1-15µm apex (Dixon, 2010; Purdy *et al*., 1983). The paraphyses can be used to distinguish between *P. melanocephala* and *P. kuenhii*, as brown rust produces more abundant capitate paraphysis with thicker walls at the apex (Dixon, 2010).

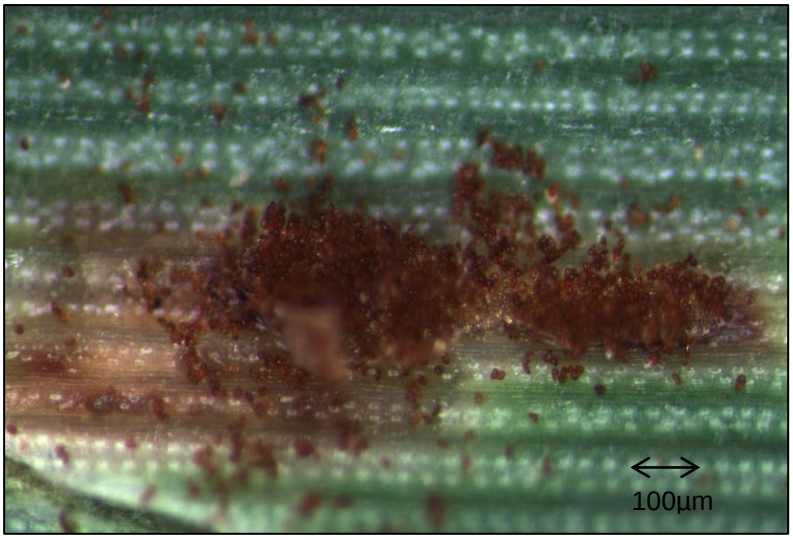

Fig 2.4: Uredinia with urediniospores erupting from a leaf surface (Photograph by Terence Mhora).

2.4.5 Symptoms of Brown Rust

Sugarcane rust is mainly a disease of the leaf and presents as reddish-brown (later dark brown) pustules (uredinia) that erupt mainly on the abaxial side of sugarcane leaves and lie parallel to the vascular bundles (Bailey 2004; Dixon, 2010; Raid and Comstock, 2000). The earliest symptoms are small, elongated yellowish spots that are visible on both leaf surfaces (Purdy *et al*., 1983; Raid and Comstock, 2000). The spots increase in length, become red-brown in colour and mature and sporulate within 10-14 days.

On highly susceptible varieties, considerable numbers of pustules may occur on a leaf, covering the entire abaxial surface and coalescing to form large, irregular, necrotic areas (Bailey, 2004; Raid and Comstock, 2000). In the later stages of disease development, premature drying-off of infected leaves can result in severe loss of photosynthetic leaf area. High rust severities may result in premature death of even young leaves. Severe infections have caused reductions in both stalk mass and stalk numbers, subsequently reducing cane tonnage (McFarlane *et al*., 2006; Purdy *et al*., 1983; Raid and Comstock, 2000).

2.4.6 Mode of Infection

Urediniospores germinate to produce a germ tube that contacts the guard cells and forms an appressorium over the stomatal aperture. A penetration peg then develops and enters the sub-stomatal cavity, where a sub-stomatal vesicle develops. Two to four infection hyphae then develop

and colonize the intercellular part of the leaf (Purdy *et al*., 1983). The infection hyphae then come into contact with the mesophyll cell. A septum cuts off the terminal hyphal cell and forms a haustorial mother cell. The haustorium mother cell, when in contact with the host cell wall forms a multi branched, lobed haustorium within the host cell (Purdy *et al*., 1983; Sotomayor *et al*., 1983). The rust fungus then grows parallel to the venation of the leaf and advances in both directions from the point of penetration. After approximately seven days, urediospores and paraphyses develop from the sporogenous hyphae and subsequently, the epidermis is ruptured by the developing urediniospore mass (Purdy *et al.*, 1983).

2.4.7 Dispersion

Rust spores are very well-suited to dissemination by air currents and its spores can be found in high concentrations in the air during dry afternoons (Brown and Hovmøller, 2002; Jackson *et al*., 2008; Viljanen-Rollinson *et al.*, 2007). On a more local scale, rust epidemics have been demonstrated to develop in the direction of prevailing winds (Viljanen-Rollinson *et al.*, 2007). Brown rust can also be disseminated by rain and irrigation splash (Anon, 2011; Raid and Comstock, 2000). Two important forms of aerial dispersion that describe rust involve the travel of spores over very long distances (possibly intercontinental) in a single step invasion, while the other is a gradual expansion of the range of a pathogen population within large territory. The single step invasion has been largely instrumental in the spread of *P. melanocephala* as meteorological data has shown that this method was responsible for the 1978 epidemic in the America's by spores from West Africa (Brown and Hovmøller, 2002; Nagarajan and Singh, 1990). *P. melanocephala* is especially suited for long distance dispersal as its spores are robust, thick walled and pigmented, giving it protection and sustained viability from the sun in its peak transport period (Raid and Comstock, 2000; Viljanen-Rollinson *et al.*, 2007).

2.4.8 Epidemiology

Leaf wetness and atmospheric temperature are the environmental factors most influential for disease development (Raid and Comstock, 2000). Several hours of free moisture on the leaf surface at a favourable temperature (25°C) is necessary for successful urediniospore germination and infection. However, though heavy rains may add to leaf wetness, they tend to remove spores from

the atmosphere, rendering them ineffective if they land on the soil (Jackson *et al*., 2008; Purdy *et al.*, 1983; Ramouthar, 2009). Urediniospore germination takes place between 15-20°C, while the subsequent formation of appresoria, a stage necessary for successful infection is optimal at temperatures between 15-30°C (Purdy *et al.*, 1983; Sotomayor *et al.*, 1983). At optimal leaf wetness and temperature conditions, urediniospores germinate within an hour, and infection hypha and haustorium develop after 36 hours (Sotomayor *et al*., 1983). Several other factors can influence rust development, and these include host genotype, plant age and soil conditions such as pH and nutrient levels (McFarlane *et al*., 2008; Raid and Comstock, 2000). Rust severity can rapidly increase within a short time, as *P. melanocephala* has a short life cycle of 14 days from germination to urediniospore production (Purdy *et al*., 1983; Raid and Comstock, 2000).

2.5 Control of Brown Rust

Brown rust has been documented to result in devastating yield losses, which makes it imperative to reduce resultant losses by either the prevention or mitigation of infection and resultant losses due to the fungus. Preventative measures are a more preferred method of controlling rust, especially as the costs and processes for mitigating the disease can be removed from the producers.

2.5.1 Varietal Control

The most economically effective way to control brown rust of sugarcane is through the use of resistant cultivars such as R570, which has maintained rust resistance for over its 20 years of extensive cultivation (Asnaghi *et al*., 2004; Bailey, 2004). This method should also be complemented by the withdrawal and withholding of susceptible varieties from cultivation (Purdy *et al*., 1983; Sood *et al*., 2009). The development of resistant cultivars has resulted in the improved control of brown rust and subsequent reduction of the resultant economic losses (Asnaghi *et al*., 2001). Overall resistance has however not been durable in certain varieties as was observed in N29, presumably because of the existence of numerous rust variants (McFarlane *et al*., 2006; Raid and Comstock, 2000). Varietal diversification has also been encouraged as it plays an important role in holding down disease pressure (Raid and Comstock, 2000).

2.5.2 Chemical Control

Fungicides have been found to be effective in the reduction of rust severity and yield losses in sugarcane (Hoy, 2008). In the USA, a systemic fungicide, pyraclostrobin (Headline® fungicide produced by BASF) has been approved for the control of brown rust (Hoy, 2010). In Southern Africa, Dithane (Mancozeb), a contact fungicide has been used with some success in increasing the yield in rust infected sugarcane (Gullino *et al*., 2010; McFarlane, 2008; Raid, 1992; Zvoutete, 2006). Abacus (epoxyconazole/ pyraclostrobin) has also been used in trials in South Africa and has shown good rust control and increase in yield (personal communication, S. A. McFarlane). The commercial application of fungicides is however not economically viable, though it has been postulated to be useful and effective in the event of a rust outbreak (McFarlane *et al*., 2006; Raid and Comstock, 2000). This is mostly due to the extended duration of the spray programs and the small yield responses obtained (Zvoutete, 2006). In such a case, it would be important to conduct regular checks on fields and apply the fungicide timeously once the early symptoms are recognised (McFarlane *et al*., 2006).

2.6 Plant Defence Mechanisms

Plants defend themselves from various forms of pathogens through defensive mechanisms which involve constitutive and induced resistance mechanisms (Dixon and Lamb, 1990; Kang *et al*., 2005; Mert-Türk, 2002). The complexity of the sugarcane genome has however meant that progress in deciphering the mechanisms of resistance to pathogens is limited. Observations have however shown syntenic relationships amongst sugarcane, sorghum and maize, leading to the discovery of genes within sugarcane, some of which include disease resistance genes (Le Cunff *et al*., 2008; Raboin *et al.*, 2006). There are basically four forms of resistance to diseases, these being innate resistance, qualitative resistance, quantitative resistance and recessive resistance (Heath, 2000; Kang *et al*., 2005; Nimchuck *et al*., 2003). In the sugarcane-rust interaction, it has been demonstrated that resistance is a result of physiological defence mechanisms triggered within the host. This has been proved true with the *Puccinia* species on some Gramineae species, as the spores germinate, but progression of the infection is decided in the developmental stage of the haustorial mother cells (Luke *et al*., 1987).

2.6.1 Innate Resistance

Innate resistance is sometimes described as non-host resistance and is characterised by its durable and complete nature, together with the fact that it is broad spectrum and displayed by an entire plant species against entire pathogen species (Heath, 2000; Nürnberger and Lipka, 2005). It is characterised by a multifaceted nature which is a result of a combination of physical, chemical and generic recognition processes which provide immunity to many potential pathogens (Dangl and Jones, 2001; Heath, 2000; Huang, 2008; Lopez *et al*., 2004). Innate resistance partly consists of pre-formed physical and biochemical (anticipin) barriers which must be circumvented by an invader, requiring appropriate infection structures and anticipin immunity or detoxification (Heath, 2000). There is also an induced response initiated by a recognition or surveillance system detecting generic conserved pathogen-derived molecules such as bacterial lipopolysaccharides, flagellin or fungal constituents such as ergosterol and chitin (Van Loon, 2009). These surveillance systems constitute of pathogen-associated molecular patterns (PAMPs) which are recognised by non-specific host pattern recognition receptors (HPRR) at plasma membrane receptors in plant cells, activating a signalling cascade which results in a basal resistance against potential pathogens (Jones and Dangl 2006; Nürnberger and Kemmerling, 2009; Thordal-Christensen *et al*., 1997; Van Loon, 2009). These HPRRs consist of an extracellular ligand binding domain, a single trans-plasma membrane domain and a cytosolic protein kinase domain that have been collectively termed as receptor-like kinases (RLKs) (Shiu and Bleecker, 2001). RLKs detect evolutionarily conserved PAMP molecules such as β-glucan and chitin from fungi leading to PAMP triggered immunity (PTI) (Kaku *et al*., 2006; Nürnberger *et al*., 2004).

The evolution of plant immunity has been proposed to follow a zigzag interplay between effector-triggered susceptibility (ETS), and effector-triggered immunity (ETI) (Chisholm *et al*., 2006; Jones and Dangl 2006; Nürnberger and Kemmerling, 2009). An example is barley (*Hordeum vulgare*), a non-host to the wheat (*Triticum aestivum*) powdery mildew fungus *Blumeria graminis* f. sp. *tritici,* whose incompatibility appears to be a PTI-based penetration resistance associated with the formation of cell-wall appositions (Collins *et al*., 2003; Trujillo *et al*., 2004). This highly effective non-host resistance contrasts with the susceptibility of many barley genotypes to the corresponding appropriate pathogen *B. graminis* f. sp. *hordei*. In this latter case, PTI is overcome by pathogen effector molecules that trigger ETS by altering the plant's physiological state to benefit pathogen colonization, or by suppressing host plant defenses that were activated by PAMP detection (Speth *et al*., 2007). In ETS, a detectable PAMP induced resistance can remain to greater or lesser extents, depending on the efficiency of ETS (Nurnberger and Lipka, 2005). This type of resistance has been

referred to as basal resistance, which is quantitative in nature (Dangl and Jones, 2001; Boller and Felix, 2009).

2.6.2 Qualitative Resistance

The transition from PTI to ETS can lead to a second group of responses known as gene-for-gene resistance or effector triggered immunity (ETI). ETI is initiated through recognition of pathogen effectors (avirulence - *avr* gene products) by resistance (*R*) gene products of the host plant (Jones and Dangl 2006; Nimchuk *et al*., 2003). *R*-genes encode receptors such as those containing an N-terminal coiled-coil structure, a central nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) region. Such NBS-LRR *R*-genes recognize fungal effectors and lead to a range of resistance responses usually associated with a hypersensitive response (HR), a cell death process that occurs at the site of attempted pathogen entry (Govrin and Levine, 2000; He, 1996; Kuta and Tripathi, 2005; Lopez *et al*., 2004). The HR is effective against obligate biotrophic pathogens such as rusts and smuts, which must obtain nutrients from living host tissue (He, 1996; Kuta and Tripathi, 2005; Lopez *et al*., 2004; Mur *et al*., 2008; Mert-Türk, 2002; Ren *et al*., 2010). An example of the effectiveness of HR was obseved in the response of sugarcane clone Ja60-5 to infection by the bacteria *Gluconaceto bacterdiazotrophicus* (Arencibia *et al*., 2006).

NBS-LRR proteins make up the majority of qualitative resistance proteins available in nature (du Preez, 2005). Qualitative resistance, also known as vertical resistance or race-specific resistance has been preferred in breeding programs due to the relative ease of transfer of single genes into well adapted cultivars by crossbreeding (Keane and Brown, 1997; McDowell and Woffenden, 2003). The gene-for-gene concept is one in which a single *avr* gene of the plant pathogen corresponds to a single resistance gene of the host (Flor, 1971; Martin *et al*., 2003). Qualitative genes are more often inherited dominantly, and are also normally found clustered together in certain chromosome arms (Ribiero do Vale *et al*., 2001). These genes have major effects and are expressed throughout the life of a plant, tending to produce a plant completely resistant to one or more strains of a particular pathogen (McDowell and Woffenden, 2003; Sakr *et al*., 2011). Their expression can however be modified by epistatic interaction, the developmental stage of the plant or the environment (Messmer *et al*., 2000; Ribeiro do Vale *et al*., 2001). Qualitative resistance is present as both durable and non-durable resistance, where resistance is considered durable if it remains effective when used for many years over a substantial area (Ribeiro do Vale *et al*., 2001). Such an example is the sugarcane variety R570, harbouring the major resistance gene *Bru1*, which has been widely grown

for over twenty years in Mauritius and the Reunion Islands and yet maintains its resistance to brown rust (Asnaghi *et al*., 2001; Asnaghi *et al*., 2004). Non-durable resistance has also been observed, such as the case where resistance of the wheat cultivar Tadorna, to *Puccinia striiformis* f. sp. *tritici* was broken down within the first year of commercial production (Johnson, 1977; Ribeiro do Vale *et al*., 2001).

Qualitative resistance provides a high level of protection, but is frequently prone to breakdown, regardless of the method of management (McDowell and Woffenden, 2003; Sakr *et al*., 2011). This occurs as new virulent pathotypes evolve, rendering previously resistant plants susceptible (Gu *et al*., 2008; Keane and Brown, 1997; Sakr *et al*., 2011).Vertical resistance most likely functions well in natural ecosystems not only because it occurs in mixed populations of the plant, but also because it is supported by quantitative horizontal resistance that has accumulated in the population in response to natural selection (Keane and Brown, 1997).

2.6.3 Quantitative resistance

In all plant species, resistance to herbivores and necrotrophic pathogens is mostly quantitative (Dangl and Jones, 2001; Boller and Felix, 2009). Resistance to biotrophs can be qualitative, a mixture of qualitative and quantitative resistances, or quantitative in nature. Quantitative resistance (QR) is defined as “a resistance that varies in a continuous way between the various phenotypes of the host population, from almost imperceptible to quite strong” (Ribeiro do Vale *et al*., 2001). QR is also considered to be horizontal, a property which makes it equally effective against all pathogen races, or race-nonspecific. QR makes up the largest proportion of genetic resistance used by sugarcane breeders and also by maize breeders, mostly due to the fact that these are outcrossing species (Buckler *et al*., 2001; Poland *et al*., 2011). Also called horizontal resistance, QR provides a lower level of resistance but importantly, is not liable to breakdown because of the absence of strong selection pressure in favour of some pathotypes and against others (Keane and Brown, 1997; Poland *et al*., 2011; Ribeiro do Vale *et al*., 2001). Resistance has also often been observed to increase as plants mature, becoming most effective when there is genetic uniformity within and between crops (Keane and Brown, 1997; Ribeiro do Vale *et al*., 2001).

QR is usually inherited additively and has an oligogenic or polygenic nature, such as that found in rice to bacterial blight caused by *Xanthomonas campestris* pv. *oryzae* and in barley leaf rust caused by *Puccinia hordei* (Ribeiro do Vale *et al*., 2001). The additive effect of QR has also been observed in the Swiss wheat variety “Forno”, which has improved resistance in the presence of six currently

documented quantitative trait loci (QTL's) (Messmer *et al*., 2000). Horizontal resistance has been known to reduce the rate and degree of infection, colonisation and sporulation, together with the rate of epidemic development due to its quantitative nature (Keane and Brown, 1997; Ribeiro do Vale *et al*., 2001). Horizontal resistance is however often avoided by breeders because it is often difficult to detect and manipulate. In recent times however, more plant breeding programs are becoming concerned with breeding for quantitatively inherited characters (Keane and Brown, 1997). This new concern for QR's has culminated from the suggestion that horizontal resistance in plants may be the reason why many minor diseases are regarded as minor (Keane and Brown, 1997). Fortunately, QR is still found abundantly in adapted cultivars and can be stacked fairly rapidly by selecting lines that have lower levels of disease severity (McIntosh, 1997; Parlevliet and Van Ommeren, 1988).

While race specific resistance relies on plant recognition of pathogen effectors or their consequences, in the majority of cases by NBS-LRR genes, non-race specific QR should involve genes with other modes of action. However, it has been hypothesized that some QRLs may be weak, or defeated, *R*-genes since they have been reported to have similar or identical linkage map positions with major *R*-genes in other various crop plants, including rice and maize (Wang *et al*., 1994; Wisser *et al*., 2005; Xiao *et al*., 2007; Young *et al*., 1995). Defeated *R*-genes could contribute toward a quantitative level of field resistance through residual effects (Durel *et al*., 2003; Pedersen and Leath 1988; Stewart *et al*., 2003).

Another form of quantitative resistance, termed basal resistance, can be considered as part of innate resistance sharing common mechanisms and remaining operative in compatible interactions to limit pathogen growth. Also included in QR are traits that likely require the action of multiple genes, including a myriad of morphological, anatomical and biochemical traits such as stomata density, biochemical composition and water repellency of surfaces, all of which can have effects on resistance (Bradley *et al*., 2003; Melotto *et al*., 2008).

QR is poorly understood and is often said to be conferred by QRLs of relatively small effect (Poland *et al*., 2011), for example as found in the study of McIntyre *et al*., (2005) on brown rust in a biparental sugarcane cross. Variations in resistance phenotypes have been attributed to the possible accumulation of numerous loci of small cumulative effect, though it is likely that some QRLs can also be of large effect (Poland *et al*., 2011). An example of a larger effect QRL is the wheat gene *Lr34* which provides resistance against leaf rust (*Puccinia triticina* formerly *recondita* f. sp. *tritici*). *Lr34* is associated with a reduced rate of haustorium formation in early stages of infection, reduced intercellular hyphal growth, increased latent period, reduced infection frequency and smaller

uredinia (Rubiales and Niks, 1995; Singh and Huerta-Espino, 1997). *Lr34* is constitutively expressed and based on its sequence, is predicted to encode an ATP-binding cassette (ABC) transporter (Krattinger *et al*., 2009). Most significantly, *Lr34* (*Yr18*/*Pm38*) provides multiple disease resistance against leaf rust, stripe rust (*Puccinia striiformis* f. sp. *tritici*) and also powdery mildew (*Blumeria graminis* f. sp. *tritici*). This resistance is still durable after more than 50 years in the field (Priyamvada *et al*., 2011).

Krattinger *et al*. (2011) showed through the construction of ABC transporter phylogenetic trees, including homologous ESTs from sugarcane and other grasses, that a sugarcane sequence is clustered together with *Lr34* orthologs. These authors considered it likely that sugarcane contains orthologous *Lr34* genes. However, they also suggested that the particular *Lr34*-haplotype found in resistant wheat cultivars may be unique, probably arising from functional gene diversification after the polyploidisation event at the origin of cultivated wheat. Among many other genes, Carmona *et al*. (2004) and Oloriz *et al*. (2012) detected increased differential ABC transporter gene expression in *Puccinia melanocephala* resistant somaclonal variants and chemically induced sugarcane mutants respectively, compared to susceptible “parent” genotypes.

2.7 Sugarcane Breeding

Genetic maps based on molecular markers have proven useful in understanding crop genome structure and for isolating important genes from many species (Jiang *et al*., 2000; Manigbas and Villegas, 2007; Raboin *et al*., 2006). The need for a saturated map is critical in the efficient localization of major genes or Mendelian factors involved in QTL (Raboin *et al*., 2006). In the past few years, these markers, in association with more powerful statistical models, have been applied to genetic analysis and breeding of several crops (Manigbas and Villegas, 2007; Maureira and Osborne, 2004; Ming *et al*., 2002). The application of these tools has however been hindered by several factors in sugarcane, such as the coexistence of alleles at each locus due to polyploidy and polysomic inheritance in the species, complicating linkage analysis and the detection of QTL (Grivet and Arruda 2001; Maureira and Osborne, 2004; Raboin *et al*., 2006).

Traditional breeding in sugarcane employs the sexual route to create clones with different genetic makeup and phenotype. The new clones are then fixed in clonally propagated cuttings for successive selection stages (Midmore, 1980; Ramdoyal *et al*., 2000; Sleper and Poehlman, 2006). These techniques have seen sugarcane breeders make advances in increasing the yield and disease resistance in the modern sugarcane cultivars derived from the early interspecific genotypes, coupled

with some cycles of intercrossing and selection (Grivet and Arruda 2001). The sugarcane breeding process is however complicated due to modern sugarcane genomes being large and having a high degree of ploidy (Asnaghi *et al*., 2001). The *Bru1* rust resistance trait has however been found to be of high narrow and broad sense heritability, assisting in the development of the existing rust resistant varieties (Asnaghi *et al*., 2001; Hogarth *et al*., 1993). Observations of marked transgressive segregation towards rust susceptibility in bi-parental crosses and selfed families have also been made, suggesting the partial dominance of the rust resistance trait (Ramdoyal *et al*., 2000).

One of the main components of plant breeding is the identification of germplasms containing genes that could improve the performance of current cultivars (Maureira and Osborne, 2004). As this activity is the cornerstone of all advanced sugarcane industries, it is important to integrate it with biotechnology to ensure that maximum advantage of the new possibilities to maintain and increase production is sustained (da Silva and Bressiani, 2005). Molecular markers are valuable tools for the breeder, as they increase the efficiency of indirect and early selection in sugarcane (da Silva and Bressiani, 2005; Huang, 2008; Ming *et al*., 2002). Biochemical and molecular markers provide a more accurate means of monitoring unifactorial inheritance in such complex genomes, resulting in varieties with high sugar content and disease resistance (da Silva and Bressiani, 2005; Grivet *et al.*, 1996).

A conventional sugarcane variety improvement cycle (breeding and selection) is normally conducted in the field and takes an average of 12 years from hybridization to cultivar release (Bischoff and Gravois, 2004; Purdy, 1985; Sleper and Poehlman, 2006). This process is however lengthy, as far as meeting industry requirements is concerned (Bailey, 2004). Extended cultivar turnover has motivated for the use of technology such as marker assisted selection (MAS), which could significantly speed up this process (da Silva and Bressiani, 2005; Ming *et al*., 2002). Comparative genetic mapping across species has revealed the organization of plant genomes and aided in the identification of several candidate genes for disease resistance (Grivet and Arruda, 2001). Genetic mapping could subsequently assist in the design of markers in sugarcane which could facilitate the screening of large numbers of new accessions in breeding programs more efficiently than field of glasshouse trials (Keane and Brown, 1997; Märländer, 2000).

2.8 Resistant Varieties

Resistant varieties arise due to the presence of either a major qualitative gene or a collection of minor genes called quantitative resistance (McDowell and Woffenden, 2003; Sakr *et al*., 2011).

These resistant varieties provide the most efficient means of control to disease and indeed, to brown rust in sugarcane (Asnaghi *et al*., 2001; Bischoff and Gravois, 2004; Keane and Brown, 1997; Ribeiro do Vale *et al*., 2001). Resistant cultivars are not only important as an efficient means of disease control, but also due to their positive environmental implications and the reduction in high costs incurred as a result of losses or in the use of alternative means of control (Bischoff and Gravois, 2004; Keane and Brown, 1997; Ramdoyal *et al.*, 2000). R570, a widely grown variety of sugarcane with resistance to *P. melanocephala* is one such example of a successfully used resistant variety (Asnaghi *et al*., 2001; Le Cunff *et al*., 2008; McIntyre *et al*., 2005; Raboin *et al*., 2004).

There have been suggestions made that the sugarcane variety R570, whose rust resistance has been attributed to the major gene *Bru1* contains other genes with smaller effects acting in a quantitative way, as the one gene hypothesis does not fully explain the susceptibility levels within some previously conducted crosses (Ramdoyal *et al*., 2000). Such information is crucial, as it would suggest breeding strategies to adopt for controlling rust. Stacking of major resistance genes alone, or together with the accumulation of minor resistance genes conferring quantitative resistance are strategies used for breeding wheat. This strategy could be incorporated into sugarcane breeding programs to improve resistance to brown rust, among other diseases (Messmer *et al*., 2000).

Suggestions have been made that tolerant varieties to brown rust have little use in the industry, as they could contribute to the development of new pathogenic races, especially when grown in large populations (Purdy *et al*., 1983). These uncertainties have called for the further development of resistant varieties, as new rust races and climate change are resulting in previously resistant/ tolerant varieties becoming susceptible (McFarlane *et al*., 2006; Purdy *et al*., 1983). The advent of novel molecular breeding techniques has resulted in resistant cultivars being produced more efficiently and rapidly for most commercial crops (Manigbas and Villegas, 2007; Märländer, 2000). The use of such technology would ensure the replacement of susceptible varieties with resistant cultivars, reducing rust outbreak risk, maximising on profit and reducing inputs (Purdy *et al*., 1983).

2.9 Rust Resistance Screening Methods

Disease screening is an important step in the breeding process, as it determines the resistance and subsequent quality of cultivars selected in the breeding program. A variety of methods are used in disease screening and these range from glasshouse experiments, field trials and *in vitro* experiments. Complete resistance to rust is generally assessed by conducting inoculations and observing the reactions on seedlings in a greenhouse; while partial resistance tests are primarily observed in the

field on plants that have reached the age at which they are most susceptible (Jackson *et al*., 2008). Rust resistance can also be assessed by inoculating plants and placing them inside dew chambers to assess disease development (Jackson *et al*., 2008). *In vitro* methods using detached leaf assays have also been formulated for the disease screening of soya beans, wheat, oats and more recently on sugarcane (Ali *et al*., 2008; Asnaghi *et al*., 2001; Hoy and Hollier 2009; Twizeyimana and Hartman, 2010). An ideal screening method would be one where the results can be obtained rapidly, with minimum use of land or space and testing material. A major advantage would be an increase in new variety turnover, as lag time spent waiting for plant growth and favourable disease conditions could be eliminated. The starting material required for an ideal method would have to be minimal, as bulking up sugarcane to sufficient levels for both field and pot trials is a lengthy process.

MAS as a rust resistance screening method has its advantages in that it requires a minimal amount of starting material, it requires no planting space and is rapid. Screening via the use of inoculation is however necessary as some resistance traits are unknown, limiting MAS to a screening function for known traits only, whereas other methods can assist in the identification of additional modes of resistance. Another challenge with MAS is that it occasionally identifies traits which are controlled by epistatic interactions which may be unrelated to the marker. Furthermore, screening for disease resistance QTL's in sugarcane using MAS is still not practical, as the sugarcane genome has not been sufficiently decoded (Le Cunff *et al*., 2008).

2.9.1 Detached Leaf Assay

The maintenance of detached leaf pieces on media containing plant growth regulators to ensure optimal health and their subsequent inoculation with pathogens is useful in the study of host-pathogen interactions for a variety of rust fungi (Browne and Cooke, 2005; Jackson *et al*., 2008; Loladze *et al*., 2005; Twizeyimana and Hartman, 2010). Detached leaf assays (DLAs) are useful in countering the large environmental influence on phenotypic estimates of disease resistance and the complex polygenic nature of fungal resistance in cultivars often experienced when conducting whole plant assays. These environmental influences are obstacles to developing resistant cultivars (Browne and Cooke, 2005). The DLA gives a technique capable of screening large numbers of breeding or mapping population lines for complete resistance. DLAs also overcome space and time constraints that are experienced when using conventional methods of assessing genotypes for disease resistance (Twizeyimana and Hartman, 2010).

This method has been successfully used in the identification of important components of resistance to *Fusarium* head blight (FHB) in European wheat germplasm (Browne and Cooke, 2005). DLA has already been proven to have similar responses to both field and seedling assays, with low standard errors among the evaluation methods (Loladze *et al.*, 2005). This makes it a potentially better method of screening for disease resistance after considering its advantages. This method however has not yet proved valuable in determining partial resistance (Jackson *et al.*, 2008).

2.9.2 Field Trials

Field based assessments have long been an important means of assessing disease resistance, proving to be useful not only in identifying resistant genotypes but as being the best known way of identifying QTL's (Nair *et al*., 2005). Field trials are normally conducted in areas with high disease pressure to allow the plants to be adequately challenged at near optimal conditions for disease infection. Inoculations are normally carried out to ensure similar treatment to each plant. Whorl inoculations and spore sprays among other techniques are used to inoculate, depending on the host and the pathogen of interest. Rust experiments have normally been carried out under conditions of high natural infection incidence and assessments made thereafter (Zvoutete, 2006). This however is not reliable as it is not possible to ascertain whether inoculum has been received and to quantify the inoculum among other parameters.

These same issues are also raised when spraying plants, as application rates and host receipt cannot be guaranteed. Whorl inoculation methods have been used in recent sugarcane rust experiments with much success (Sood *et al*., 2007). This method not only provides an assurance that inoculum is delivered evenly to the plant, but can also be quantified. Whorl inoculation has also been successfully used with other crops such as maize in the identification of QTLs and resistant genotypes (McCammon *et al*., 1985; Nair *et al*., 2005). Whorl inoculation also has the advantage of being able to maintain controls, unlike other experiments conducted using airborne pathogens (Shokes *et al*., 1996).

2.9.3 Glasshouse Trials

Glasshouse trials have been used in the rapid screening of a large number of cultivars (Sood *et al*., 2009). This method is advantageous in that it provides controlled conditions under which disease

development and pathogen/ host interactions can be observed. The increased severity of infection in glasshouse trials makes them ideal in the identification of complete resistance. This is due to conditions which are frequently optimal for the development of rust, which on the contrary is an inaccurate assessment, as such conditions are rarely found in the field. This factor is one such limitation in the use of glasshouse experiments for identifying QTLs, as plants seldom respond as they would under natural conditions, where several other factors can influence the outcome of disease expression.

2.10 Molecular Marker Based Systems

Molecular markers used in MAS for identifying a wide array of traits have been obtained from a variety of techniques which include amplified fragment length polymorphisms (AFLP), random amplified polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) amongst other methods (Hoarau *et al*., 2001; Le Cunff *et al*., 2008). These markers have also been used in genetic map technology where they have been useful in the identification of genes such as *Bru1* (Asnaghi *et al*., 2000; Asnaghi *et al*., 2004). More recently, molecular markers from technologies such as Subtraction Subtractive Hybridization (SSH) and NBS profiling have been useful in the improvement of existing genetic maps by saturating them and increasing their resolution (Alsop *et al*., 2011).

2.10.1 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a highly reproducible method of characterising genomic DNA that can be used to generate numerous informative genetic markers for DNA of any origin or complexity (Vos *et al*., 1995). This ability to consistently diferentiate individuals using the AFLP method was proved by Jones *et al*. (1997) in a proficiency test amongst a network group of laboratories in Europe. This method is generally used for fingerprinting and has been successfully used to differentiate between individuals and independently evolving lineages. AFLP has also been found to be very useful in the analysis of genetic variation, even below the species level and has been successfully used to analyse population structure and differentiation (Mueller and Wolfenbarger, 1999). The strength of AFLP has been demonstrated in its use in the differentiation of near-isogenic lines of soybean differing at single to small regions in their genome (Mueller and Wolfenbarger, 1999).

AFLPs have also shown their capability to be used for QTL mapping, as they are capable of generating many genome wide polymorphic markers (Mueller and Wolfenbarger, 1999). Linkage maps for QTL analysis have been used to analyse agronomic traits which include disease resistance

and salt tolerance (Asnaghi *et al*., 2004; Mueller and Wolfenbarger, 1999; Raboin *et al*., 2006). Maps constructed with AFLP markers have shown a much higher resolution than other methods and show extended genome coverage and decreased distance between markers when added to existing maps. This was demonstrated by Le Cunff *et al*., (2008), who used AFLP markers to get to within 0.14 and 0.28 cM of the *Bru1* rust resistance gene, whose closest markers were previously located at 1.9 and 2.2 cM on either side of the gene (Asnaghi *et al*., 2004). AFLPs have also been used for Linkage Disequilibrium mapping on a sugarcane breeding population in an association analysis study. This study revealed the presence of negatively correlated markers between smut and eldana association, but most importantly, improved the method of identifying cross combinations resistant to both smut and eldana (Butterfield, 2007).

2.11 Detection of Differential Gene Expression

Identification of host genes involved in disease resistance is a critical step in understanding resistance mechanisms in host pathogen interactions (Sutton and Shaw 1982; Xiong *et al*., 2001). Suppression Subtractive Hybridization (SSH) is a screening method used for distinguishing between two closely related cDNA samples (Diatchenko *et al*., 1996; Rebrikov *et al*., 2004; Roelofs *et al*., 2007). The use of infection and stress induced cDNA libraries has been successfully used to identify defence related genes in a number of plants including rice, wheat and sugarcane using the SSH protocol (Oloriz *et al*., 2012; Watt 2003; Xiong *et al*., 2001; Yan *et al*., 2009). This method is frequently used to analyse differentially expressed genes between samples by using extracted poly-A+ RNA transcribed into cDNA from both sets of interest (Distler *et al*., 2007). The current SSH method has been improved and uses fewer steps than that initially described by Diatchenko *et al*., (1996). This more recent SSH protocol has the ability to enrich for genes (and mRNA) with low expression levels and also to identify differentially expressed genes, while eliminating the homologous cDNAs (Distler *et al*., 2007; Li *et al*., 2002; Oloriz *et al*., 2012; Roelofs *et al*., 2007; Triplett *et al*., 2006). These different SSH analyses have yielded interesting results, as they have turned up combinations of genes responsible for signal transduction, transcriptional regulation and hypersensitive responses (Yan *et al*., 2009). SSH analysis in sugarcane has also been successfully conducted for the sugarcane- smut interaction and has shown the presence of putative receptors involved in the signalling of resistance mechanisms, transcription factors and enzymes in resistant genotypes (Borrás-Hidalgo *et al*., 2005; Heinze *et al*., 2001). Such results are important as they not only elucidate host-pathogen interactions, but can be subsequently used in the development of markers to aid in the development of resistant cultivars.

The use of AFLPs in the analysis of differential gene expression has also been employed and has shown great versatility in that it can be used to elucidate important differences in combination with other genome analysis methods (Carmona *et al*., 2004). cDNA-AFLP analysis has been used in the analysis of differential gene expression in the interaction between somaclonal variants of the sugarcane genotype B4362 and *P. melanocephala* (Carmona *et al*., 2004). This method has also been used to analyse the differentially expressed genes in the sugarcane- smut interaction of sugarcane hybrids and has revealed the presence of putative chitin receptor kinases, a Pto ser/thr protein kinase interactor and an active gypsy type LTR retro-transposon in a resistant variety (Thokoane and Rutherford, 2001). This method has more recently been used to analyse the differential expression of cDNA produced by two sugarcane cultivars after inoculation with *Sporisorium scitamineum* (Syd.) M. Piepenbr., M. Toll & Oberw (LaO *et al.,* 2008). The research by LaO *et al.* in 2008 demonstrated the high differentiating abilities of AFLP, as it indicated that the method can even be used to show expression differences in the same individual before and after its interaction with a pathogen.

2.12 Summary

Puccinia melanocephala, a major pathogen of sugarcane, has resulted in severe losses to the international sugarcane industry and to the economies of affected countries where sugarcane is a major economic contributor. The effects of brown rust have made it imperative to ensure that methods of controlling the disease are constantly formulated and improved. The use of resistant varieties against rust has by and large been the favoured method of control as it not only reduces losses due to rust infection, but also comes with the benefits of reduced expenditure in the form of fungicides and related control measures. These factors are important in this modern world, as sugarcane has taken on the added role of being a source of energy for the industrial sector in the form of ethanol, an environmentally feasible fuel option, considering climatic challenges that are being faced globally. The reduced use of agrochemicals can subsequently aid in a reduction of the carbon footprint in sugarcane production.

The identification of QRLs and alternative major genes of resistance to rust is a crucial exercise in the goal to increase production of resistant cultivars for the sugarcane industry. As QRLs have been identified as a durable source of resistance due to their non-specificity, their identification and accumulation into new resistant genotypes will increase resistance to brown rust in new cultivars. An increase in the number of cultivars with added resistance would see the withdrawal of susceptible cultivars, and a massive reduction in the threat the disease currently poses. The

development of resistant varieties would have benefits which include increased productivity, lower production costs, reduction in the carbon footprint and positive contributions towards mitigating environmental pollution and climate change.

Both field and pot trials have been traditionally utilised for the identification of genes of resistance for use in breeding programs. These tools have proved valuable as they have yielded most of the varieties still in use today. The use of genetic analysis tools has augmented these traditional procedures, validating them and ensuring that there are fewer inaccuracies. These methods have significantly reduced the time it takes for screening protocols to be conducted and promise to reduce the sugarcane variety improvement cycle, as they become more accurate and efficient. Molecular techniques also provide an avenue towards the more rapid identification of resistance mechanisms through the use of technology such as cDNA-AFLP and SSH. These possibilities give rise to the aim of this project, which is to uncover the genomics of rust resistance within the LD2 breeding population.

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Chapter 3

Rust resistance screening methods: A whorl inoculation technique for discriminating among resistant, intermediate and susceptible genotypes from a sugarcane population.

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Abstract

Brown rust of sugarcane is a disease best controlled by the use of resistant cultivars. The production of resistant cultivars is however highly dependent on the efficiency of a breeding program, as major drawbacks pertaining to rust related losses have been due to escapes in these programs. Escapes have resulted in unforeseen losses such as those experienced when the rust tolerant N29 sugarcane variety became susceptible. An inoculation technique for the efficient large scale screening of sugarcane cultivars in the South African Industry was subsequently developed using whorl inoculation. Whorl inoculation was conducted on the 80 genotype LD2 breeding population in two pot trials conducted under different weather conditions. *Bru1*, a major rust resistance gene was found in 54 of the genotypes in the LD2 population. Lesion development, sporulation and an overall symptom rating system were parameters used to analyse the effects of whorl inoculation. The Restricted Maximum Likelihood procedure showed significant differences between the pot trials, an occurrence attributed to the different weather conditions under which the trials were conducted. This underlined the impact of genotype by environment (GE) interactions when conducting screening exercises. The first pot trial was conducted in rust optimal conditions, with an average temperature of 20^{o}C. Rust symptoms were subsequently more severe than in the second trial, in which the average temperature was a sub-optimal 17.9^{o}C. Correlation coefficient analysis showed significant correlations between both pot trials. Sporulation however did not show a significant correlation between the trials when the *Bru1* negative genotypes in LD2 were analysed. The *Bru1* positive genotypes maintained rust resistance. The *Bru1* negative genotypes 96E0212, 96E0895, 97E0589, 97W0181, 93F0234 and 97E0410 were consistently highly resistant in both screening methods, suggesting either an alternative major rust resistance gene or strong quantitative resistance. These trials demonstrated that whorl inoculation can be successfully used to screen large populations in pot trials, with minimum escapes when conducted under optimal conditions.

3.1 Introduction

Brown rust of sugarcane is a disease capable of inflicting severe yield losses on a harvest, and has previously resulted in losses of greater than 50% (Purdy *et al*., 1983). Losses are dependent on cultivar susceptibility and prevailing environmental conditions (Bailey, 2004; Hoy and Hollier, 2009; Taylor *et al*., 1986). Recent losses in the South African sugarcane industry amounted to 26% yield reductions in the N29 variety (McFarlane *et al*., 2006). Research has shown that the application of fungicides can be successfully used to mitigate yield losses and increase yields (Hoy and Hollier, 2009). These yield improvements have however been negated by the cost of fungicides, which impact negatively on net profit, resulting in the use of fungicides being suitable only as a contingency measure, in the event of an unexpected outbreak (Hoy and Hollier, 2009; Sood *et al*., 2009; Zvoutete, 2006).

The use of resistant cultivars and good cultural practices was proven to be the most effective means of control, not only from a practical aspect, but also from an environmental perspective. Resistant cultivars have been used successfully since the global rust outbreak in the late 1970's and early 80's, resulting in a rust screening step being incorporated into most breeding programs (Purdy *et al*., 1983; Sood *et al*., 2009). These breeding programmes have seen the replacement of rust susceptible varieties with resistant ones, resulting in reduced losses from brown rust.

A variety of techniques are employed in the screening of rust resistance in breeding populations, with field trials being more widely used, as the results are a better representation of crop responses in the actual conditions the crops will be propagated (Anand *et al*., 2003; Izanloo *et al*., 2008; Sood *et al*., 2009). Field analyses also have the advantage of being able to screen for both quantitative and/ or qualitative resistance as compared to glasshouse screening which is more inclined towards selecting for qualitative resistance (Jeger and Viljanen-Rollinson, 2001; Nair *et al*., 2005). This has been proved in the successful breeding of disease resistant potato cultivars, using field trials to identify horizontal/ quantitative resistance (Roane, 1973). Field analyses are normally conducted in areas of high disease pressure and infection rates, analysed and used to assign resistance ratings to cultivars within breeding populations (Asnaghi *et al*., 2001; Sood *et al*., 2007; Tai *et al*., 1981). Field trials however have limitations in that they are affected by a host of variables, of which most are not under human control (Izanloo *et al*., 2008; Sood *et al*., 2009). Efforts have been made to control some of these variables under field conditions, with inoculation of field plants being common practice to ensure disease pressure is both present and uniform among test genotypes. Field trials have also been carried out in conjunction with glasshouse pot trials to mitigate field trial limitations, which include lack of temperature and humidity control (Izanloo *et al*., 2008). The stringency of the

glasshouse method can also have the advantage of ensuring a high throughput (Sood *et al*., 2009). This exercise, though expensive, has not only aided in confirming field data, but has also aided in the discovery of other sources of vertical resistance (Hu *et al*., 1996).

The stacking of resistance mechanisms has been found to improve the efficacy and durability of pathogen resistance in crops (McIntosh, 1997; Parlevliet and Van Ommeren, 1998). The stacking of resistance genes confers stronger and more durable resistance when compared to the use of single gene resistance mechanisms. Single gene resistance is prone to breakdown due to the presence of strong selection pressure in favour of virulent pathotypes, which are characterised by a high genetic plasticity (Hu *et al*., 1996; Keane and Brown, 1997; Ribeiro do Vale *et al*., 2001).

Whorl inoculation of sugarcane has recently allowed the reliable, mass screening of large numbers of sugarcane cultivars (Sood *et al*., 2009). This method fares better for sugarcane and for inoculations using obligate pathogens such as *P. melanocephala,* which do not produce large quantities of spores (Sood *et al*., 2009). This characteristic of *P. melanocephala* eliminates the possibility of spraying as an inoculation technique, as large amounts of inoculum would be required to spray numerous cultivars that are screened in a cultivar development programme (Sood *et al*., 2009). The ability to screen large numbers of genotypes for rust resistance will result in more resistant genotypes being available for use in breeding programmes and the subsequent release of more rust resistant commercial cultivars.

To reach a stage where the threat posed by rust is minimal, ideal screening methods must be employed to identify resistant genotypes. This ideal method should be one in which the technique used gives high throughput, eliminates escapes and ensures an accurate expression of resistance. Ultimately, this screening method should give reproducible disease expression when compared to known cultivars and must also be able to correlate with field observations under natural conditions (Bugdee and Sappenfield, 1967). The need to reach this ideal has increased this past decade, with the reminder of how much of a threat rust still is, after the devastating outbreak experienced on the partially rust resistant variety N29. This occurrence has led to the objectives of this study, which aim to adopt and optimise a glasshouse pot trial for the mass screening of large populations in the South African sugarcane industry. This study also aims to uncover the rust resistance levels present in a breeding population (LD2), for use in further studies and subsequent release of resistant varieties. These objectives can be reached by the use of leaf whorl inoculations in the screening stages of the SASRI breeding program.

3.2 Materials and Methods

Two pot whorl inoculation trials were conducted at different time intervals and under different weather conditions using an LD2 breeding population consisting of 80 genotypes and three controls, namely N12 (resistant), N29 (susceptible) and N39 (intermediate).

3.2.1 Pot trial preparation

Pots with a 20l volume were filled with a mixture of sandy soil and vermiculite, labelled from 1-80 for each LD2 genotype and three pots labelled N12, N29 and N39 for the controls. The pots were placed into steel troughs half filled with water. Five seedlings of each genotype were transplanted into their corresponding pot and watered daily using a sprinkler. The speedlings were fertilised with 100g of 2.3.2 granular fertiliser (Grovida Horticultural products, KZN) once a month for two months before inoculation. Temperature ranged between 19- 25.5°C and relative humidity between 61.3-73.6% (Fig 3.2.1).

3.2.2 Urediniospore collection and inoculum preparation

Rust infected leaves from the cultivars N29 and N39 were harvested from the field, cut into small pieces and the spore infested areas placed into a 500ml SHOTT DURAN® glass bottle containing 300 ml distilled water and glass beads. The bottle was shook vigorously till the spores were removed from the pustules in the leaves, turning the water into a murky brown colour. The leaves were removed from the glass bottle and the process repeated using water from the previous extraction. The spore extraction water was occasionally topped up to 300 ml with water used to rinse previously extracted leaves. The process was repeated till the solution was completely opaque.

The spore solution was immediately quantified using a Neubauer counting chamber (Marienfield Superior, Germany) under a Nikon Eclipse 50*i* light microscope (Sood *et al*., 2009). The suspension was adjusted to a concentration of 10^6 spores/ ml and transferred to a glass beaker. Germination counts were conducted on 1% (w/v) water agar, prepared using bacteriological agar (Merck, Gauteng) in distilled water and set in plastic Petri dishes. Spore suspension (100 µl) was added to the agar surface and spread by swirling sterile glass beads inside the petri dishes. Germination tests were conducted on five plates and incubated overnight in the dark at 20°C (Braithwaite, 2005). Percentage germination was obtained by counting spores under a Nikon Eclipse 50*i* light microscope

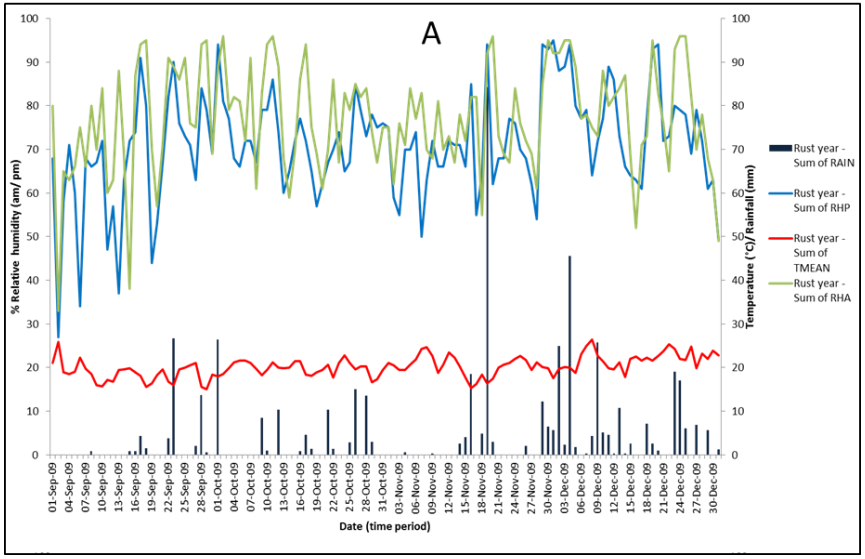
and using the formula: Percentage germination = (germinated spores/ total spore count) * 100. Spore solutions with at least a 15% germination rate were used for whorl inoculation.

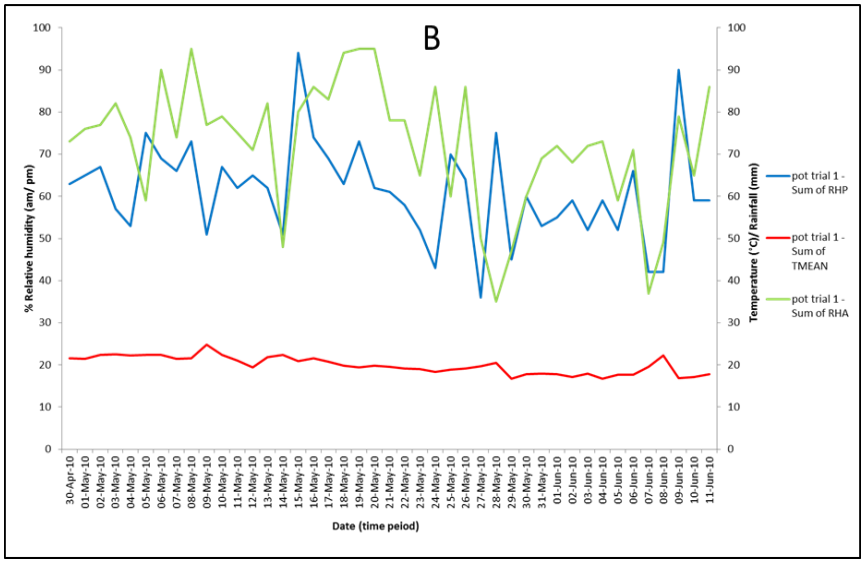
3.2.3 Plant preparation

After four months, the sugarcane had tillered and four stalks were selected per genotype from different stools in each pot. Each of the four stalks was tagged using danger tape to monitor the inoculated plant. The innermost leaves, including the top visible dewlap (TVD) were cut off with a pair of scissors to expose the whorl and aid in identification and monitoring of the leaves in direct contact with the rust inoculum. Parafilm (Pechiney plastic packaging, Chicago, IL.) was wrapped around each whorl to secure it from inoculum loss and ensure equal exposure to the inoculum.

3.2.4 Screening of LD2 population for rust resistance by leaf whorl inoculation

Spore suspension (1 ml) was dispensed into the leaf whorl of each tagged and secured plant using an Eppendorf micropipette. Inoculation was done towards sunset to allow dark incubation of the inoculated plants. The parafilm was removed during the first week to allow unobstructed growth of the plants. Observations were conducted daily and the recording of observations commenced from the first to the sixth week. Disease reactions were rated using lesion development and sporulation intensity as observed under a Leica MZ 12^5 dissecting microscope at 80X magnification. Lesion rating was based on a modified 1 to 5 scale (Fig 3.2.2a) as described by Sood *et al.* (2009), while sporulation was based on a 0-3 scale, 0 denoting no sporulation, 1= slight, 2= medium, and 3= heavy sporulation (fig 3.3.2b). An overall rust resistance rating scale was calculated by multiplying the combined average lesion ratings by the average sporulation ratings for each individual genotype in both pot trials.





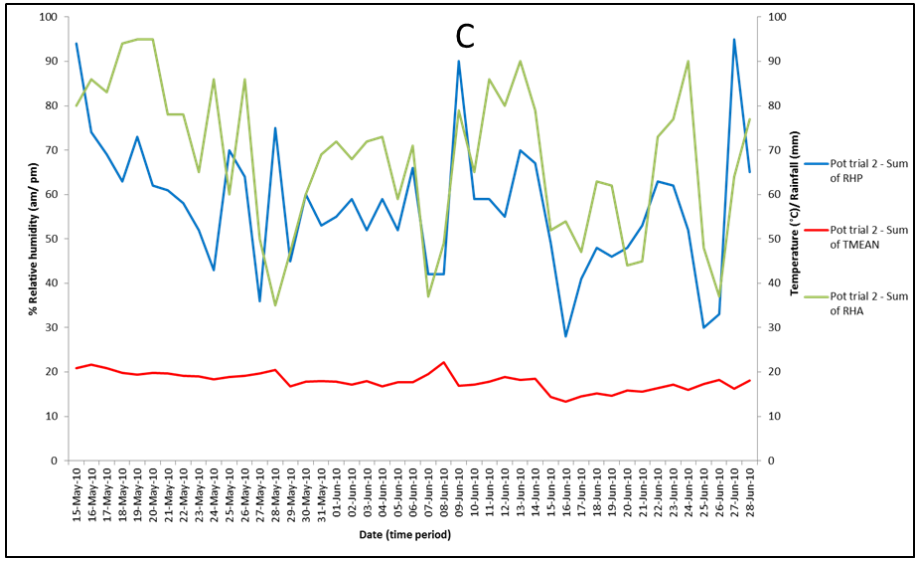
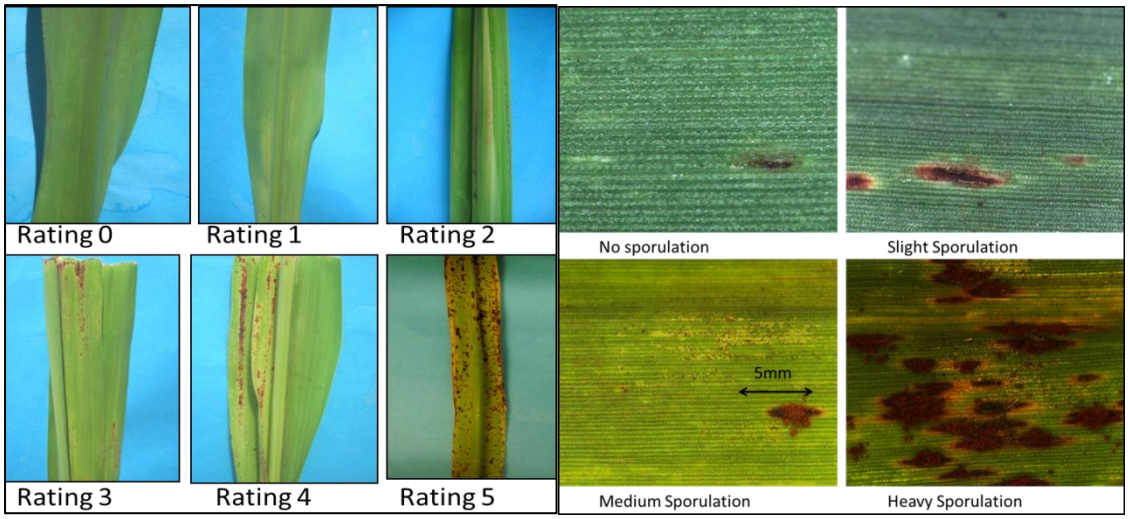


Fig 3.2.1: Weather data during the rust inoculation trials compared to (A) late 2010, when a rust epiphytotic broke out in the field. These trials include the first pot trial (B) and the second pot trial (C). The weather data consisted of rainfall averages, percentage relative humidity taken at 0800hrs (RHA) and at 1400hrs (RHP) and daily average temperature (°C).



(a) **(b)**

Fig 3.2.2: Visual rating scale used for rust symptoms after whorl inoculation with *P. melanocephala* spores. A lesion rating scale (fig a) were used together with sporulation ratings (fig b).

3.2.5 Statistical analysis

Prior to analysis, data were tested for normality using the Shapiro-Wilk test. Each trial was analysed using the rust ratings within a trial over time, which were then compared to each other using analysis of variance (ANOVA) with repeated measures. Analyses were also conducted between the two pot whorl inoculation trials using the Restricted Maximum Likelihood (REML) procedure - Meta Analysis (Genstat ver.13). Correlations were performed using SAS, to analyse the similarities between trials, using Spearman's correlation coefficient and Pearson's Product Moment Correlation Coefficient (PPMCC). The two methods of calculating correlation were compared to each other to ensure validity of the results (Shokes *et al*., 1996).

3.3 Results

The lesion rating scale of 0- 5 (Fig 3.2.2) was used to rate rust resistance in the trials in which ratings of 0- 1.5 were considered resistant; 1.51- 2.5 were intermediate and ≥ 2.51 were susceptible. Sporulation ratings were used in conjunction with the lesion ratings in which the ratings 0-1 were considered resistant, 1-2 intermediate and 2-3 susceptible. The sporulation ratings were usefull in the formulation of an overall rust resistance rating (Section 3.2.4). The whorl inoculation technique showed the efficiency of the *Bru1* gene as a major rust resistance gene, as only 8% of the genotypes testing positive for both markers were rated as susceptible while 51% were resistant and most of the intermediate rated genotypes narrowly missed the upper resistance "cut off" rating of 2.5. The *Bru1* negative genotypes however showed a greater proportion (25%) of susceptible genotypes. Surprisingly, 42% of the *Bru1* negative genotypes screened via whorl inoculation demonstrated resistance to brown rust, suggesting the presence of strong quantitative resistance, or an alternative major rust resistance gene in the population. Among the genotypes with 0.5 of the *Bru1* marker (one of either markers), all were susceptible, except the genotype 92M1397 which was of intermediate resistance.

REML variance components analysis showed significant differences when the different rust infection trials were compared to each other over time and among genotypes. Significant (P<0.001) differences were observed through the first and fourth weeks, with increasing rust severity. Visual ratings for the fifth and sixth weeks were not significantly different from week four readings. It was decided to use week six data to explain the remaining trials, to ensure that no symptoms were missed. ANOVA (repeated measures) showed that the individual trials were significantly (P<0.001) different among genotypes and during the different time points that visual analyses were carried out.

The data for both whorl inoculation trials are represented in Figures 3.3.1 and 3.3.2 in which the overall rust reaction ratings are represented by black bars, while *Bru1* marker presence is represented by the grey diamond shapes in the same graphs. The number "1" on the right hand y axis represents presence of both markers, implying *Bru1* presence, "0.5" represents the presence of one or the other of the two markers, a result which was interpreted as not confirming *Bru1* presence and zero represents absence of both markers, an indication of *Bru1* absence. Overall rust ratings between 0- 3.5 were regarded as resistant, 3.51- 6.5 were regarded as intermediate and 6.51- 10 as susceptible. The first pot trial showed 35% of the *Bru1* negative genotypes to be rust resistant, while 23% were susceptible (fig 3.3.1). The

remaining genotypes were of intermediate resistance, with overall rust ratings close to the cut off point for resistance, indicating a relatively high level of resistance. These observations underlined the particularly virulent conditions experienced during the first pot trial. Of the *Bru1* positives, 63% were highly resistant and the remaining 37% appeared intermediate, with overall ratings of slightly over the 3.5 threshold for resistance. In the second pot trial, only 5% of the *Bru1* positive genotypes were above the 3.5 rating, with the highest being rated 4.0, half a unit outside the resistance range. A high proportion of these intermediate genotypes were marginally missing the upper resistance cut off point, yet again demonstrating the *Bru1* gene's efficacy. The genotypes 96E0212; 96E0895; 97E0589; 97W0181; 93F0234 and 97E0410 were *Bru1* negative and consistently resistant in both pot trials.

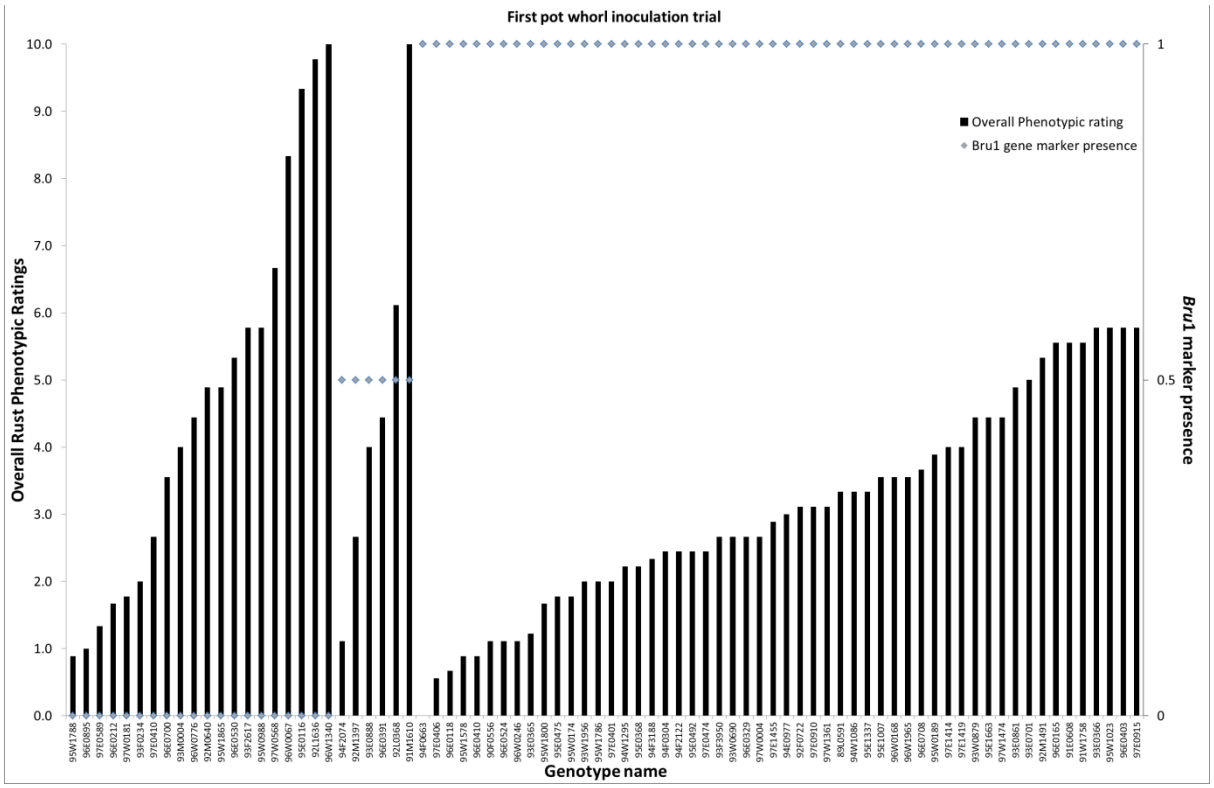


Fig 3.3.1: Overall rust reaction ratings of the LD2 breeding population in Pot Trial One six weeks after whorl inoculation with *P. melanocephala* spores.

The second pot trial on the same genotypes gave a similar trend for most of the observed genotypes, with the *Bru1* negative genotypes 96E0212; 96E0895; 97E0589; 97W0181; 93F0234 and 97E0410 being resistant, as in the first pot trial. The second pot trial showed a higher proportion of *Bru1* negative resistant genotypes, with 73% being under the 3.5 rating and 12%

being susceptible. There was also a significant (*P*= 0.05) increase in the percentage of resistant *Bru1* positive genotypes, with 95% being resistant, while the remaining 5 % were all rated below 4.0, showing near resistance among the intermediate group. The genotype 91M1610 was the most susceptible in both pot trials, an observation made previously in the field.

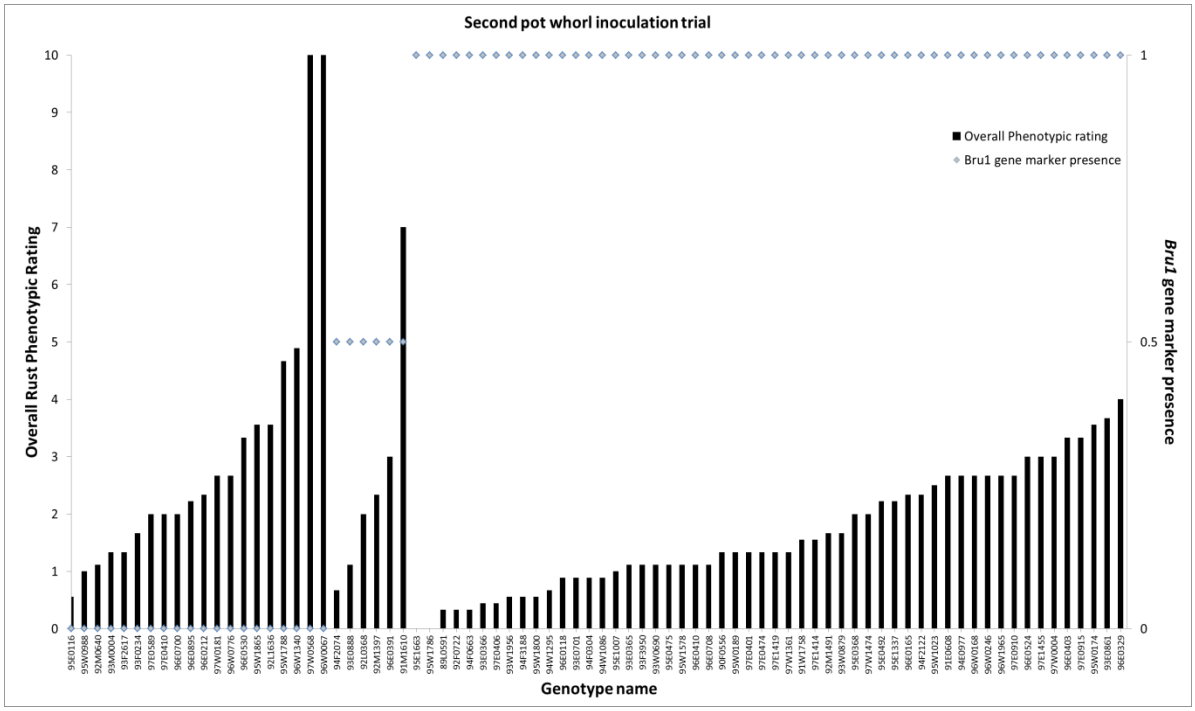


Fig 3.3.2: Overall rust reaction ratings of the LD2 breeding population six weeks after whorl inoculation with *P. melanocephala* spores in a second pot trial.

The differences noted between the pot trials' overall rust ratings showed that the first pot trial had more severe symptoms than the second pot trial. The different weather conditions experienced in both trials (Fig 3.2.1) could have resulted in the differences in severity of the symptoms. Genotypes in both pot trials generally maintained their resistance rating ranges, with genotypes 95E0116, 92L1636 and 96W1340 however being the most divergent between both trials with highly contrasting ratings. Between the pot trials, 95% of the *Bru1* positive genotypes were in similar overall resistance rating groups (groups consisting of resistant, intermediate and susceptible); while 5% of similar *Bru1* positive genotypes between the pot trials were grouped differently due to slight differences of between 0.06-0.5 rust rating units between genotypes.

Comparisons between the *Bru1* negative genotypes in both pot trials showed a 66% similarity in resistance group between genotypes. Only 2.5% of the genotyes in the entire population

gave completely divergent ratings in both trials, with the genotypes 92L1636 and 95E0116 being resistant in the first pot trial, but being susceptible in the second trial.

Correlation coefficients were calculated for the different parameters recorded between the two pot trials. The Pearson's product moment correlation coefficient (PPMCC) and the Spearman's rank correlation coefficient were used simultaneously to ascertain the similarity between the whorl inoculation data. It was found that in terms of the overall rating scale (fig 3.3.3) and lesion rating scale (fig 3.3.4), the genotypes gave a similar response (*P*= 0.05) between the pot trials, with both correlation methods showing statistically significant correlation coefficients. There were however no significant similarities in genotype response when sporulation was used as a parameter and analysed using the Spearman correlation coefficient. The PPMCC for sporulation was however significant, with *r* = 0.03 (Fig 3.3.5).

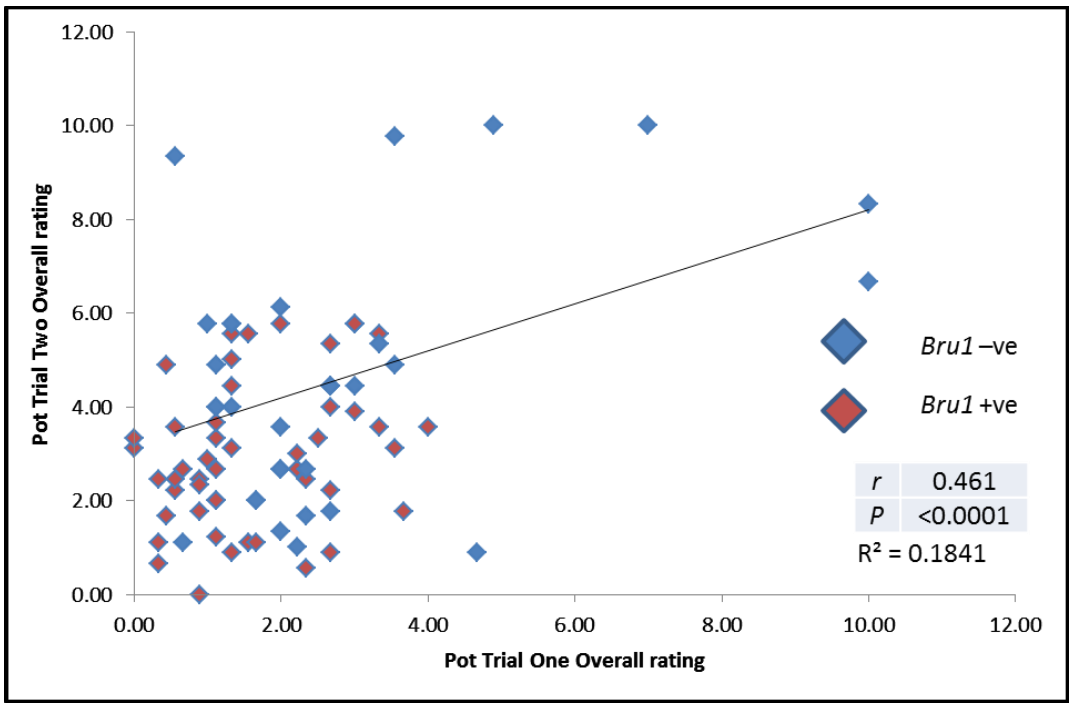


Fig 3.3.3: Correlation between the overall ratings of the first and second pot trials. The letter *r* represents the Pearson product moment correlation coefficient while the letter *P* denotes the probability of the correlation coefficient being significant (*P*= 0.05). n=80

The overall rating system had a higher and more significant correlation coefficient than the other rust symptom parameters measured in the LD2 population using both correlation

analysis methods (Fig 3.3.3). The *Bru1* negative genotypes however had a slightly more significant correlation between the trials when compared to the overall rating correlations, with the *r* for sporulation not being significant. The *Bru1* positive genotypes showed symptoms of brown rust infection in the form of varying lesion intensity, which was however confounded by the absence of heavy sporulation which resulted in them having low overall rust ratings (Fig 3.3.3). Three of the *Bru1* positive genotypes, one in Pot Trial One and two in Pot Trial Two did not sporulate at all in any of the replicates, resulting in a sporulation rating of zero, and a subsequent overall rating of zero.

The sporulation correlations were slightly significant when all the LD2 genotypes were compared (Fig 3.3.5), likely due to the resistant nature of the *Bru1* positive resistant genotypes. However, sporulation correlations were not significant for the *Bru1* negative genotypes (Fig 3.3.8), whereas the lesion ratings were significant (fig 3.3.7). This was probably due to different effects weather has on the extent/ severity of rust symptom development.

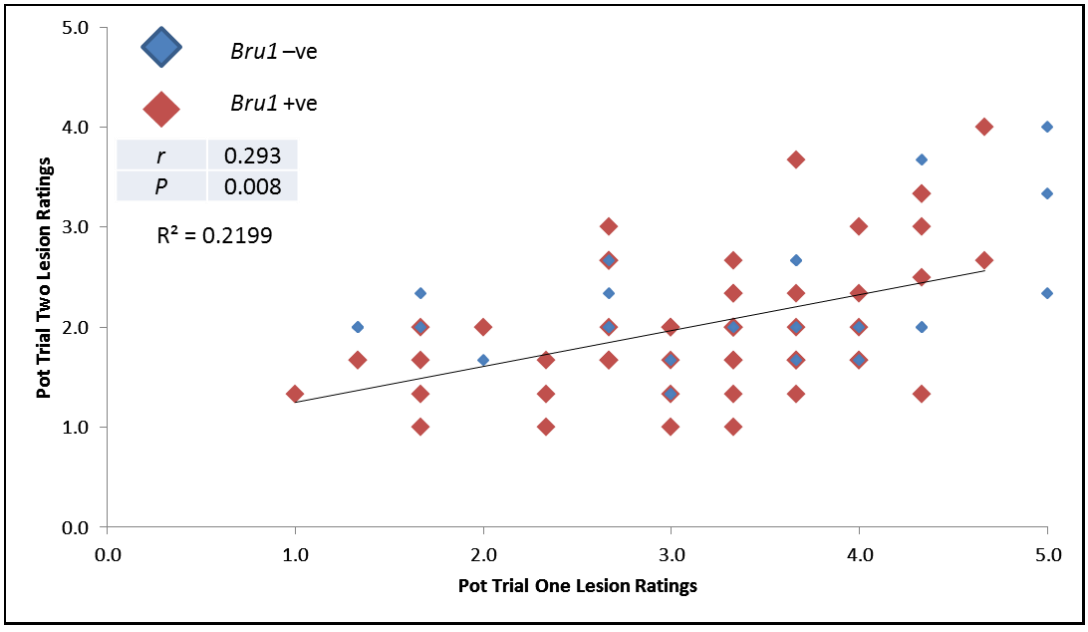


Fig 3.3.4: Correlation between the lesion ratings of the first and second rust screening pot trials. The letter *r* represents the Pearson product moment correlation coefficient while the letter *P* denotes the probability of the correlation coefficient being significant (P= 0.05). n=80.

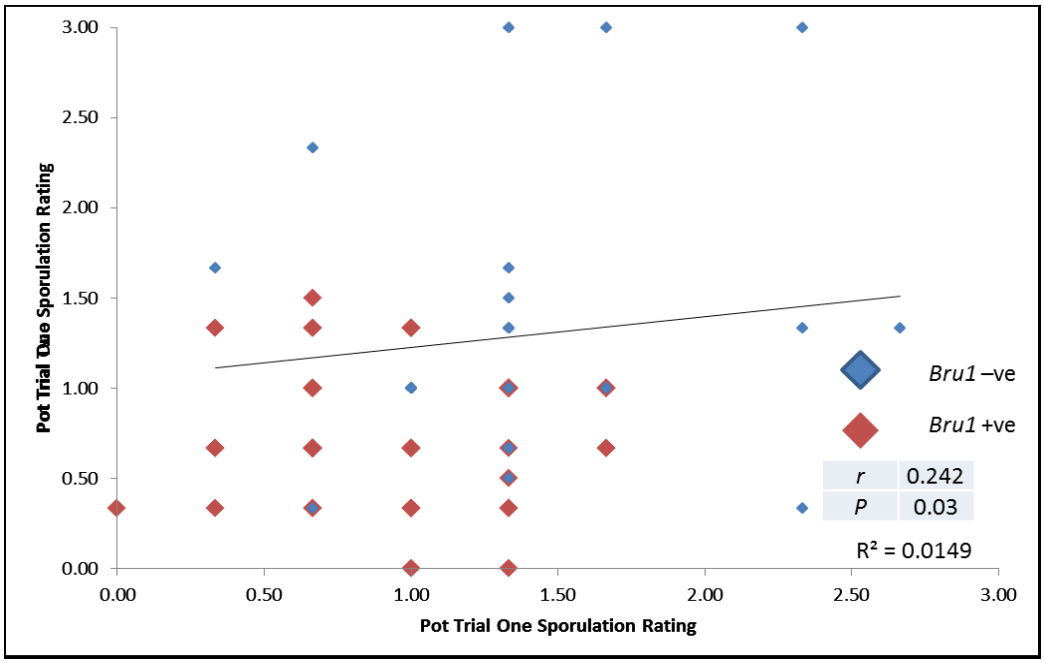


Fig 3.3.5: Correlation between the sporulation ratings of the first and second rust screening pot trials. The letter *r* represents the Pearson product moment correlation coefficient while the letter *P* denotes the probability of the correlation coefficient being significant (P= 0.05). n=80.

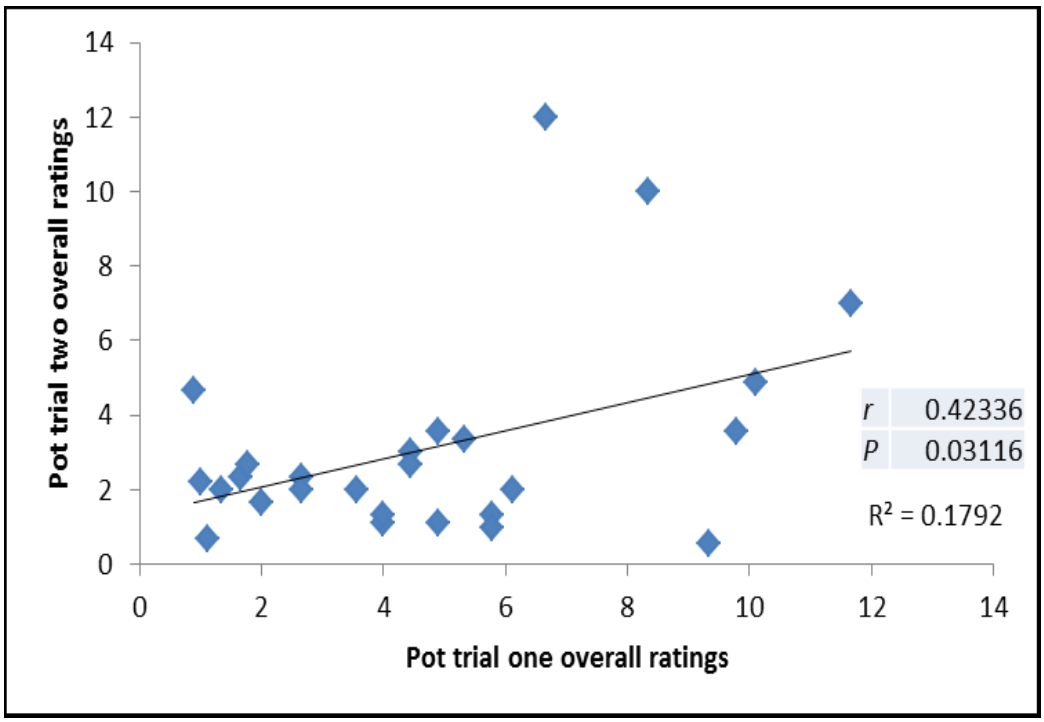


Fig 3.3.6: Correlation between the overall ratings of the *Bru1* negative genotypes in the first and second pot trials. The letter *r* represents the Pearson product moment correlation coefficient while the letter *P* denotes the probability of the correlation coefficient being significant (P= 0.05). n=26.

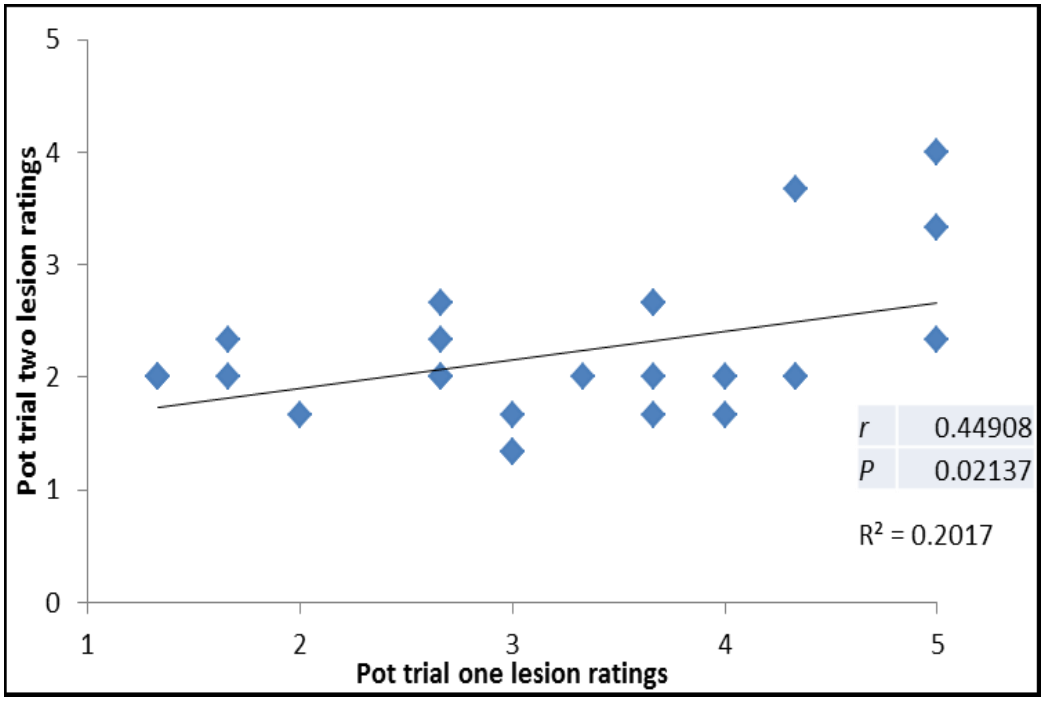


Fig 3.3.7: Correlation between the lesion ratings of the *Bru1* negative genotypes in the first and second pot trials. The letter *r* represents the Pearson product moment correlation coefficient while the letter *P* denotes the probability of the correlation coefficient being significant (P= 0.05). n=26.

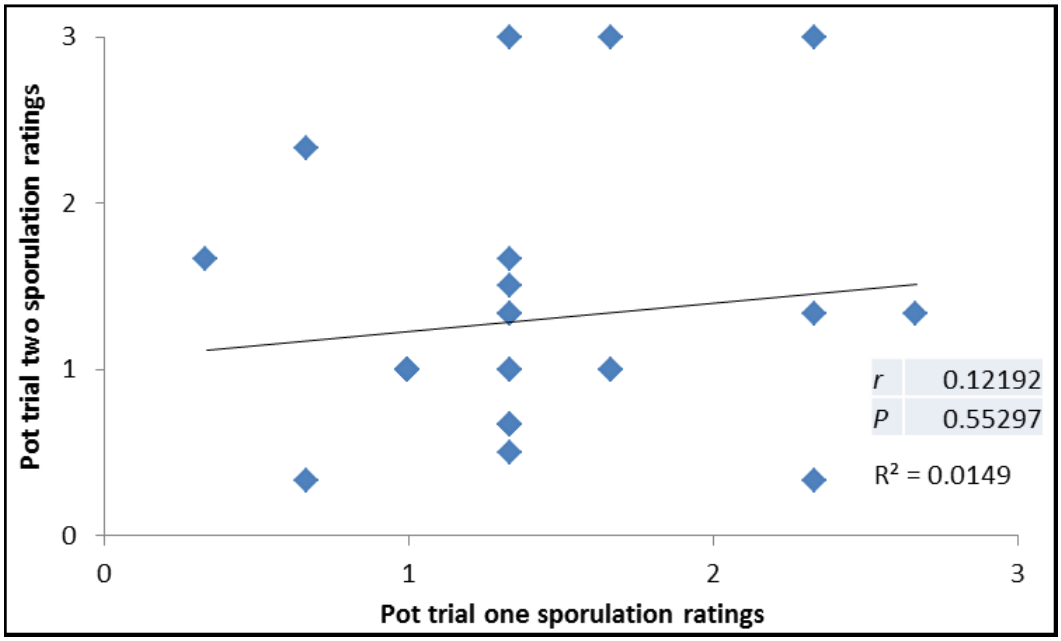


Fig 3.3.8: Correlation between the sporulation ratings of the *Bru1* negative genotypes in the first and second pot trials. The letter *r* represents the Pearson product moment correlation coefficient while the letter *P* denotes the probability of the correlation coefficient being significant (P= 0.05). n=26.

3.4 Discussion

The whorl inoculation technique demonstrated its effectiveness by its ability to generate reproduceable observations in the sugarcane- *P. melanocephala* interaction in the two pot trials. The effectiveness of the whorl innoculation technique has also been observed in some sugarcane trials in Florida (Sood *et al*., 2007; Zhao *et al*., 2011). Though the observations from both pot trials significantly correlated to each other, there were some phenotypic differences between similar genotypes, with 25% of the genotypes used in the trials (both *Bru1* positive and negative) being placed into different resistance groups; albeit due to slight differences in rating units. Only 2% of the genotypes were extremely different in terms of resistance groups (resistant to susceptible). This observation was due to the influence of genotype by environment (GXE) effects on rust development which resulted from the different weather conditions experienced during the separate pot inoculation trials (Asnaghi *et al*., 2001; Hu *et al*., 1996; Tai *et al.*, 1981; Twizeyimana *et al*., 2007). GXE interaction on rust development was further shown by differences in the correlations between the first and second pot trial for all the parameters analysed, with the sporulation parameter not being significantly correlated when the *Bru1* negative genotypes were analysed. Environmental conditions, which are major contributing factors in GXE interactions, include temperature, humidity (Fig 3.2.1) and the simultaneous presence of other pathogens (Twizeyimana *et al*., 2007). The differences, mainly in the temperature and humidity between the first and second pot trial, were thought to have resulted in Pot Trial Two displaying less severe rust infestation than Pot Trial One in terms of both lesions and sporulation as shown in Figures 3.3.5 and 3.3.6. The temperatures for Pot Trial One were between 19-22°C consistently throughout the trial period; while Pot Trial Two had temperatures consistently between 16-19°C, with the temperature below 18°C for extended periods. These lower temperatures could have resulted in the rust pathogen being less virulent and subsequently resulting in the less severe observations that were made (Braithwaite *et al.*, 2005; Sood *et al*., 2009). Relative humidity was also substantially different between the trials, as Pot Trial Two had much lower RHA and RHP, which also fluctuated more than in Pot Trial One.

Some of the varieties consistently maintained a low range of resistance ratings in both pot trials, indicating high resistance to rust. This result was coherent with previous results, which confirmed the ability of the method to produce consistent, reproducible and reliable results (Sood *et al*., 2007). When weather data was observed, it was found that previously, the most

severe infections had occurred when there were relatively low average temperatures observed (20^{o}C), coupled with high relative humidity as experienced in late 2009, when the most recent rust epiphytotic occurred (Fig 3.2.1 A). The weather data from late 2009 was compared to that of the different rust trials (Fig 3.2.1 B and C). Brown rust has been observed to germinate and develop optimally at temperatures between 19-25°C (Braithwaite, 2005; Purdy *et al*., 1983; Ramouthar, 2009). Temperatures of 19-21°C, coupled with the presence of leaf surface wetness have been shown to be crucial for rust development on the first day of inoculation. Temperatures can then fluctuate to a maximum of 25°C afterwards, with no positive or negative effects on rust development (Braithwaite, 2005). The temperature conditions during the pot trials were close to optimal, as temperatures were consistently between the ranges of 18-20°C, though the average temperature for the second pot trial was below average optimal temperature, at 17.9^{o}C.

The whorl inoculation screening results proved that this method can be successfully used to screen for rust resistance. Leaf whorl inoculations were conducted in the late evening in all trials. This ensured that spore solutions were constantly in contact with the leaf surface in favourable conditions for sufficient periods to allow rust germination and establishment (Sood *et al*., 2009). Whorl inoculation has been postulated to work better as a screening method as the leaf whorls may act as a barrier to temperature and moisture fluctuations, ensuring conditions for spores are conducive for germination and infection (Sood *et al*., 2009). This method resulted in the LD2 population being successfully screened for rust resistance in two separate pot trials. A significant number of genotypes in the population which tested resistant in one whorl inoculation trial were consistently resistant in the other whorl inoculation trial. This confirmed the effectiveness of whorl inoculation as a screening method and its ability to reveal susceptible genotypes which otherwise escaped detection under natural infection conditions in the field (Sood *et al*., 2009). The effectiveness of whorl inoculation over other methods was also observed in maize inoculated with *Cochliobolus heterostrophus* Drechsler, the causal agent of southern corn leaf blight (Simmons *et al*., 1998).

The *Bru1* positive genotypes analysed by whorl inoculation in Pot Trial One showed slight sporulation in some of the leaf replicates of nearly all the genotypes, while those in Pot Trial Two exhibited less severe sporulation and on fewer relicates than in Pot Trial One. Only three genotypes in both trials did not sporulate in any of the replicates (94F0663 in Pot trial one, 96E1663 and 95W1786 in Pot trial Two), resulting in an overall rating of zero which was

calculated by multiplying the average lesion rating by the average sporulation rating. The genotype 94F0663 had an average overall rating of 0.2, while 96E1663 and 95W1786 were both rated 1.6, after averaging the overall ratings from both pot trials. Slight sporulation on *Bru1* positive genotypes has been previously noted during *in vitro* analyses on the rust resistant cultivar R570, which exhibited lesion formation and sporadic incidence of sporulation after inoculation with *P. melanocephala* (Asnaghi *et al*., 2001). Similar observations were also made in a maize cultivar which is resistant to *Cochliobolus heterostrophus* Drechsler, the causative agent of leaf blight. These resistant maize cultivars are known to produce sterile lesions due to the possible activation of defence systems and necrosis of affected areas, in what is sometimes referred to as lesion mimicry (Anand *et al*., 2003; Asher and Thomas, 1987; Honée, 1999; Simmons *et al*., 1998). This highlighted the need to be able to ascertain the sporulation or sterility of rust lesions when making observations, as resistant genotypes could be discarded or overlooked during screening due to lesion development. The use of the whorl inoculation technique will greatly assist in ensuring the release of truly rust resistant varieties as such an efficient method will reduce the number of genotypes which escape detection, subsequently decreasing the number of situations were resistant varieties become susceptible after commercial release (Hoy and Hollier, 2009; McFarlane *et al*., 2006; Pillay *et al*., 2005).

Correlation analysis demonstrated the ability of whorl inoculation to give reasonably consistent responses among the LD2 genotypes. This was shown by the consistency of the genotypes which maintained rust resistance throughout both trials, even though favourable conditions in Pot Trial One resulted in the genotypes exhibiting more severe rust symptoms than in Pot Trial Two. The significant correlation between the trials demonstrated that even with environmental conditions contributing to differences, the other factors involved such as genotype resistance mechanisms, aided in keeping the trials significantly similar. These results were useful in that they were able to give an indication of the approximate period and ideal conditions in which rust screening tests should be conducted, to ensure the least number of escapes, as was observed in Pot Trial One. Climate change has resulted in weather patterns becoming less predictable, making timing of trial initiation more difficult, even with the use of whorl inoculation. This was highlighted by the relatively low correlation coefficients, though significant and the non-significant correlation coefficient when the sporulation parameter was used. The coefficients obtained from analysis of these trials showed the variability that arises due to different weather conditions (Hu *et al*., 1996; Nair *et al*., 2005).

Pot trials have the advantage of having the capacity to be conducted under more controlled conditions to cater for changes which might occur in outside weather conditions. The ability to control conditions is beneficial as it can complement the advantages of whorl inoculation to give more consistent observations. This exercise is however costly, as it requires equipment such as air conditioners, humidifiers, pots and also additional labour to conduct this type of trial. The use of pot trials where optimal infection conditions are prevalent, such as in the first pot trial is recommended. Such a setup would greatly reduce the number of escapes by encouraging infection under the pathogens optimal conditions, while eliminating the risk that arises from conducting trials in the field, where conditions might confound the actual resistance status of a particular genotype (Izanloo *et al*., 2008).

More rapid detection methods such as marker assisted selection and detached leaf assays would also be beneficial in augmenting and improving screening exercises (Braithwaite, 2005; Nair *et al*., 2005; Twizeyimana *et al*., 2007). The use of other parameters that can be used to measure disease response such as photosynthetic capacity, chlorophyll content, and enzyme activity can be investigated within these inoculation trials to ascertain their usefulness in developing more accurate, robust and time saving screening analyses.

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Chapter 4

A PCR diagnostic and AFLP based approach for the identification of *Bru1* containing genotypes and a possible means of detecting alternative resistance in a sugarcane population.

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Abstract

Puccinia melanocephala, the cause of brown rust of sugarcane, is known to result in losses of up to 50% in susceptible varieties. The use of resistant genotypes is the most effective method of control. Production of resistant varieties is however time consuming and labour intensive, with breeding programs taking up to 12 years to release a variety. Marker assisted selection (MAS) has been successfully used in other crops to substantially reduce cultivar release times, increase yields and reduce losses to pests and disease. Amplified fragment length polymorphism (AFLP) is a technique which has been used to develop genetic maps which can elucidate the genome of an individual and allow for the identification of major genes and quantitative trait loci (QTLs). A breeding population (LD2) was characterised using AFLP markers and screened for the presence of *Bru1*, a major rust resistance gene. LD2 was grouped according to *Bru1* presence or absence and AFLP marker data was correlated to LD2's rust phenotypic responses as the dependent variable. AFLP markers for quantitative resistance/ susceptibility and major resistance/ susceptibility were subsequently selected using these correlations. Linear regression was conducted to test the significance (P=0.05) of these correlations. Genotypes were grouped using scatter analysis and PCA to ascertain the effect of these selected markers. Strongly correlating AFLP markers were identified from the correlation analyses and managed to significantly segregate the differently rated genotypes. Relationships were observed between, and among markers such as the marker pair 82M56 and 18M30 (r=0.79) which were highly correlated to each other and also to both 53M41 and 34M24S. Stepwise multiple linear regression was then used to select markers with which a resistance model was created for the different phenotypic measures assessed, these being the lesion

ratings and the overall phenotypic rust ratings. The lesion rating models displayed lower standard error of observation (SEE) in a 5 unit lesion rating scale. The overall phenotypic rating model comprising eight markers allowed 84% (SEE = 0.9) of the variance to be accounted for in the *Bru1* negative genotypes, compared to 96.1% (SEE = 0.163) in the lesion rating model. Overall, this information suggested the existence of an alternative major rust resistance gene and a number of useful QTLs which can be used to strengthen rust resistance in sugarcane. The data also showed potential to generate markers for MAS in the breeding program.

4.1 Introduction

Modern cultivars of sugarcane are inter-specific hybrids between S*. officinarum* and *S. spontaneum* (Cordeiro *et al.*, 2007; Grivet *et al*., 1996; Pan *et al*., 2004). Sugarcane has a highly complex genome which is a highly polyploid and aneuploid inter specific hybrid, with between 2n=100 and 2n=130 chromosomes (Aitken *et al*., 2006; Edmé *et al*., 2005; Grivet and Arruda, 2001; Raboin *et al.*, 2006). Most of the chromosomes in the modern cultivars originate from the sugar-producing species *S. officinarum* (2n=80), while 15 to 25% are derived from the wild species *S. spontaneum* (2n=40 to 128) (Asnaghi *et al.*, 2004; Pan *et al*., 2004). A small percentage of the genome has also been contributed from inter-specific recombinations and by the other *Saccharum* species and related grass genera (Hoarau *et al*., 2001; Le Cunff *et al*., 2008; Ming *et al.*, 2006). *S. saccharum* has contributed to the high sugar content of the modern sugarcane cultivars, while *S. spontaneum* has greatly contributed towards improved disease and insect resistance, stubble vigour and longevity of the modern sugarcane cultivar (Pan *et al*., 2004). Recombination between homoeologous chromosomes in the modern cultivars has resulted in between 5-10% of the genome in modern cultivars consisting of recombinant and/ or translocated chromosomes between *S. officinarum* and *S. spontaneum* (Cuadrado *et al*., 2004; Grivet and Arruda, 2001).

As sugarcane is such an important economic contributor to many countries, it is vital for its genome to be better understood, as this would allow modern breeding techniques to be utilised, resulting in increased and cost effective cultivar production. Sugarcane's economically important traits have been found to be mostly quantitative in nature. These quantitative traits can however be masked by genotype x environment (GxE) interactions, resulting in a large number of them being discarded in the early stages of breeding programs (Dreher *et al*., 2000;

Pan *et al*., 2004; Zhao *et al*., 2011).The genomic setup of sugarcane is such that many of these quantitative trait loci (QTL's) are clustered in a particular locus, and the effect of an allele is only visible when it exceeds the average effect of all other segregating alleles in the background (Raboin *et al*., 2006). A more objective approach to cultivar selection is required, and the development of molecular markers has aided in this regard, with several marker types being developed for sugarcane breeding since the mid-nineties. These molecular markers include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), AFLP and microsatellite markers (Aitken *et al*., 2006; Asnaghi *et al*., 2000; da Silva and Bressiani, 2005; Daugrois *et al*., 1996; Pan *et al*., 2004; Raboin *et al*., 2006). Such molecular markers have greatly aided in the construction of genetic maps, which have elucidated the genome of sugarcane, and in the process mapped the position of the major rust resistance gene, *Bru1* (Asnaghi *et al.*, 2004; Maureira and Osborne, 2004; Raboin *et al.*, 2006).

AFLP has been successfully used to differentiate sugarcane cultivars and to map the sugarcane genome. This method has a major advantage in that it can distinctly differentiate among closely related genotypes and individuals (Agarwal *et al*., 2008; Belaj *et al*., 2003; Mueller and Wolfenbarger, 1999; Powell *et al*., 1997). AFLP has the advantage of being multiplex, which offers the potential to improve the efficiency and throughput of marker data compilation (Vos *et al*., 1995; Vuylsteke *et al*., 2007).These properties of AFLP have all contributed to the breakthroughs in sugarcane genomics, where maps constructed using AFLP have uncovered numerous QTLs, including the *Bru1* gene (Asnaghi *et al*., 2000; Hoarau *et al*., 2001; Le Cunff *et al*., 2008).

DNA probes derived from these marker technologies have also been used to show the presence of synteny clusters between sugarcane and some other members of the *Andropogoneae* tribe (Le Cunff *et al*., 2008). Syntenic positions in the *Andropogoneae* genomes have been useful in the detection of numerous QTLs and the accurate location of the *Bru1* gene (Asnaghi *et al*., 2000; Raboin *et al.*, 2006). The use of AFLP and bulked segregant analysis resulted in a more detailed genetic map around *Bru1* (Asnaghi e*t al.*, 2004). Flanking markers located 0.14 and 0.28cM from *Bru1* were subsequently identified (Le Cunff *et al.,* 2008).

Bru1 has been found to have moderate to high heritability, despite the complexity of the sugarcane genome (Asnaghi *et al*., 200; Asnaghi *et al*., 2004). *Bru1* is stable and durable as it has not broken down in over 20 years that the R570 cultivar has been cultivated (Asnaghi *et*

al., 2004). This gene has also been postulated to be a monogenic and dominant allele, as it gave a 3:1 segregation ratio of resistance in the selfed progeny of R570 (Asnaghi *et al.*, 2000).

It is with this background that the objectives of this paper were set. Flanking primers based on AFLP markers and mapping data for detecting the *Bru1* gene were obtained from CIRAD and used to diagnose for *Bru1* presence in the LD2 plant breeding population at the South African Sugarcane Research Institute. An older population, LD1, was also screened for *Bru1* in order to get a better idea of the overall incidence of this gene in South African germplasm. The *Bru1* marker data, together with the AFLP data for the LD2 population and pot trial rust resistance data were correlated.

4.2 Materials and Methods

4.2.1 DNA extraction

DNA was extracted from the young leaves of each of 80 genotypes from the LD2 breeding population, using the Qiagen DNeasy® Plant Mini Kit (Hilden, Germany). The DNA was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific Inc.) and diluted to 25ng/µl. Previously extracted DNA from the LD1 population was also diluted to 25ng/µl. DNA from both breeding populations was used to perform *Bru1* diagnostic polymerase chain reaction (PCR), using an optimised CIRAD protocol described in section 4.2.2.

4.2.2 *Bru1* PCR1 and PCR2 reagents and cycling conditions

KapaTaq DNA Polymerase (Cape Town, South Africa) was used for all PCR reactions. Magnesium chloride ($MgCl_2$), PCR grade water, 10X buffer and deoxyribonucleotide triphosphate (dNTP's) were all supplied in the KAPA Biosystems kit. Primers were designed by the French Research Organisation, CIRAD and synthesised by Inqaba Biotech (Costet *et al.*, 2012). PCR reactions were carried out in an Applied Biosystems PCR System 9700 thermal-cycler. The CIRAD protocol however had to be optimised and the procedure is described below.

4.2.2.1 Optimisation of PCR protocol

A *Bru1* positive sample, a *Bru1* negative sample, a random subsample of the LD2 genotypes and a negative control were used in PCR optimisation. The following optimisation steps were carried out repeatedly and concurrently with the standard protocol and the results compared:

1. The number of cycles in the thermal cycling profile was increased from 35 to 40.
2. The temperature of the denaturation steps was increased to 95°C.
3. A touchdown protocol was employed. The maximum temperature used was within 1°C of the Tmax of the primers used (60°C for the primer set 9O20-F4 (alias PCR1) and 61°C for the primer set R12H16 (alias PCR2)); a stepwise 2°C decrease in temperature was employed until the optimum temperature was reached (55°C) for both primers.
4. A 2% (v/v) dimethyl sulfoxide (DMSO) was added as an enhancer to the *Bru1*-PCR2 master mix.

The CRAD based protocol for both primer pairs made use of 50 ng of DNA mixed with 1xPCR buffer, 2 mM MgCl2, 0.2 mM, dNTP, 0.2 µM forward primer, 0.2 µM reverse primer, 0.5 U DNA polymerase in a final volume of 25 µl for the R12H16 primer whereas 9O20-F4 was made up to a final volume of 50 µl (Costet *et al*., 2012). The PCR profile used was: one step of 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s.

The protocol was optimised for the prevailing laboratory conditions by using 25 ng of DNA and 1.6 U of Taq DNA polymerase in both reactions. DMSO (1 µl) was added only to the R12H16 reaction whereas 2 mM of $MgCl_2$ was excluded from the 9O20-F4 reaction. In addition to these changes in the PCR master mix, the thermal cycling conditions were changed to a touchdown protocol which was as follows: 95 °C for 5 min followed by 3 two cycle stages with 2 °C decrements in the annealing temperature in the cycle 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 45 s. This was followed by 34 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. A final elongation step of 72 °C for 8 min was conducted.

The touchdown thermal cycling protocol was subsequently selected for screening the breeding populations using both the PCR1 and PCR2 flanking primers. In addition, DMSO (1 µl) was

incorporated into the PCR2 master mix as it greatly enhanced the PCR reaction. In the PCR1 optimisation process, it was found that the touchdown protocol without additional $MgCl_2$ and DMSO was the best. This was due to the enhancer interfering with Rsa1 during the endonuclease digestion step, whereas the additional $MgCl_2$ had no overall effect on the reaction.

4.2.2.2 PCR diagnostics of breeding populations using optimised protocol

Previously extracted DNA from both breeding populations were screened for the presence of *Bru1* using the optimised PCR protocol described in section 4.2.2.1. PCR reaction products (20µl) from each reaction were mixed with 3µl of Fermentas 6X orange DNA loading dye (Carlsbad, CA, USA). This mixture was loaded into a 2.5% (w/v) agarose gel containing 3.3 mg/ml ethidium bromide. A DNA molecular weight ruler (O'GeneRuler™ 100bp DNA Ladder Plus, Carlsbad, CA, USA) was also loaded into the gel as a reference for band size identification. The gel was electrophoresed in a 1X TBE buffer (54 g/l Tris base, 27.5 g/l boric acid and 0.01 M EDTA, pH 8.0) at 80V for three hours. The gel was immediately viewed in the AlphaImager™ 2200 transilluminator (Alpha Innotech Corporation) at 302nm and the images were captured and printed. A 570bp fragment was expected for *Bru1* positive samples using the PCR2 primer, while amplification of multiple fragments was expected in all samples with the PCR1 primer. All the *Bru1* diagnostic PCR reactions were carried out at least twice for each genotype in both populations analysed. Each gel was scored independently for each time the reaction was conducted.

4.2.3 Rsa1 endonuclease digests

PCR products from the PCR1 reaction were cleaved using the Fermentas FastDigest® Rsa1 endonuclease. A 200 bp digest product was expected for *Bru1* positive samples. Twenty microliters of the 9O20-F4 PCR products were digested with 2.5 U of Rsa1 (Fermentas), 1x Rsa1 fast digest buffer and made up to a final volume of 30 µl. The reaction mix was briefly mixed and incubated in a heating block at 37°C for five minutes. The tubes were immediately put on ice, after which 3 µl of Fermentas 6X orange DNA loading dye (Carlsbad, CA, USA) was added

to each tube and loaded into a 2.5% (w/v) agarose gel containing 3.3 mg/ml of ethidium bromide. The gel was electrophoresed in 1X TBE buffer at 80V for three hours.

4.2.4 AFLP markers

AFLP profiles for the LD2 population were obtained from the SASRI Plant Breeding Department, who used the Invitrogen™ Life Technologies AFLP kit on genomic DNA extracted from the leaves of each genotype. This genetic marker data was obtained using an 8x8 primer combination with slight modifications as suggested by Hoarau *et al*. (2001) and the manufacturer's instructions for γP^{33} labelling, using the Gibco BRL kit. The individual AFLP primers used are shown in Table 4.2.1.

Table 4.2.1: AFLP primers used for marker generation. 8 x 8 = 64 combinations were used.

EcoR1 primer		**Mse1 primer**	
Code	Selective nucleotide	Code	Selective nucleotide
1	AAC	1	CAA
2	AAG	2	CAC
3	ACA	3	CAG
4	ACC	4	CAT
5	ACG	5	CTA
6	ACT	6	CTC
7	AGC	7	CTG
8	AGG	8	CTT

The whole plant inoculation data (lesion and spore ratings) were then converted to overall rust ratings for each genotype (Fig 4.2.1). Overall rust ratings ranged between 0 – 10. The range 0-3.5 was assigned as resistant; 3.51- 6.5 assigned as intermediate; and 6.51-10 as susceptible. The LD2 population was then divided into the *Bru1* negative and *Bru1* positive groups. The AFLP profiling data for the *Bru1* negative genotypes were correlated with the respective genotypes' whorl inoculation data using the Pearson product-moment correlation coefficient (PPMCC). The inoculation data obtained in chapter three of this thesis were used as the

dependant variables. This procedure was conducted so as to reveal markers linked to quantitative and possibly qualitative resistance in the *Bru1* negative genotypes (Otsen *et al*., 1996; Vandenberghe *et al*., 2003). Regression analyses were carried out on markers of potential quantitative trait loci and possible alternative qualitative genes to determine if the correlations were significant at a confidence interval of 95%.

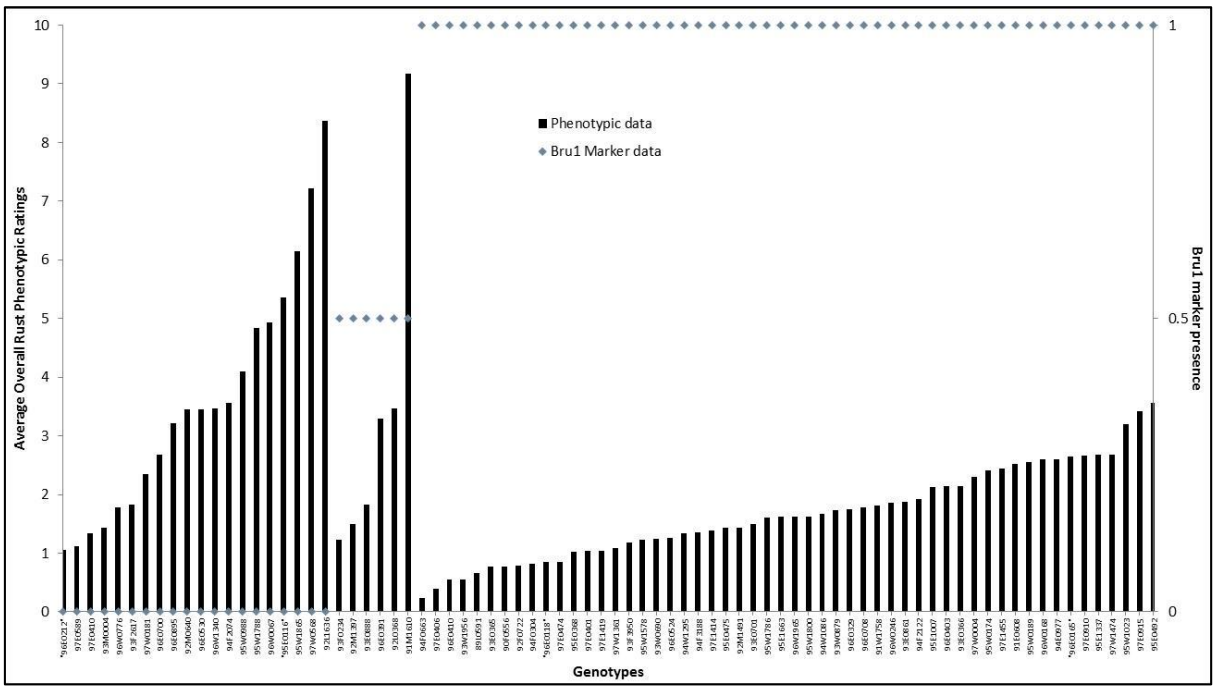

Fig 4.2.1: Average overall rust phenotypic ratings of the LD2 breeding population from two pot trials, six weeks after whorl inoculation with *P. melanocephala* spores.

4.2.5 Data analysis

Three marker categories were determined, based on the PPMCC (*r*) and a significant regression *P* value at a 95% confidence interval. These marker categories were defined as quantitative resistance, quantitative susceptibility and qualitative resistance markers. Markers for qualitative resistance were determined using a rule which stated that "the selected markers should be present in resistant genotypes, be totally absent from the susceptible genotypes and be present in at most one of the intermediate genotypes". Markers for resistance and susceptibility were then selected to build a quantitative rust resistance prediction model using stepwise multiple linear regression (MLR) analysis, a method which uses a mixture of the backward elimination and forward selection (Jansen, 1993; Kao *et al*., 1999). This prediction model subsequently developed was based on the LD2 *Bru1* negative genotype regression

analyses. Two models were derived using the overall phenotypic ratings and the lesion ratings. This was after considering the correlation observed between two whorl inoculation pot trials which showed significant correlations for both the lesion and overall phenotypic parameters (Chapter Two). The *Bru1* positive genotypes were then analysed using these models to assess their possible rust responses in the event of the brown rust resistance gene *Bru1* being broken down. Dendrograms were computed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method and Euclidean distances used to show the relationships among the markers (Zhang *et al*., 2004). The LD2 population was then analysed based on the AFLP marker data generated from each genotype using principle component analysis (PCA) using the ADE-4 multivariate analysis software (Thioulouse *et al*., 1997).

4.3 Results

4.3.1 *Bru1* diagnostic PCR

Optimisation of the *Bru1* marker diagnostic PCR protocol allowed more accurate and consistent results to be obtained, as shown by the differences between the results before and after optimisation (Fig 4.3.1). Optimisation allowed the entire LD1 and LD2 populations to be screened for the presence of the *Bru1* gene. The *Bru1* gene analysis gave an overview of the presence of the *Bru1* gene within the breeding populations in the South African industry (Fig 4.3.2). The screening results in Chapter Three suggested the possibility of an alternative rust resistance gene, as eight of the genotypes from LD2, lacking the *Bru1* major rust resistance gene were highly resistant and were from the same genealogy. Previously conducted rust phenotypic analysis also gave inferences on how much alternative rust resistance mechanisms to *Bru1* exists in these populations. The results indicate breeding improvements from LD1 to LD2 as there are fewer genotypes showing absence of the *Bru1* markers in LD2, and a subsequent increase in the *Bru1* containing genotypes in the LD2 population as compared to the LD1 population, which is the predecessor.

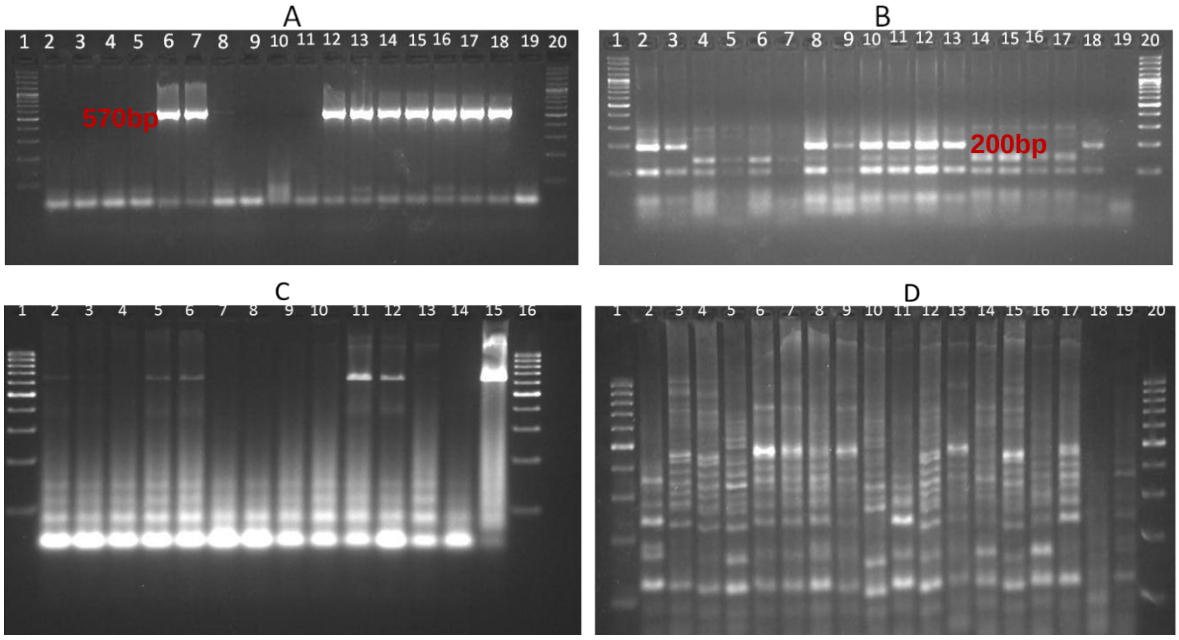

Fig 4.3.1: PCR diagnostics for the presence of the *Bru1* gene using two flanking markers. The markers used were: (A) PCR 2 primers, with an expected 570bp product for *Bru1* positive genotypes and (B) PCR 1 primers, whose PCR product was digested with Rsa1 endonuclease to give a 200bp product for *Bru1* positive genotypes. Lanes 1 and 20 on both gels (A and B) contained the 100bp ruler while lanes 12 and 13 on both gels are examples of *Bru1* positive genotypes. Lanes 18 on images A and B are positive controls, while lanes 19 are negative controls. The gel images A and B represent the optimised PCR reaction, while C and D show the inconsistency of the reaction before optimisation.

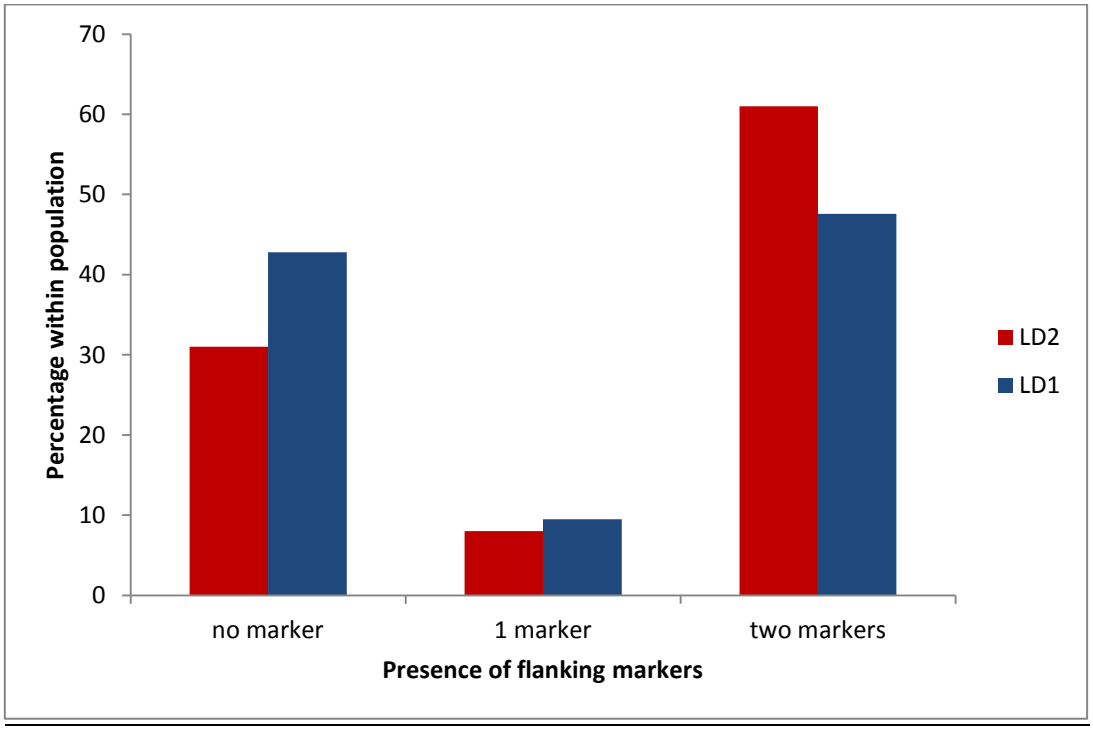

Fig 4.3.2: Presence and absence of the *Bru1* gene in the breeding populations LD1 and LD2.

4.3.2 AFLP correlations

Results from correlations conducted using AFLP and whorl inoculation data indicated the presence of markers which have the potential to be used as markers of rust resistance, due to their high correlation with the phenotypic responses obtained from pot trial whorl inoculation (Tables 4.3.1-3). These correlations also allowed the identification of markers for possible quantitative and qualitative resistance and susceptibility traits. The genetic marker and phenotype correlations were conducted on the *Bru1* negative genotypes in order to obtain markers specific to the alternative resistance mechanisms these genotypes possess.

Markers were associated with possible quantitative resistance based on a significant correlation coefficient (*r*) at a 95% confidence interval ($r \geq 0.381$) (Table 4.3.1). The rating scale used resulted in negative correlations being associated with rust resistance, whereas positive correlations were associated with rust susceptibility. The markers 53M31, 34M24S and 24M17S were all present in the resistant and intermediate genotypes, but were absent in the susceptible genotypes. The markers found to be associated with quantitative rust resistance were found in the highly resistant *Bru1* positive genotypes, whose average rust phenotypic ratings were below 2.0, indicating the markers' association with traits of strong rust resistance. The markers 18M30, 87M02, 55M27 and 27M03S were all present in the resistant genotypes but were absent in both the intermediate and susceptible genotypes. The *Bru1* positive genotypes these markers were associated with were also highly resistant, with average phenotypic ratings of less than 1.80 and as many as 42 genotypes possessing one of these markers (marker 87M02). The marker 55M27 (average rust rating of 1.13) had the lowest mean phenotypic rating in the *Bru1* positive clones, suggesting its association with a highly effective resistance trait which could most likely be an alternative major rust resistance gene, as it was absent in both the susceptible and intermediate genotypes. The markers 53M31, 16M54, 84M04, 46M47, 18M39 and 82M56 also displayed low average phenotypic ratings when found in combination with *Bru1*. The marker 26M07S was found to be present in most of the *Bru1* negative genotypes, but found in only 4% of the *Bru1* positive genotypes. The marker 24M17S was found to be the marker present in the highest proportion in the *Bru1* positive genotypes as well as in the resistant *Bru1* negative genotypes.

Markers associated with a quantitative form of susceptibility were selected based on significant correlation coefficients at *P*> 0.005 (Table 4.3.2). The marker 41M10, associated with quantitative susceptibility was present in 75% of the *Bru1* positive clones, suggesting that this marker was only effective in instances where there were no other forms of resistance. 41M10 was also found to be present in only one of the *Bru1* negative resistant genotypes.

Markers were also selected as possible indicators of an alternative source of qualitative resistance to *P. melanocephala*. Markers for qualitative resistance were determined using the criteria stated in section 4.2.5 (Table 4.3.3). There were 15 markers found to adhere to this rule, of which nine of these markers were completely absent in the Intermediate group and were present between the range of 17- 50% in the *Bru1* positive clones. The intermediate clones which contained the selected markers for qualitative resistance showed phenotypic rust rating ranges which were in the low to medium severity range, suggesting the usefulness of these markers in selecting for highly resistant clones in future breeding programmes. The markers 77M02S and 27M25S were however found in intermediate genotypes whose resistance ratings were higher, and closer to the susceptibility rating cut off point.

Table 4.3.1: AFLP markers associated with quantitative rust resistance.

Marker	Pearson correlation coefficient	*P-value*	Number of *Bru1* negatives with marker			Number of *Bru1* positives with marker (out of 54)	Average phenotypic rating for *Bru1* positives with the marker
			Resistant (out of 17)	Intermediate (out of 6)	Susceptible (out of 3)		
53M31[b]	-0.651	0.0002	14	2	0	26	1.59
34M24S	-0.651	0.0002	14	2	0	23	1.58
24M17S	-0.625	0.0006	14	2	0	35	1.69
86M24	-0.538	<0.001	13/16[a]	4	0/2	30/48	1.64
55M39	-0.476	0.0011	14	3	1	30	1.59
34M01S	-0.540	0.0026	11	3	0	22	1.64
71M13S	-0.480	0.0041	15	15	1	32	1.74
82M56	-0.574	0.0042	13	1	0	24	1.74
18M30	-0.604	0.0047	13	0/5	0	22/51	1.66
35M12S	-0.556	0.0049	14	3	0	36	1.67
62M12S	-0.613	0.0059	17	6	1	51	1.67
88M37	-0.544	0.0059	16	4/5	1	42	1.81
86M04	-0.537	0.0059	16	5	1	28/50	1.61
26M16S	-0.506	0.0059	16	6	1	47	1.67
18M39	-0.484	0.0059	16	5/5	1	39/51	1.74
38M01S	-0.512	0.0075	17	3	2	6	1.66
87M02	-0.506	0.0084	9	0	0	42	1.76
43M58	-0.398	0.0106	8	4	0	33	1.74
76M07S	-0.461	0.013	10/16	3/4	0	29/51	1.78
16M54	-0.457	0.014	14/16	4	1	47/53	1.70
55M37	-0.394	0.0154	12	4	1	38	1.72
18M17	-0.473	0.0169	17	2/5	2	32	1.59
46M47	-0.382	0.0188	15	4	2	39	1.55
26M07S	-0.451	0.0208	17	3	2	2	1.78
17M37	-0.458	0.0209	13	2	1	33	1.76
44M57	-0.402	0.0238	12	4	0	32	1.63
73M02S	-0.451	0.0247	14	4	1	32	1.74
54M34	-0.433	0.0273	13	2	1	26	1.58
55M27	-0.426	0.0300	5	0	0	7	1.13
71M06S	-0.478	0.0304	12	1	1	20	1.76
27M25S	-0.418	0.0335	7	1	0	33	1.77
43M44	-0.393	0.0468	6	0	0	12	1.53
58M23	-0.409	0.0471	10	1/ 4	0	53	1.84
27M03S	-0.392	0.0474	4	0	0	9	1.53
84M04	-0.404	0.0493	13	3	1	20/52	1.41

[a] **Missing data**: Number of genotypes with the marker/ total number of genotypes scored i.e. 13/16 would mean 13 genotypes with the marker recorded out of 16 scored instead of 17 scored.

[b] **Highlighted markers** represent those selected by MLR in the final eight marker models**.**

Table 4.3.2: AFLP markers associated with quantitative rust susceptibility.

Marker	Pearson correlation coefficient	*P-value*	Number of *Bru1* negatives with marker			Number of *Bru1* positives with marker (out of 54)
			Resistant (out of 17)	Intermediate (out of 6)	Susceptible (out of 3)	
47M04[b]	0.531	0.0006	5	3	3	17/51
18M20	0.491	0.0006	6	2/5	3	25/51
72M16S	0.490	0.0028	3	2	3	21/50
47M12	0.492	0.0047	7	3	3	24/51
45M52	0.435	0.0047	4	1	3	20
43M32	0.552	0.0052	3	2/5	3	16/53
25M02S	0.474	0.0057	7	3	3	27
54M03	0.512	0.0075	0	1	2	28
85M23	0.494	0.0082	3	3	2	12
11M43	0.443	0.0115	3	2	2	17
82M52	0.408	0.0149	4	2	3	21
31M02S	0.468	0.0160	6	4	3	27
15M55	0.462	0.0175	0	1	1	3/29
16M30	0.495	0.022	4/14 [a]	1/4	3	11/50
82M49	0.447	0.0222	1	1	1	37/51
15M36	0.389	0.0237	8	2	3	24/53
22M04S	0.406	0.0265	3	2	3	27
34M21S	0.432	0.0277	7	4	3	47
41M10	0.474	0.028	6/16	5	2	39/52
84M29	0.427	0.0294	6	4	2	45
54M05	0.450	0.0307	2	2	3	27
56M09	0.423	0.0314	2	2	2	1/53
47M16	0.389	0.0339	4	2	2	11/52
58M22	0.431	0.0356	6	2/4	3	17/51
62M10S	0.413	0.0358	5	5	3	39
34M16S	0.431	0.0382	3	1	3	10

[a-] **Missing data:** Number of genotypes with the marker/ total number of genotypes scored i.e. 4/14 would mean 4 genotypes with the marker recorded out of 14 scored, instead of 17 scored.

[b-] **Highlighted markers** represent those selected by MLR in the final eight marker models**.**

Table 4.3.3: AFLP markers associated with qualitative rust resistance

	Bru1 negatives					*Bru1* positives
Rating Range	0- 3.5		3.51- 6.5		6.51- 10	
Marker	Number of **resistant** genotypes with marker (out of 17)	Mean phenotypic rating for resistants with marker	Number of **intermediate** genotypes with marker (out of 6)	Mean phenotypic rating for Intermediates with marker	Number of **susceptible** genotypes with marker (out of 3)	Number of genotypes with marker (out of 54)
18M30[a]	13	2.19	0/5	-	0	22/51
87M02	9	2.02	0	-	0	27
67M15S	7	2.69	0	-	0	16
43M44	6	1.97	0	-	0	12
45M45	6	2.09	0	-	0	12
12M58	6	2.18	0	-	0	18
48M35	6	2.56	0	-	0/2	20/50
54M08	6	2.67	0	-	0	16
71M14S	6	2.78	0	-	0	9
82M56	13	2.18	1	4.93	0	25
53M09	7/16	2.39	1/5	3.56	0	17/50
54M15	7	1.98	1	4.93	0	17
27M25S	7	1.71	1	5.35	0	24
51M13	6	2.03	1/5	4.93	0	18
77M02S	6	1.94	1	5.35	0	20

[a-] **Highlighted markers** represent those selected by MLR in the final eight marker models.

4.3.3 Multiple Linear Regression Analysis

The regression model selected 50% of the markers from either resistance group in the overall phenotypic rating model and stepwise elimination resulted in marker pairs consisting of a resistant and susceptibility linked marker being eliminated consecutively during the marker reduction step. Stepwise linear regression revealed eight markers which were significantly correlated to rust resistance or susceptibility and in turn were highly significant when constructing a regression model for rust response. These markers when reduced by backward elimination resulted in models being determined for eight markers down to one (Fig 4.3.3). Both the lesion and overall models were highly significant, with r^2 values ranging between 0.77 for eight markers and 0.41 for one marker in both the overall phenotypic and lesion rating models.

Eight markers were able to explain 96.1% of the percentage variance as compared to 38.3% when one marker remained after backward elimination in the lesion rating model (Fig 4.3.3), whereas in the overall phenotypic rating model, eight markers explained 84% while one marker explained 40% of the variance. These results showed that these markers work well in combinations, as the percentage variance explained rose from 40% to 61.8% in the overall rating model with the addition of the marker 25M02S to 53M31 (fig 4.3.4b). Lesion ratings when used to predict models for rust resistance using stepwise MLR generally showed a much higher percentage variance accounted for with the two marker model, right up to the eight marker model of the phenotypic rating (fig 4.3.3). The lesion rating models also had much smaller standard errors of observation (SEE) when compared to the overall phenotypic rating models, demonstrating increased accuracy of prediction (fig 4.3.4a-b and fig 4.3.6 a-b).

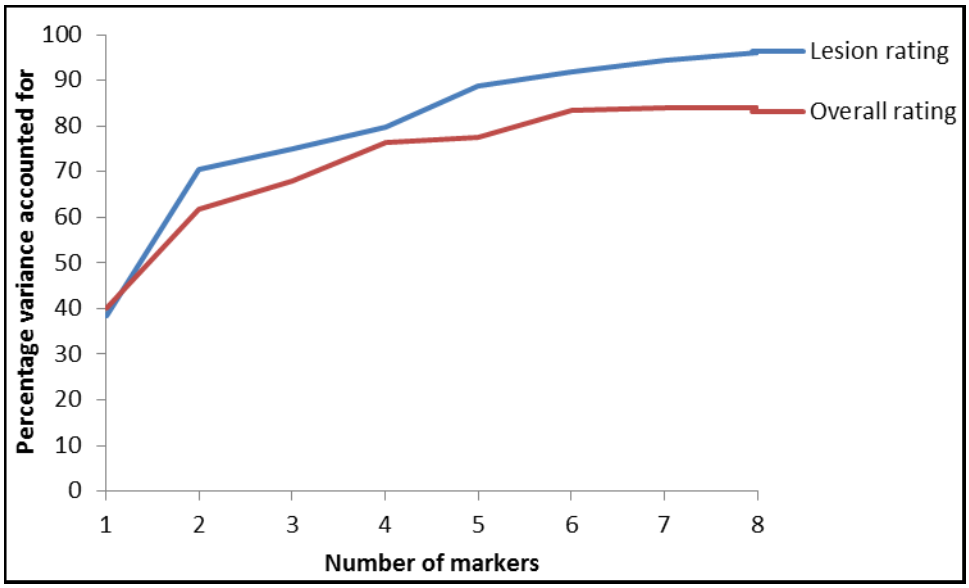

Fig 4.3.3: The change in percentage variance accounted for in the overall phenotypic rating and lesion rating models with an increase in markers obtained by stepwise multiple linear regression analysis.

When the models were used to analyse the *Bru1* positive population, with the assumption that *Bru1* was absent, it was found that the genotypes lacking markers for quantitative/qualitative resistance were predicted to be susceptible, according to the models (fig 4.3.5a-b and fig 4.3.7a-b). According to the different models, the implications are that between 50% and 70% of the *Bru1* positive genotypes could be potentially susceptible to brown rust in the event of the *Bru1* gene breaking down (Fig 4.3.5 and Fig 4.3.8). An example of such genotypes were the *Bru1* positives 94F0663; 97E0406 and 90F0556; which were highly resistant post inoculation,

but when analysed using the model, were predicted to be susceptible due to the absence of AFLP markers associated with quantitative resistance. These results suggest that in the event of the efficacy of the *Bru1* resistance gene being lost, such genotypes are potentially highly susceptible. The observation that genotype 94F0663 was initially highly resistant when compared to the other *Bru1* positive genotypes suggests the presence of other factors which may not have been accounted for in this model, but contributed in conferring greater resistance to this genotype. The genotypes 97E0910, 97E0915 and 95W1023 on the contrary, were among the least resistant of the *Bru1* positive genotypes when phenotypic ratings were conducted. These genotypes were also predicted to be susceptible when fit into the Overall rating and lesion rating models consisting of the range from eight to one marker.

The predictions for the *Bru1* positive group when using the overall rating model, was not significantly correlated to the phenotypic responses in any of the models (Fig 4.3.5a and b), an observation also noted in the lesion rating models (Fig 4.3.7a and b). As all the genotypes produced lesions, a model was also considered where all the genotypes were incorporated into one model, regardless of *Bru1* presence. The stepwise MLR was also used, but the models showed non significant correlations between the actual lesion ratings and the predicted ratings (Fig 4.3.8). The overall phenotypic rating and the lesion rating models consisting of eight markers or less had 53M31 as the only common marker between them. Five markers were however similar between the models at the 20 marker stage, but were eliminated through stepwise MLR at different stages of both the overall and lesion rating models. These markers were 12M58, 24M17S, 34M01S, 45M52 and 53M31 (Table 4.3.4). The marker 53M31 had the highest *r* for the quantitative resistance linked markers. 45M52 was the only quantitative susceptibility marker while the rest were qualitative resistance linked markers.

Table 4.3.4: Common markers between stepwise multiple linear regression derived models for the overall rating and for the lesion rating.

Marker name	Overall rating analysis *r*	Lesion rating analysis *r*
53M31	-0.65	-0.55
12M58	-0.34	-0.38
24M17S	-0.63	-0.55
34M01S	-0.54	-0.465
45M52	0.435	0.462

4.3.4 Cluster Analysis

The dendrogram topology revealed marker relationships relative to each other and grouped the markers according to how closely they gave similar rust infection responses in the LD2 population. Cluster analysis is a common exploratory classification method employed in most diversity analyses. It is known to be particularly useful in discovering natural groupings among entries or items without assumption on the number of groups or group structure (Arro, 2005). Markers were initially selected using the PPMCC and grouped into their respective categories. Stepwise multiple linear regression was then used to select markers from these combined categories, which were then highlighted using coloured blocks according to their resistance group (Fig 4.3.9). Overall, the markers selected by the model were found to be distributed evenly along the length of the dendrogram. The markers were generally clustered according to their resistance groups, with the resistance markers 24M17S and 35M12S being clustered together.

The segregating units in the dendrograms were percentage similarity, an example being shown in Table 4.3.5 (part of a table containing similarity data shown by the dendrograms). The markers 45M52 and 34M16S were very similar to each other (91%), but significantly different from 85M23 (5% similar to 45M52 and 9% to 34M16S). This trend is shown on the dendrogram, where the markers are found in different clusters, suggesting markers of different susceptibility genes.

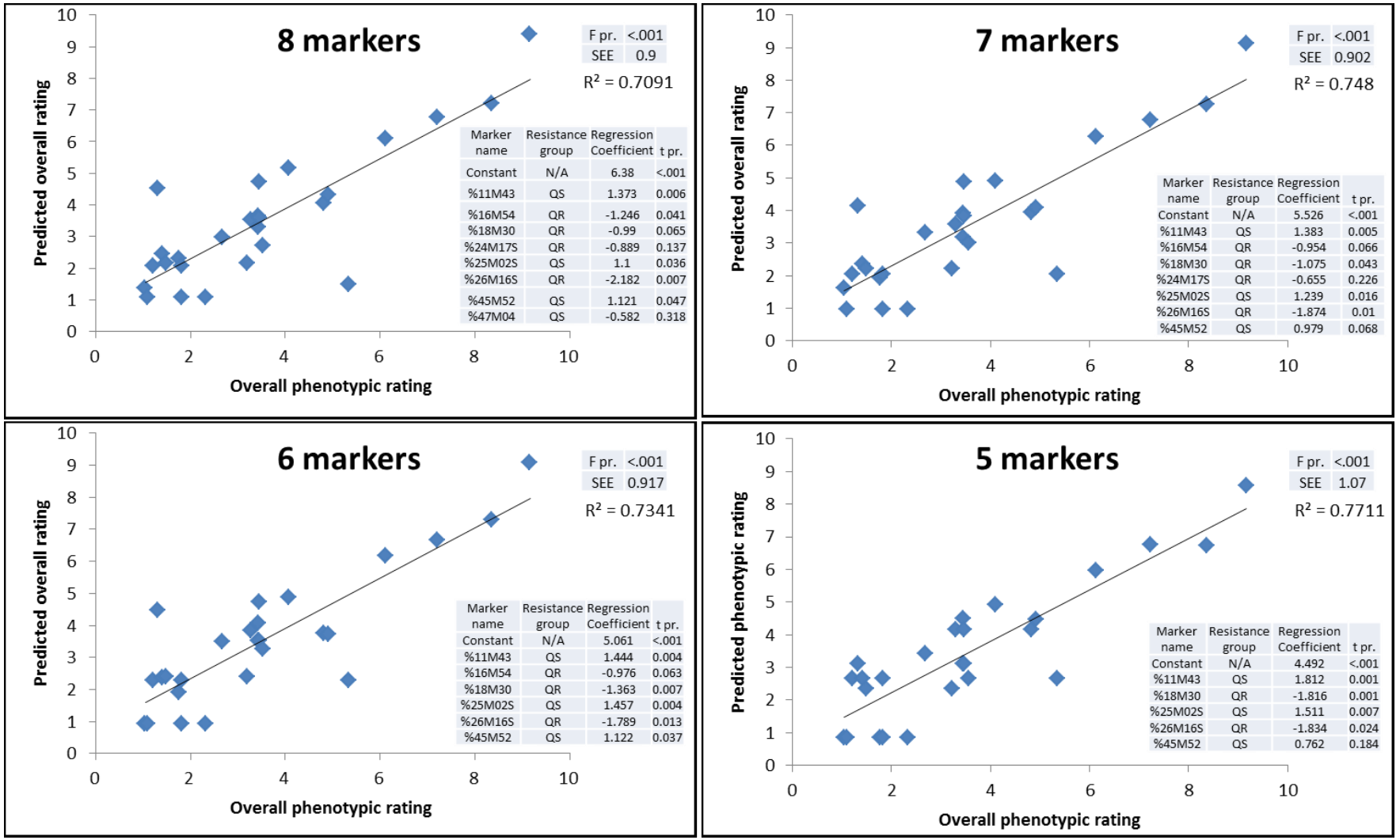

Fig 4.3.4a: Model derived from stepwise multiple linear regression of AFLP markers with the overall phenotypic rating of the *Bru1* negative LD2 genotypes after inoculation with a *P. melanocephala* spore suspension (n=26).

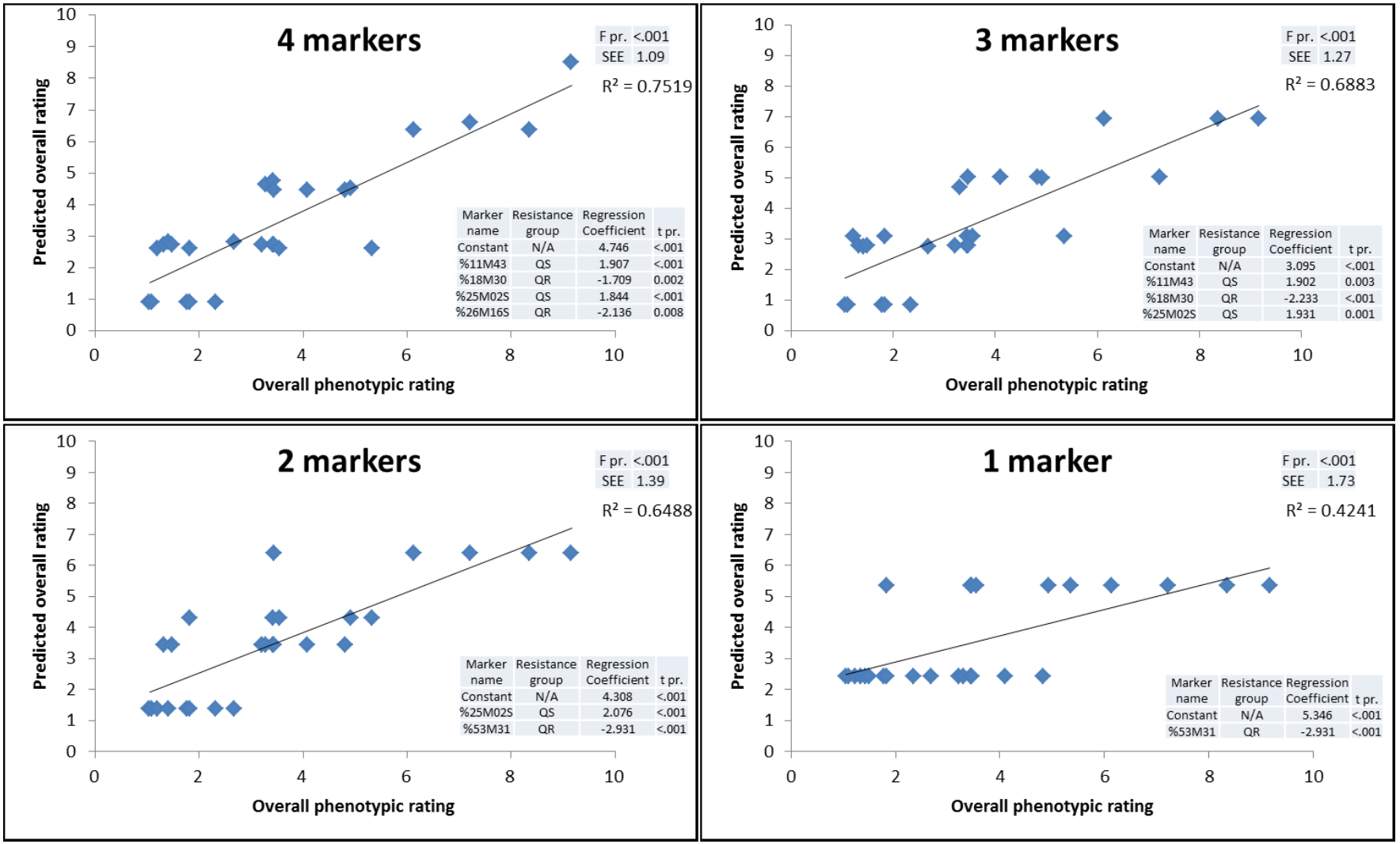

Fig 4.3.4b: Model derived from stepwise multiple linear regression of AFLP markers with the overall phenotypic rating of the *Bru1* negative LD2 genotypes after inoculation with a *P. melanocephala* spore suspension (n=26).

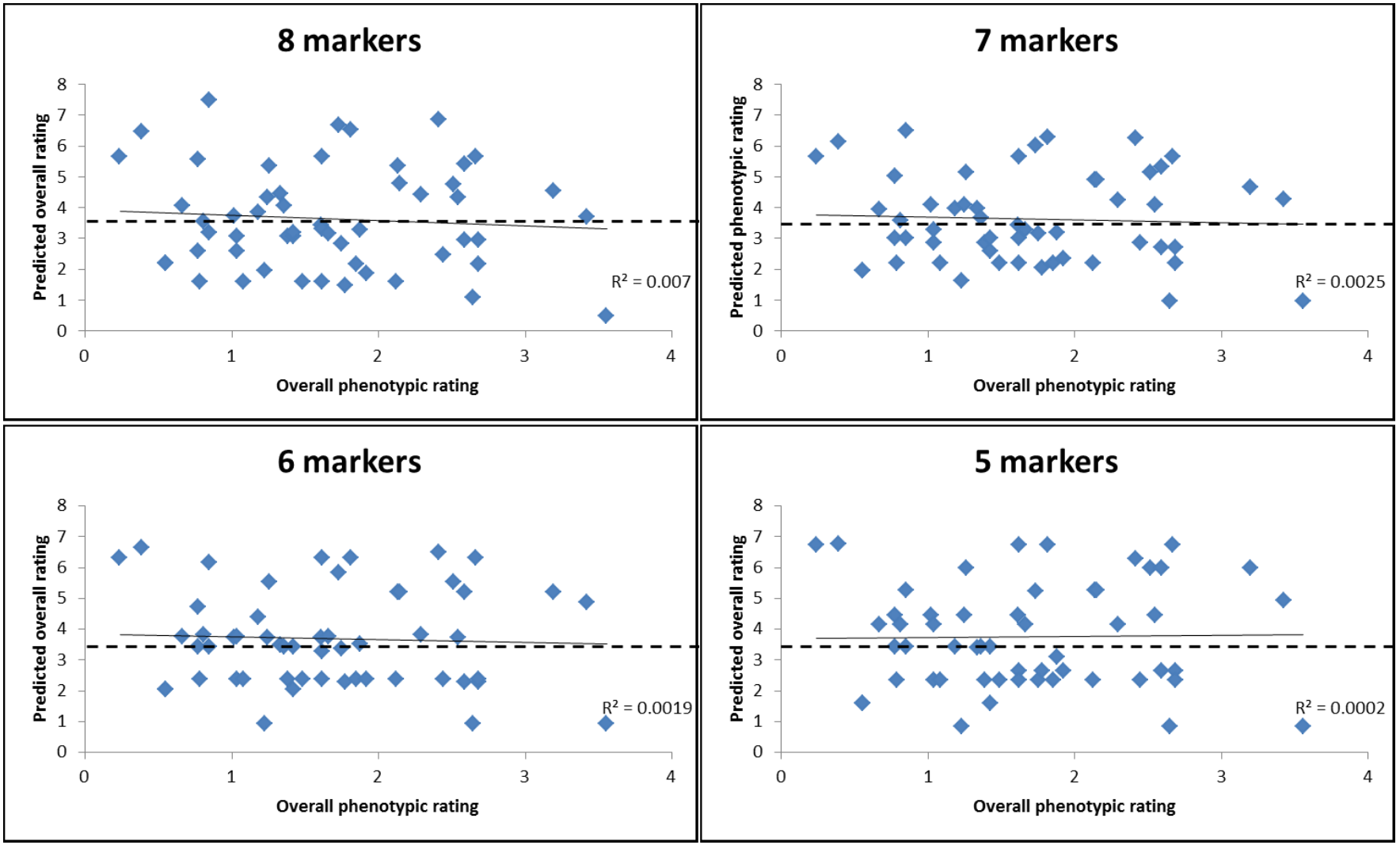

Fig 4.3.5a: Model of predicted overall resistance ratings of the *Bru1* positive genotypes using formulae derived from stepwise linear multiple regression analyses of the *Bru1* negative genotypes (n=54). The black dotted line represents the cut off point for resistance.

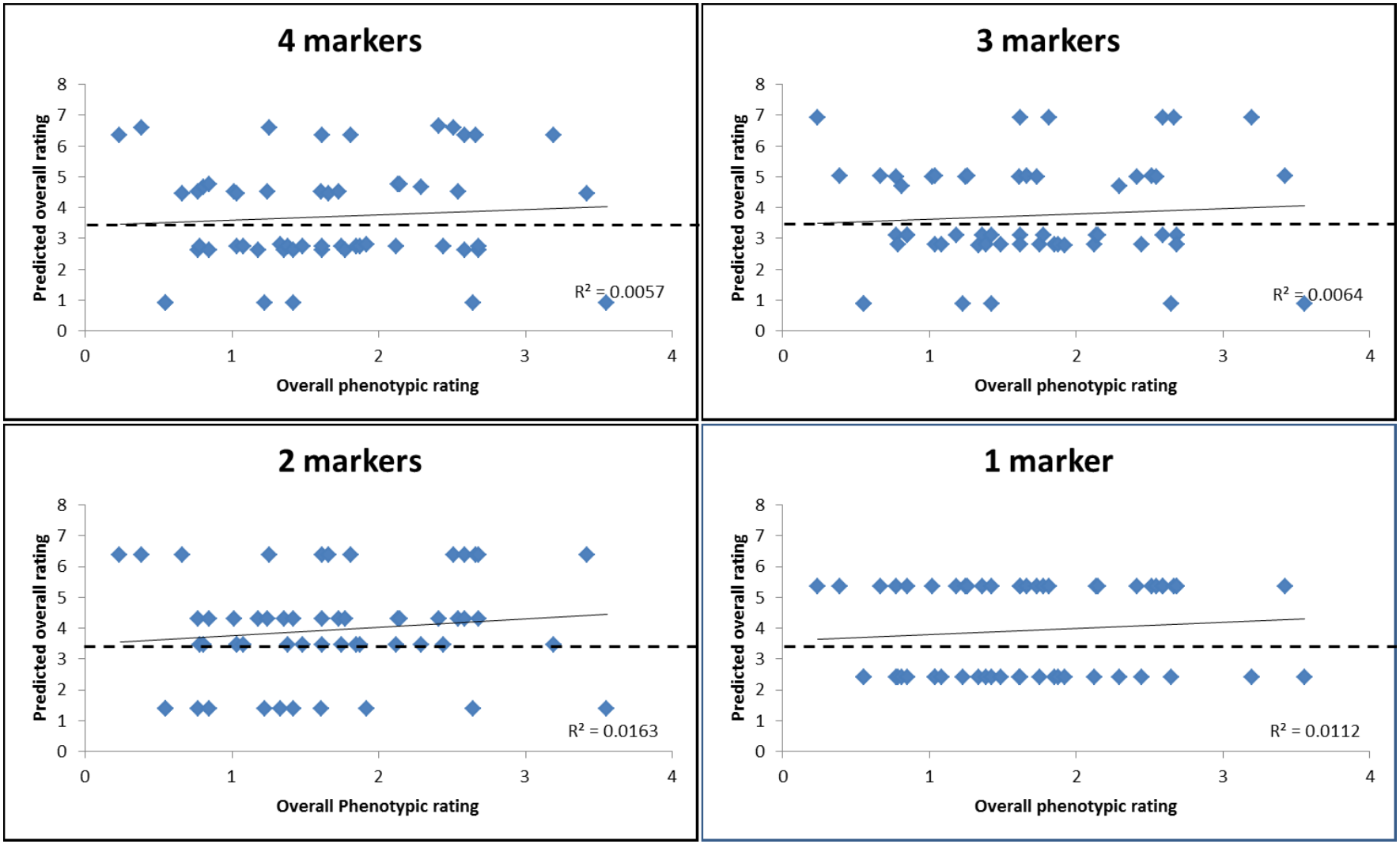

Fig 4.3.5b: Model of predicted overall resistance ratings of the *Bru1* positive genotypes using formulae derived from stepwise linear multiple regression analyses of the *Bru1* negative genotypes (n=54). The black dotted line represents the cut off point for resistance.

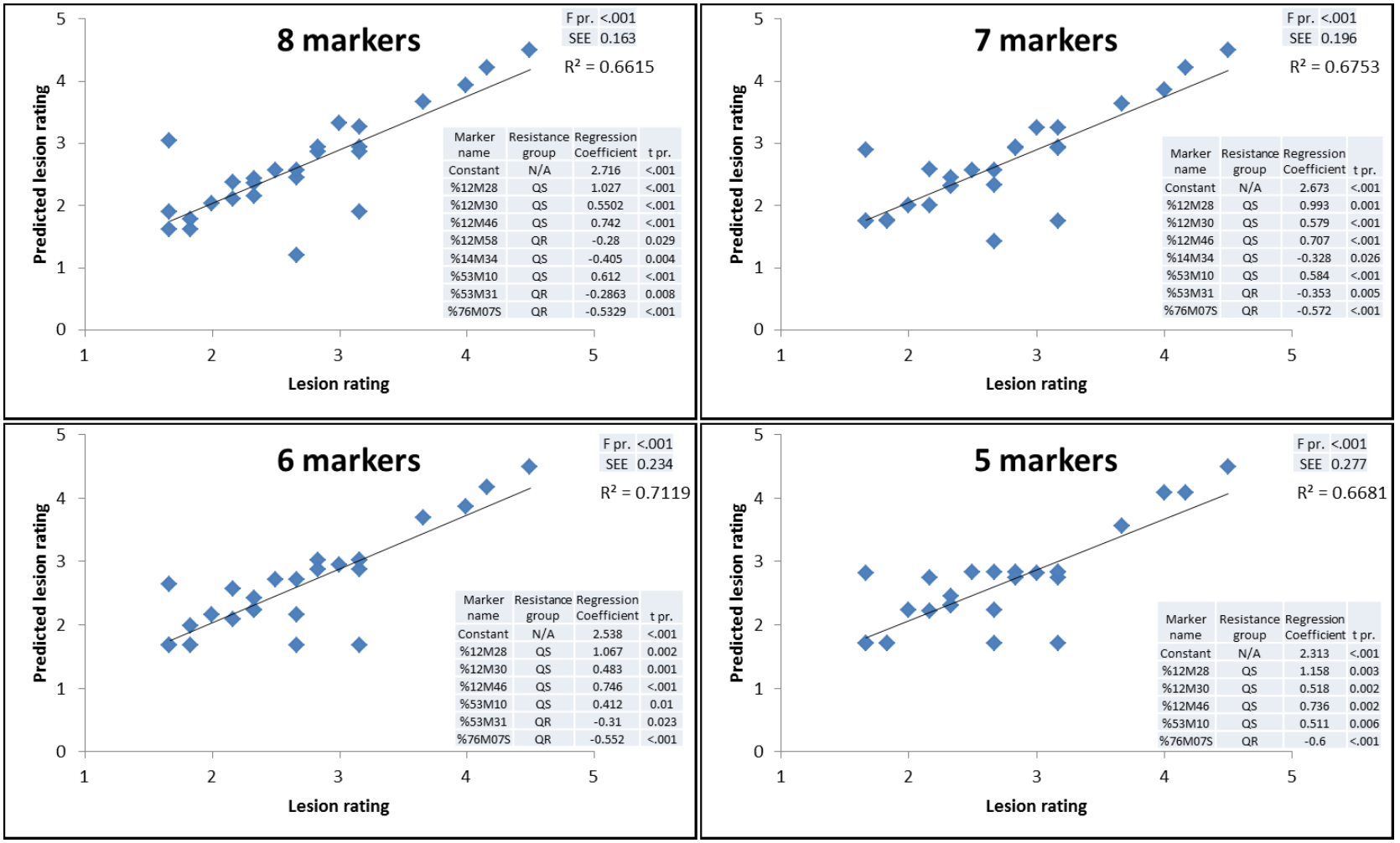

Fig 4.3.6a: Model derived from stepwise multiple linear regression of AFLP markers with the lesion ratings of the *Bru1* negative LD2 genotypes after inoculation with a *P. melanocephala* spore suspension (n=26).

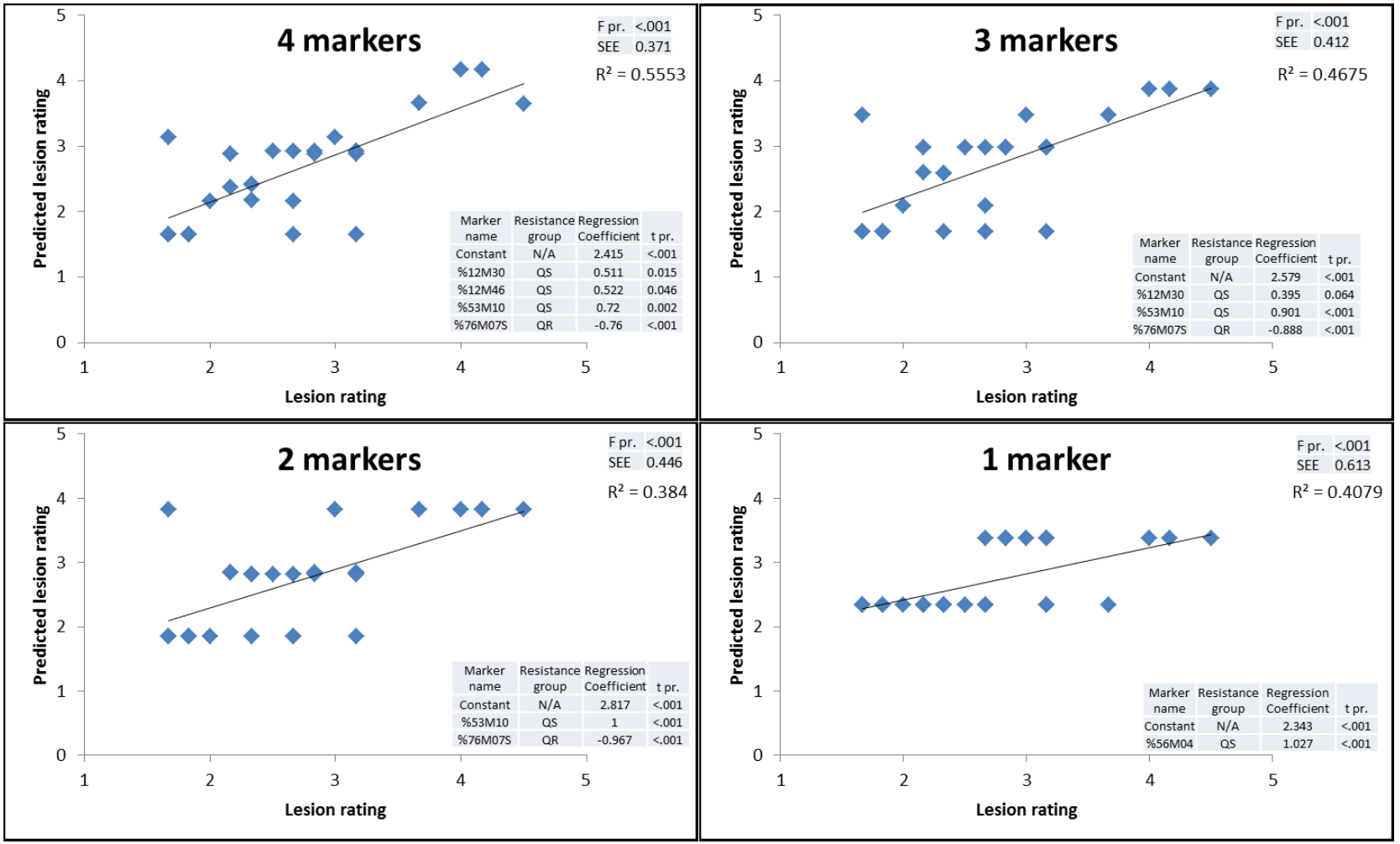

Fig 4.3.6b: Model derived from stepwise multiple linear regression of AFLP markers with the overall phenotypic response of the *Bru1* negative LD2 genotypes after inoculation with a *P. melanocephala* spore suspension (n=26).

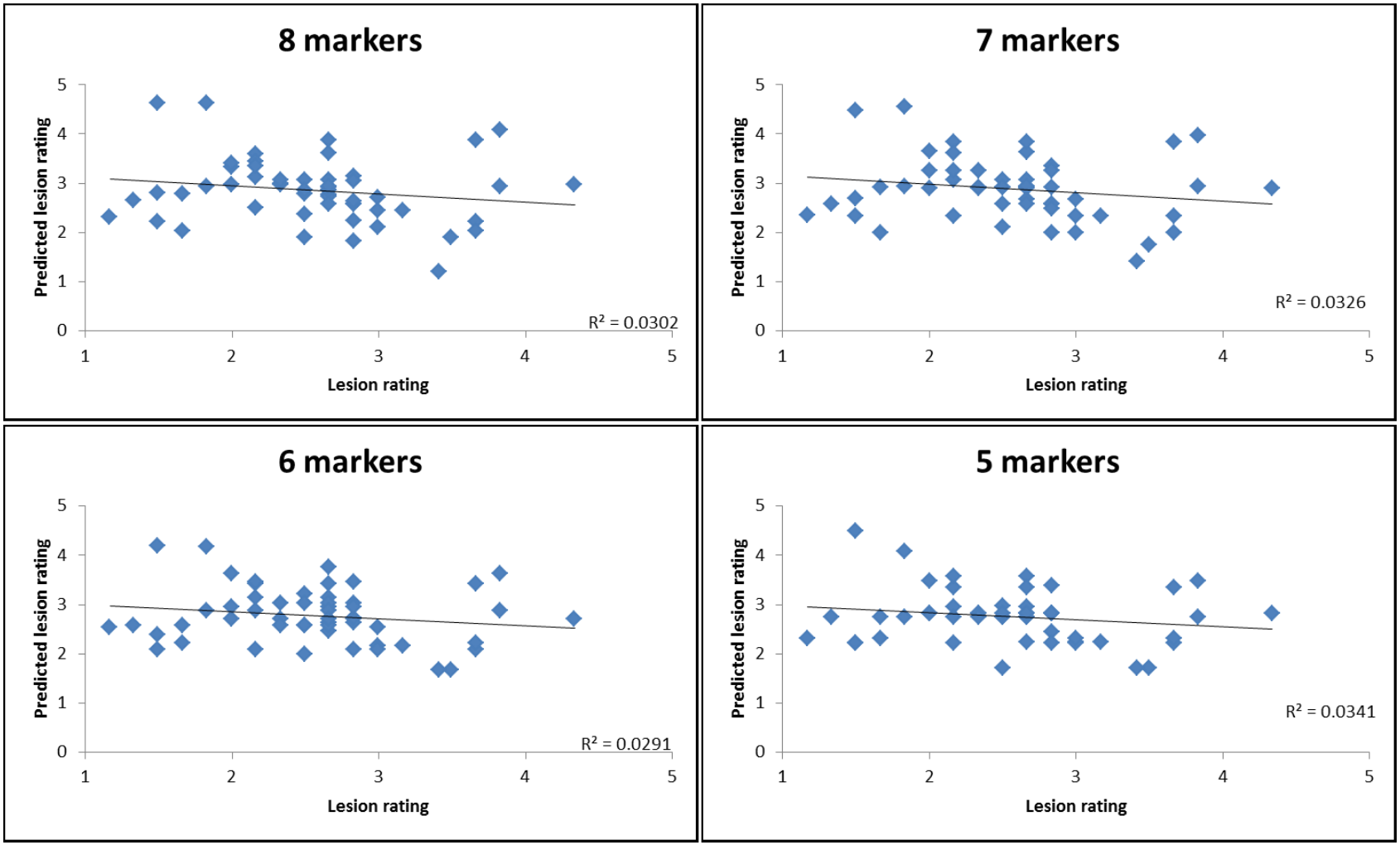

Fig 4.3.7a: Model of predicted lesion ratings of the *Bru1* positive genotypes using formulae derived from stepwise linear multiple regression analyses of the *Bru1* negative genotypes (n=54).

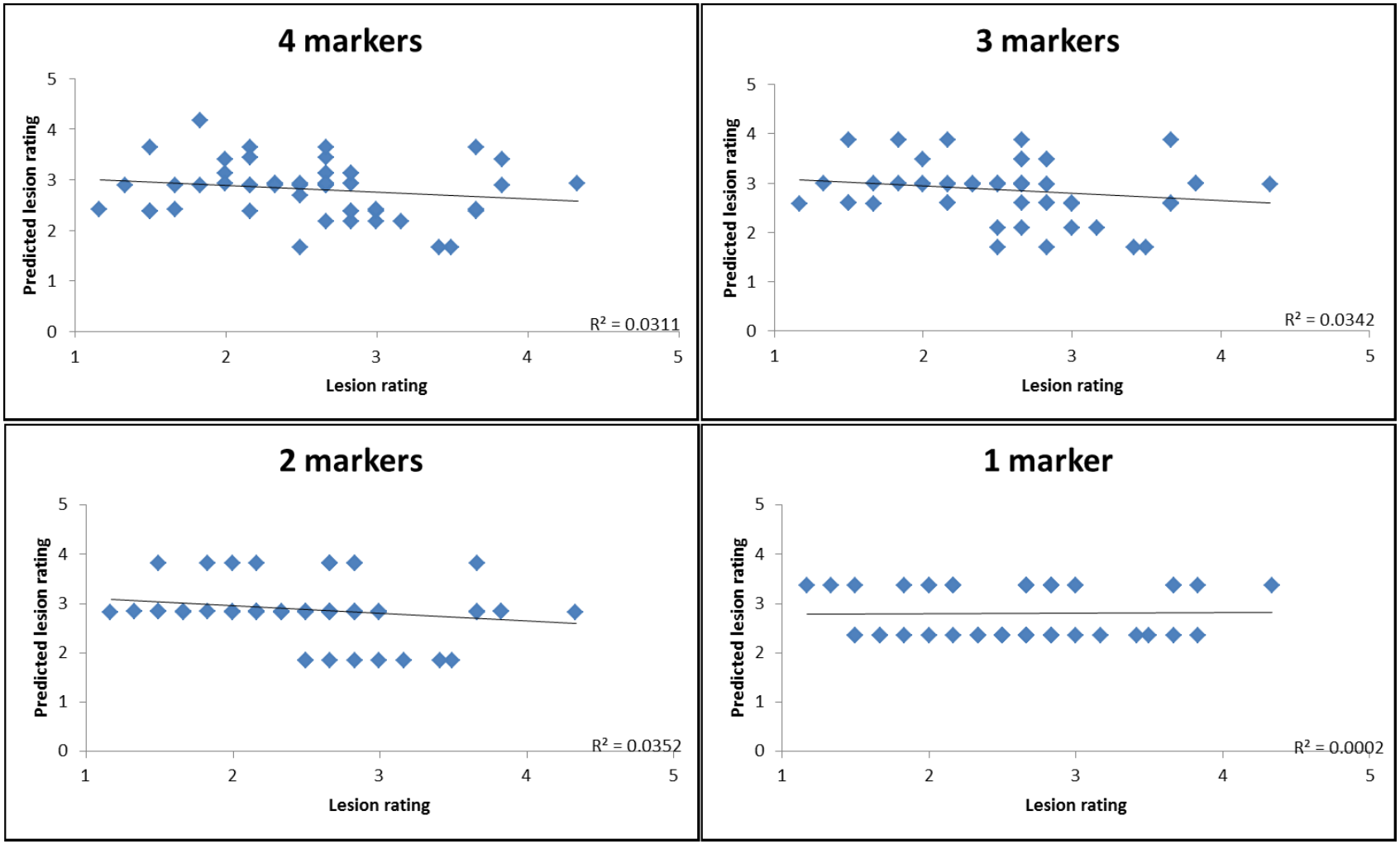

Fig 4.3.7b: Model of predicted lesion ratings of the *Bru1* positive genotypes using formulae derived from stepwise linear multiple regression analyses of the *Bru1* negative genotypes (n=54).

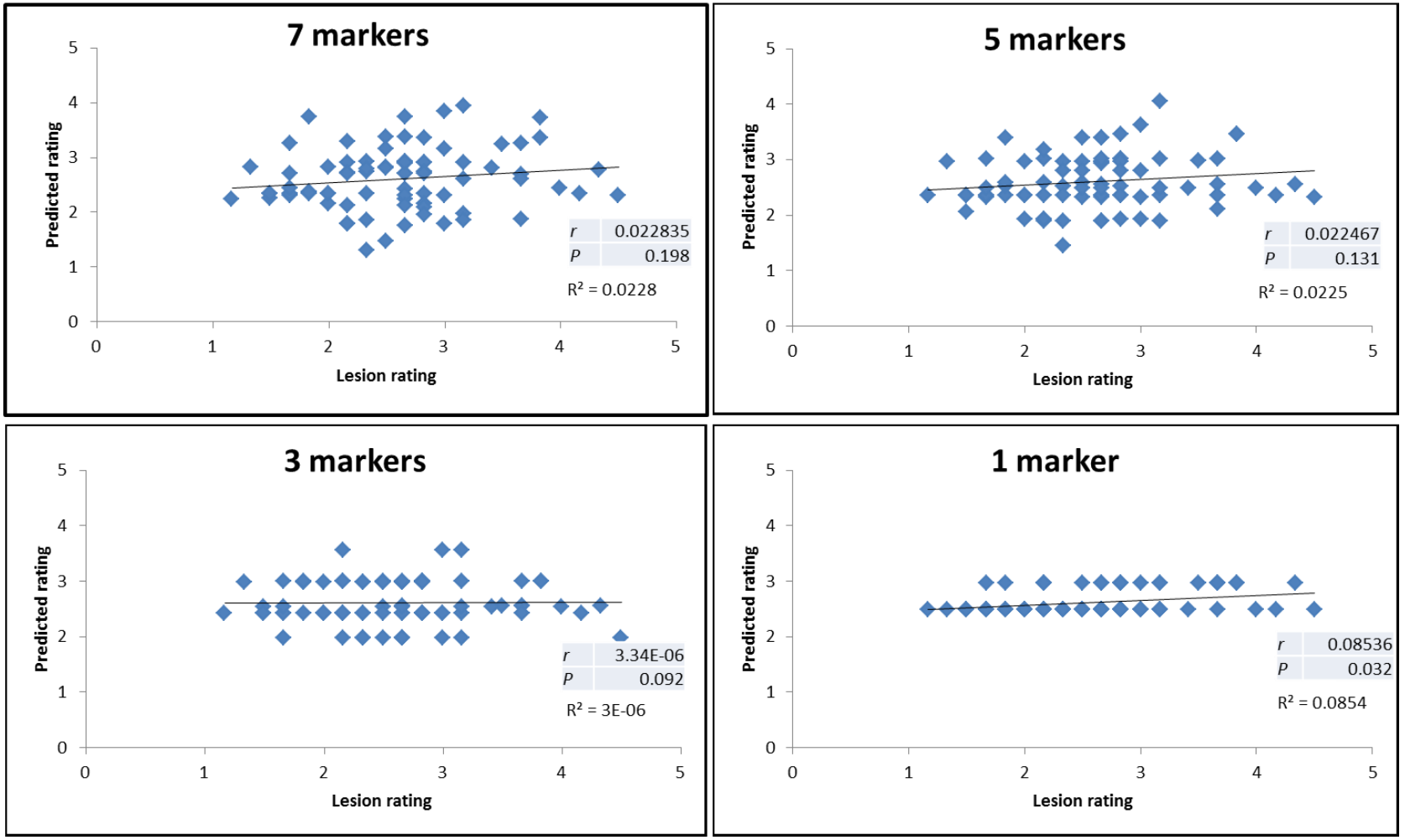

Fig 4.3.8: Model of predicted lesion ratings versus observed lesion ratings of all the LD2 genotypes using formulae derived from stepwise linear multiple regression analyses (n=80).

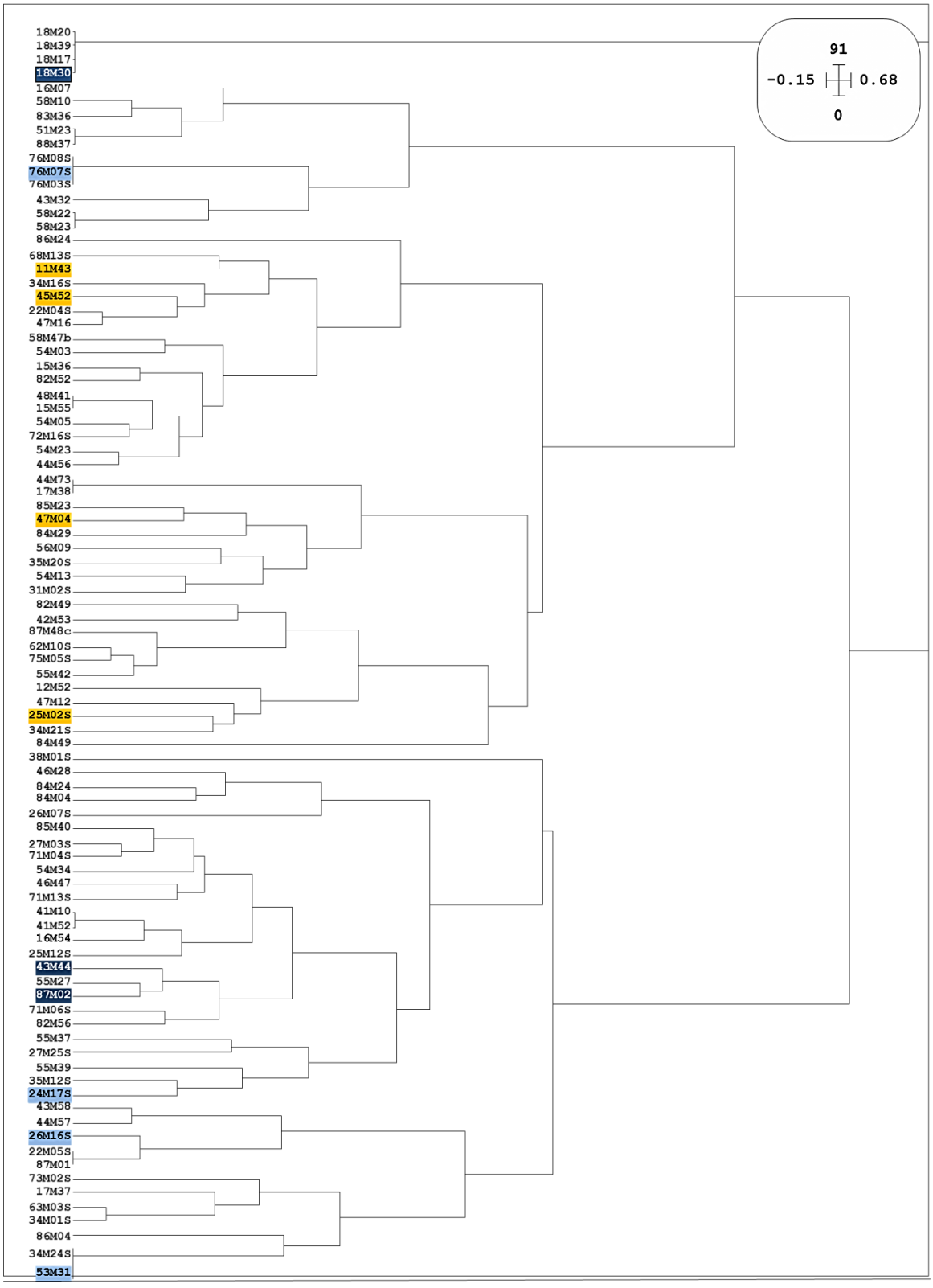

Fig 4.3.9: **Dendrogram topology showing marker relationships for the quantitative marker sets.** The markers in yellow ( ) denote markers for quantitative susceptibility; the markers in light blue ( ) represent markers of quantitative resistance; and the makers in dark blue ( ) are possible markers of qualitative resistance.

Table 4.3.5: Percentage similarity between markers of quantitative susceptibility

Marker	45M52	25M02S	85M23	11M43	82M52	22M04S	54M05	34M16S
45M52	100							
25M02S	66	100						
85M23	5	24	100					
11M43	13	19	63	100				
82M52	25	22	43	78	100			
22M04S	85	71	0	3	12	100		
54M05	32	30	45	75	92	21	100	
34M16S	91	63	9	20	33	76	40	100

4.4 Discussion

The optimised *Bru1* marker analysis showed its ability to accurately and consistently diagnose for the presence of *Bru1*. Genotypes found to be positive for the presence the *Bru1* gene had correspondingly low overall rust lesion and sporulation ratings (an average of 1.67 ranging from 0.24 to 3.56) after inoculation. These observations were expected, as genotypes possessing *Bru1* were anticipated to be resistant to rust. PPMCC correlations revealed markers associated with rust resistance among the rust resistant genotypes diagnosed as being *Bru1* negative. These markers, when analysed using PCA, showed their ability to discriminate the genotypes into distinct groups (*Bru1* negative resistant, *Bru1* positive resistant, susceptible and intermediate). Most importantly, these markers showed their ability to discriminate all the resistant groups from the susceptible group. Some of the markers selected for resistance were found in the intermediate and sometimes even in the susceptible groups. This phenomenon was explained by Zhang *et al*. (2004), who found that important traits related to habitat adaptation in sugarcane, exhibited enormous variability in sugarcane germplasm and were also shown to be both complex and quantitatively inherited.

MLR analysis using the lesion response of LD2 after rust inoculation resulted in an eight marker model which accounted for 96.1% of the variation within the *Bru1* negative genotypes, as compared to the overall rating model which had 84% of the variance explained. The number of markers in the model was subsequently reduced to one marker using stepwise MLR, with all models having relatively high and significant r^2 values and an F. probability of <0.001. The five

maker lesion rating model was a strong candidate for further use, as it had fewer markers and still accounted for 88.7% of the variance and a standard error of observation of only 0.28. The overall phenotypic rating MLR model had lower percentage variance accounted for, relative to the lesion rating models. The overall rating model also had greater standard errors of observation compared to the lesion rating model, suggesting reduced accuracy of prediction. These observations resulted in the lesion rating model being considered as a strong candidate for further use due to its more accurate prediction power.

These observations underlined the fact that it is critical when using data modelling techniques, to select an optimum number of variables. This prevents redundancy in the X-variables and over fitting of the model, which often results in poor prediction results (Butterfield, 2007). The selection of fewer markers bears the risk of under fitting the model and subsequently a large number of samples must be used to develop the model in order to be able to obtain better predictions (Kao *et al*., 1999; Naes *et al*., 2002). This particular study was characterised by such limitations, as the available *Bru1* negative genotypes from the LD2 population were only 26. These 26 genotypes selected for modelling also had an uneven distribution within the resistance groups, resulting in a skewed distribution in which there were only three susceptible genotypes and six intermediate genotypes. The limited sample number and skewed ratings of the available population resulted in the need to obtain additional genotypes from the variety collection, so as to not only increase the sample size, but to even out the distribution of resistance groups and obtain a wider spread of resistance ratings.

The models determined by the stepwise MLR were then used to predict the possible resistance status of the *Bru1* positive genotypes, with the assumption that *Bru1* major rust resistance gene breaks down. The model showed that 50- 70% of the *Bru1* positive genotypes did not possess sufficient markers associated with resistance to be able to maintain resistance in the event of *Bru1* breakdown. This demonstrated the usefulness of using the model to select genotypes containing these resistance conferring traits for rust resistance, in addition to *Bru1*. This would enable the development of even more resistant genotypes, as it has been previously documented that pyramiding of resistance elements often results in more durable and effective resistance (Keane and Brown, 1997; Parlevliet and Van Ommeren, 1988; Poland *et al*., 2011). Other studies have also shown that such marker based models could result in the discovery of new gene combinations for specific traits (Butterfield, 2007).

Markers selected as those possibly conferring quantitative resistance were loosely grouped into three sets of distinctly different markers, which suggested the presence of more than one form of quantitative resistance in the *Bru1* negative resistant group. These markers displayed the potential to be useful, if they are found to indeed tag different resistance conferring traits. Identification of such markers could allow these traits to be more easily identified and subsequently be incorporated into genotypes for use in the breeding program, as it has been demonstrated that combining vertical and horizontal resistance can confer greater resistance to disease (Keane and Brown, 1997; Lagat *et al*., 2008; Parlevliet and Van Ommeren, 1988; Poland *et al*., 2011). The markers 34M24S, 53M31, 82M56 and 18M30 had a high *r* when correlated to the phenotypic ratings and were also aliased to each other during MLR. These markers were also grouped together after cluster analysis, with the markers 53M31 and 34M24S showing the closest similarity on the dendrogram, suggesting that they could tag the same trait. In addition, these makers, when found in *Bru1* positive genotypes, gave the lower range of phenotypic ratings, displaying the associated gene's ability to confer additional resistance to brown rust. This effect of stacking resistance genes in order to obtain a stronger and more durable resistance against disease has been successfully used in other crops such as maize and continued research will be able to confirm these observations in sugarcane (Lagat *et al*., 2008; McIntosh, 1997; Parlevliet and Van Ommeren, 1988; Poland *et al*., 2011).

Markers associated with quantitative susceptibility suggested that the traits they tag are recessive, as their presence in the resistant groups did not necessarily result in susceptibility of these genotypes to brown rust. These markers were however occasionally found associated with the more susceptible of the intermediate genotypes. This suggested that the genes tagged by these markers could result in susceptibility in the absence of rust resistance traits found in the resistant groups. PCA analysis showed that these markers segregate the genotypes such that the susceptible group is located further from the other three groups, which are situated more closely together (Fig 4.4.1). This is an undesirable effect as this could result in the less resistant intermediate genotypes not being selected out, resulting in retention of susceptibility traits in these genotypes. The observation with the markers of quantitative susceptibility was in contrast with that of the resistance markers, where there was a wider spread in the groups, showing the variability of genotype resistance.

Markers associated with qualitative resistance were found to be associated with genotypes of stronger resistance when found in the intermediate group. The markers 43M44 and 57M38 were the most closely correlated (*r*= 0.4), with an 86% similarity to each other. This effect indicates two possibilities, which are that the two may be separate groups which correspond to one linkage group or haplotype due to sharing a probe (Butterfield 2007; Barnes and Bester, 2000); or that they are part of a single cosegregation group which is likely to be revealed with the use of sufficient markers in a mapping exercise (Barnes and Bester, 2000).

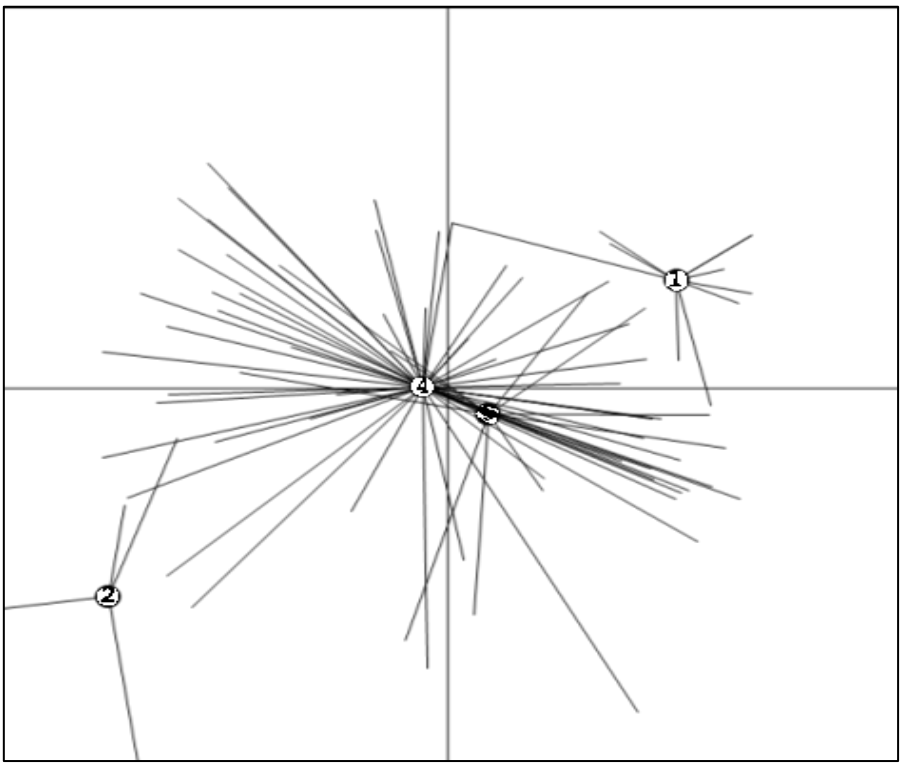

Fig 4.4.1: Principle component analysis of the quantitative resistance marker set relative to the overall phenotypic responses after whorl inoculation. The numbers in the PCA represent: 1= *Bru1* negative resistant; 2= *Bru1* negative susceptible; 3= *Bru1* negative Intermediate; 4= *Bru1* positive.

Most markers for susceptibility to rust were found to be highly correlated to each other, suggesting that the markers could all tag a similar trait. This would imply that only one or a few genes are responsible for susceptibility. The markers 44M38, 45M42 and 45M57, interestingly had a correlation coefficient of one among each other and were not present in any of the other genotypes outside the susceptible group of LD2. This observation led to the conclusion that these markers tag a gene which is responsible for rust susceptibility and will not be found when major rust resistance genes are present. These makers also suggested that the gene

tagged by these markers could be found on the same locus as the major resistance gene/s, and could probably be a recessive gene. An alternative explanation to the presence of many of these related markers is the anonymity of AFLP markers (Butterfield, 2007).

AFLP markers are “anonymous in nature, making it difficult to postulate any potential allelic relationships between these markers without a genetic map through which homology relationships between linkage groups can be identified (Butterfield, 2007).” This is more difficult as some of these markers may represent allelic diversity at the same locus, a phenomenon arising from genetic duplication and recombination which is a known occurrence in modern sugarcane cultivars (Butterfield, 2007; Hoarau *et al*., 2001; Le Cunff *et al*., 2008). The need to map the populations in order to arrive at markers which will be useful in breeding programs is further highlighted by the fact that the presence of one resistance allele can be nullified by the presence of multiple copies of susceptibility alleles (Butterfield, 2007). A genetic map is a solution to this situation, and allows for relationships between markers to be taken into account, increasing the efficiency and reliability of using these markers for marker selected breeding (Butterfield, 2007; McIntyre *et al*., 2005).

Results from these analyses demonstrated and confirmed the ability of AFLP to effectively evaluate a population with precision (Garcia *et al*., 2004). The successful implementation of MAS can result in the reduction of cultivar release cycles and the realisation of other breeding objectives (Dreher *et al*., 2000; Garcia *et al*., 2004). Increasing the number of genotypes and ensuring equal representation of the resistance groups within the model described will refine it and increase its accuracy (Butterfield, 2007), as was shown by this study which used a skewed population consisting of a limited number of genotypes for modelling.

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Chapter 5

Rust resistance screening methods: Detached leaf assays as a means of selecting for resistance to *Puccinia melanocephala*.

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Abstract

Resistant cultivars are the most efficient method of mitigating losses resulting from pests and disease. Breeding programs are tailored to improve the release of cultivars with high resistance and high yield potential, among other desirable agronomic traits. Screening methods which are cost effective, have high throughput and save time are desirable in the sugarcane industry, whose average release time for a cultivar is 12 years. The detached leaf assay (DLA) was used to screen for rust resistance in selected genotypes from the LD2 breeding population. Selected genotypes were grouped as *Bru1* negative resistant, *Bru1* positive, *Bru1* negative susceptible and *Bru1* negative intermediate. DLA conducted on water agar (large square plates and Petri dishes) and Magenta® plant culture boxes containing water were compared. Leaves were inoculated with *Puccinia melanocephala*. Visual analyses were conducted through counting of lesions and sporulation frequency and intensity. Chlorophyll fluorescence (3000µmol m^2/s light intensity) and soil plant analysis development (SPAD) were measured. These parameters ascertained the effects of rust on chlorophyll *a* activity and chlorophyll content, with a view to using them as an early diagnostic tool. Visual analysis proved to be a reliable screening method, as it clearly differentiated the resistant and susceptible genotypes within 12 days. Greater lesion formation and sporulation were observed in the susceptible group. Effects of rust on chlorophyll indicated differing rust reactions from the different resistance groups of plants between four to five days. Overall, chlorophyll related observations showed a decrease in the performance index (PI_{ABS}) and chlorophyll content over time, a common characteristic of detached leaves. The differences however were more pronounced in inoculated leaves when compared to un-inoculated leaves. The PI_{ABS} in the un-inoculated region of inoculated leaves also showed greater activity relative to that of the

control, suggesting a compensatory effect to counter the effect of rust. These observations resulted in resistance groups being differentiated within four days as compared to the 12 required when conducting visual analysis. The chlorophyll analyses also resulted in screening being conducted before the onset of contamination related leaf necrosis and death as compared to the visual analysis method. These studies showed that DLA can be used as a rapid screening tool in a breeding program.

5.1 Introduction

Breeding commercial cultivars with desirable agronomic traits and durable resistance to disease has been a practice on-going for over a century (Bremer, 1961). Cultivars with disease resistance properties are the most effective in terms of disease control and have become an integral component of modern agriculture (Asnaghi *et al*., 2004; Bailey, 2004; Bürling *et al*., 2011). These cultivars not only control disease effectively, but reduce carbon footprints and potential environmental issues associated with fungicides.

Disease resistance genes have been sourced from the wild species of *Saccharum*, such as *S. spontaneum* and *S. robustum* (Baucum *et al.*, 2009; James, 2004; Kelly *et al.*, 2009). Resistance traits have been traditionally identified for integration into the genome of offspring from crosses by field screening techniques which often take several years to conduct and require high financial input (Bürling *et al*., 2011; Purdy *et al*., 1983; Sood *et al*., 2009). These screening techniques can be highly subjective and have the disadvantage of being seasonal, being limited by uncontrollable variables such as the environment and being affected by the availability and uniform exposure to inoculum in instances where natural infection is relied upon (Bürling *et al*., 2011; Sood *et al*., 2009; Twizeyimana *et al*., 2007).

The need to develop efficient, objective and rapid methods of disease screening with results comparable to field screening techniques is crucial in plant breeding (Bürling *et al*., 2011; Purdy *et al*., 1983). Assays conducted on detached leaves have been considered such a method and have been successfully exploited for their ability to be used in a diagnostic manner in screening plant genotypes against a variety of fungi (Browne and Cooke, 2005; Jackson *et al*., 2008; Pettitt *et al*., 2011; Twizeyimana and Hartman, 2010). The use of screening methods

which employ plant-pathogen interactions have proved to be invaluable as they can more accurately expose the resistance status of genotypes selected by marker assisted selecton, as some resistance genes may be controlled by epistatic interactions which might be unrelated to the target gene (Le Cunff *et al*., 2008).

A fundamental part in conducting DLAs is the maintenance of leaf health, which can be attained by the use of plant growth regulators (Jackson *et al*., 2008). This is an important step, as maintenance of healthy leaves allows differentiation to be made between infected and uninfected leaves. The maintenance of healthy leaves in a DLA allows the identification of susceptible genotypes and non-virulent pathogen species, as the effect of scenescence on detached leaves is negated, giving rise to the ability to use negative controls without risk of factoring in scenescence related leaf death to screening results (Twizeyimana *et al*., 2007). Maintenance of healthy leaves can be attained through the use of a variety of plant growth regulators, which include benzylaminopurine (BAP), benzimidazole and the cytokinin kinetin (Jackson *et al*., 2008; Twizeyimana *et al*., 2007). Fungicides and lactic acid have also been commonly used in media amendments to protect detached leaves from saprophytic fungi and bacteria (Twizeyimana *et al*., 2007).

Visual analysis is commonly used to make observations on disease resistance when using DLAs and parameters such as lesion coverage and sporulation have been used to determine cultivar resistance and pathogen virulence (Jackson *et al.*, 2008; Twizeyimana *et al*., 2007; Twizeyimana and Hartman, 2010). Other parameters such as chlorophyll fluorescence have been widely studied in the detection of fungal pathogen resistance in host plants. The method has also been used to discriminate between genotypes differing in pathogen resistance (Bürling *et al*., 2011; Rolfe and Scholes, 2010; Scholes and Rolfe, 2009).

The use of photochemical efficiency as a means to diagnose disease or pest effects on crop yield is one which has the potential not only to predict and understand yield responses to disease, but also to act in a diagnostic manner (Lopes and Berger, 2001). This is made possible by the effects of pests and pathogens on crop carbon flow processes which can be summarised as being the effects on radiation interception and radiation use efficiency (Lopes and Berger, 2001; Zhao *et al*., 2011). This makes the quantification of these effects a possible tool in the prediction of yield reductions when coupled to crop growth simulators (Lopes and Berger, 2001). Chlorophyll fluorescence has the ability to detect slight changes in the photosynthetic metabolism of a plant which can be brought about by infection of plant tissues by a pathogen

(Rolfe and Scholes, 2010). This capability has been demonstrated by the early detection of changes in the photosynthetic profile of an infected plant even before visible symptoms have been observed (Rolfe and Scholes, 2010; Scholes and Rolfe, 2009).

The use of chlorophyll fluorescence has been employed to screen for disease resistance in a variety of plants, including sugarcane (Rolfe and Scholes, 2010; Zhao *et al*., 2011). Chlorophyll fluorescence analyses the response of the PSII reaction center in a dark adapted leaf by illuminating it with a saturated light pulse (Strauss *et al*., 2007; Van Heerden *et al*., 2003). The intensity of chlorophyll *a* fluorescence undergoes characteristic changes, known as the Kautsky effect. The fast rise of the Kautsky transient represents the primary reactions of photosynthesis, which subsequently provides information on the photochemical activity of PSII (Strauss *et al*., 2007). The JIP test is then applied to the fluorescence data, deriving parameters such as the performance index (PI_{ABS}). The PI_{ABS} is a multiparametric expression that combines the three main functional steps of photosynthetic activity by a PSII Reaction Center complex (Strauss *et al*., 2007; van Heerden *et al*., 2003).

The relationship between disease severity and yield loss is also greatly influenced by Genotype X Environment (GxE) factors (Lopes and Berger, 2001). This makes *in vitro* screening techniques valuable as susceptible genotypes can be identified before being released, avoiding situations where varieties can suddenly be found to be susceptible. GxE interactions also need to be strongly considered as they play a major role in rust resistance (Ribeiro do Vale *et al.*, 2001). Studies have shown that genotypes found to be susceptible or of intermediate resistance when using screening techniques outside the field such as glasshouse trials and *in vitro* analyses could well be resistant in the field (Asnaghi *et al*., 2001; Dixon *et al*., 2002). Research has frequently demonstrated how certain crops can be disease resistant in an environment and then become susceptible in a different one as was shown by Dixon *et al*. (2002) on the differing responses of cassava to different pests and diseases under different environmental conditions.

With this background, the objectives of the study in this paper were conceived. An *in vitro* method of detecting resistance of sugarcane cultivars to *P. melanocephala* was formulated around the commonly used visual analysis. The visual results were then compared to an analysis of the response of the PSII reaction center (PI_{ABS}) of infected leaves from a breeding population so as to ascertain the best approach towards screening for rust resistance using the DLA. Chlorophyll content of the leaves was also measured using the soil plant analysis

development (SPAD) meter to relate chlorophyll content of infected leaves to their photochemical efficiency (PI_{ABS}). These two parameters were measured so as to better understand the effects *P. melanocephala* has on infected leaves and to more rapidly screen genotypes relative to the visual analysis of the detached leaf assay. Another objective of this paper was to ascertain if these methods could be used in a high throughput screening system.

5.2 Materials and Methods

A detached leaf assay was conducted using the method described in Mhora *et al*. (2011). Genotypes from a Linkage Disequilibrium breeding population at the South African Sugarcane Research Institution (SASRI) called LD2 were selected based on previous resistance information obtained from whorl inoculation, *Bru1* marker data and AFLP correlation. These selected genotypes were put through the screening technique to ascertain the effectiveness of the previously developed assay.

5.2.1 Germination and pathogenicity tests on *P. melanocephala* urediniospores

5.2.1.1 Spore germination tests

Optimal spore germination conditions were assessed by comparing the effect of 1-nonanol (SIGMA-ALDRICH®, Steinheim, Germany) to water on *P. melanocephala* spores (Braithwaite, 2005; French and Gallimore, 1972; Zhao *et al*., 2011). Concentrations of 0.001% (v/v), 0.002% (v/v) and 0.005% (v/v) of 1-nonanol were compared to water, to determine the best germination conditions for the spores. *P. melanocephala* spores were harvested from N29 leaves obtained from the field. Spores on the leaves were suspended in a 100ml SHOTT DURAN® glass bottle containing 50ml each of the different concentrations of sterile 1-nonanol in its different concentrations and glass beads. In a separate glass bottle, spores were suspended using 50ml of sterile water and glass beads. Germination counts were conducted on 1% (w/v) water agar prepared using bacteriological agar (Merck, Gauteng) in distilled water and set in plastic Petri dishes. Spore suspension (100 µl) was added to the agar surface and spread using glass beads. The germination tests were conducted on 5 plates per spore concentration and incubated overnight in the dark at 20°C (Braithwaite, 2005). Germination

counts were conducted under a Nikon Eclipse 50*i* light microscope by counting the spores and obtaining a percentage germination using the formula: Percentage germination = (germinated spores/ total spore count) * 100.

Prior to analysis, the data were subjected to a normality test using the Shapiro-Wilk test. The data were found to be normal and were subsequently subjected to an analysis of variance test (ANOVA) followed by the Holme Sidak post-hoc test.

5.2.1.2 Pathogenicity tests

Infectivity tests were conducted using *P. melanocephala* spores suspended in 0.001% (v/v) 1-nonanol and in water as described in section 5.2.1.1. Leaves from the mid-section of the top visible dewlap (TVD) of the rust susceptible variety N29 were cut into 3-4cm long pieces and placed, abaxial side up onto 1% (w/v) water agar containing 0.33 g/l pentachloronitrobenzene (PCNB). Inoculum (100 µl) was applied to the abaxial side of the leaves according to the corresponding treatment. The leaves were dark incubated at 20°C overnight before being transferred to an 18-hour photoperiod room where they were monitored daily for symptoms of rust infection till the third week. Lesion and spore formation on N29 leaf pieces were recorded and compared between the two treatments. Statistical analysis was conducted using the two sample T-test.

5.2.3 Inoculum preparation for DLA

Distilled water was chosen as the sole constituent of the spore suspension medium. Inoculum was prepared in 300ml of distilled water using the protocol described in 5.2.1.1. The spore solution was immediately quantified using a Neubauer counting chamber (Marienfield superior, Germany) under a Nikon Eclipse 50*i* light microscope (Sood *et al*., 2009). The solution was adjusted to a concentration of 10^6 spores/ ml and transferred to a glass beaker. Germination counts were conducted on 1% (w/v) water agar as described in section 5.2.1.1.

5.2.4 Media preparation

Media preparation was conducted using a combination of plant growth regulators, fungicides and lactic acid as described in Mhora *et al*. (2011). Media containing water and PCNB (SIGMA-ALDRICH®, Steinheim, Germany) in 1% (w/v) agar was selected as the best media for maintaining leaf health. This media was subsequently used in all DLAs. The media was autoclaved at 121°C for 20 minutes and poured into plates, where it was allowed to set before being stored at 4°C. Sterile water was used to maintain the leaves in Magenta® plant culture boxes using the alternative method (Braithwaite, 2005).

5.2.5 Leaf material and inoculation

Twelve genotypes were selected from LD2, planted and maintained as described in Chapter 2. Leaves from the TVD were harvested from each genotype after three months. The mid sections of the leaves were cut into 6cm pieces, retaining the midrib and then dark acclimatised for an hour. The leaves were then attached onto a humidifier consisting of a pipette tip box containing distilled water using a rubber band; and inoculated with 100µl of inoculum. The leaves were incubated overnight at 20°C in a tip box humidifier (Fig 5.3.3a), placed into their respective media and maintained in an 18-hour photoperiod room. The experiment was conducted using square plates and Petri dishes containing water agar and PCNB and also in Magenta® plant culture boxes containing water. Three leaves were inoculated in each Magenta® box and square plate experiment, while two leaves were used as negative controls. Nine leaves were used in the Petri dish experiment for inoculation and nine as a negative control.

5.2.6 Chlorophyll fluorescence and (SPAD) measurements

Cut leaves which had been dark acclimatised for an hour as described in section 5.2.5 were measured for chlorophyll fluorescence and chlorophyll content prior to inoculation. Chlorophyll *a* fluorescence measurements were taken from each dark-acclimatised leaf cutting using a Hansatech Handy Pea fluorescence meter at a light intensity of 3000 µmol m^2/s. Two readings were taken from the leaf area directly in contact with the inoculum, while another

two were taken from an area which did not get into contact with the inoculum, making a total of four readings on each leaf. Chlorophyll content was also measured immediately after the fluorescence readings, using a SPAD chlorophyll meter (Konica Minolta, USA) (Zhao *et al*., 2011). Four readings were taken on each leaf, in a similar way to the fluorescence readings.

The leaves were then embedded into their respective treatments, incubated overnight in the dark at 20°C, and then transferred to an 18-hour photoperiod growth room. Chlorophyll *a* fluorescence and SPAD measurements were carried out every 24 hours for eight days. The chlorophyll fluorescence data was processed using the JIP test (Strasser *et al*., 2000), which led to the calculation of the PI_{ABS}, which is regarded as a reliable indicator of electron transport efficiency and photosynthetic capacity during stress (Strauss *et al*., 2007). ΔPI_{ABS} was calculated using the formula: $\Delta PI_{ABS} = PI_{ABS}$ (day 0) - PI_{ABS} (day x).

5.2.7 Visual analysis

The leaves were monitored daily for 15 days to observe any visual rust symptoms (lesions and spores). The lesions on each leaf cutting were counted and assessed for sporulation. The degree of sporulation on each lesion was assessed using the sporulation rating scale shown in fig 5.2.1. Lesion ratings were also carried out using the rating scale described in Table 5.2.1 for the larger leaf pieces used in the method by Braithwaite (2005).

Table 5.2.1: A lesion rating scale used to separate differing rust infection symptoms

Rating scale	Symptoms
0	no lesions/ chlorotic flecks
1	one to a few lesions
2	more than a few lesions
3	numerous lesions
4	numerous coalescing lesions
5	severe symptoms with numerous coalescing lesions and necrosis

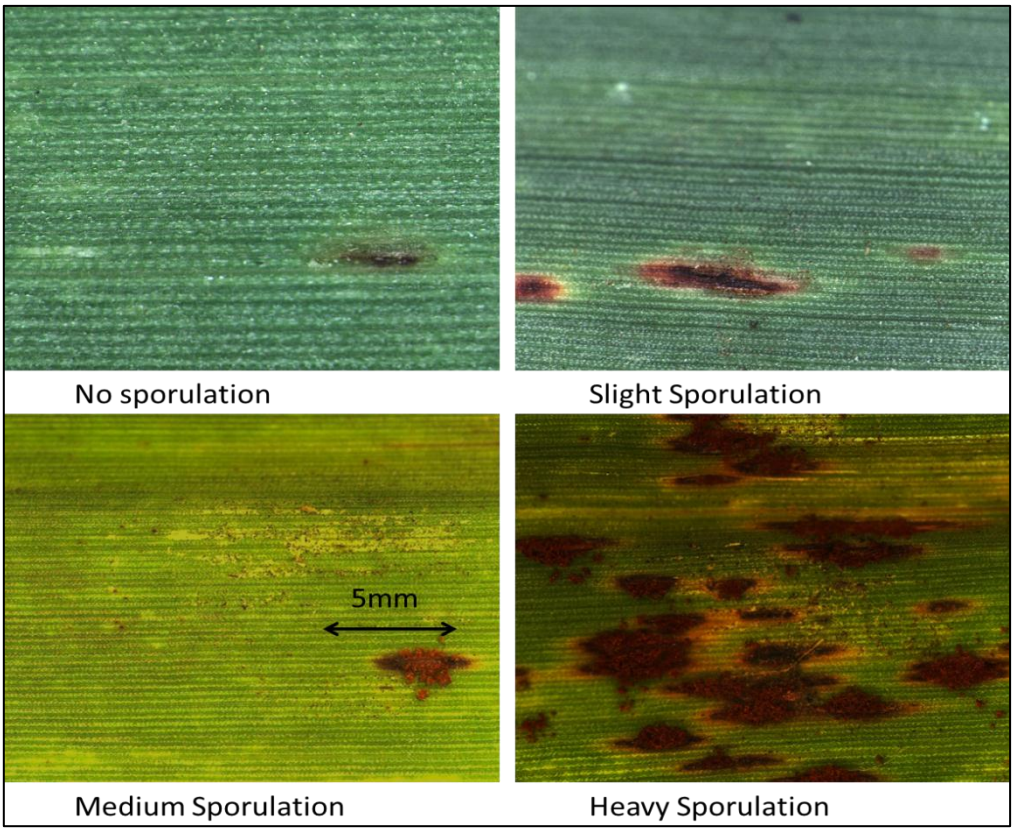

Fig 5.2.1: The sporulation rating scale used in the visual assessment of sporulating lesions. No sporulation= 0, slight sporulation= 1, medium sporulation= 2 and heavy sporulation= 3. (Pictures by T. T. Mhora).

5.2.8 Data analysis

Data were analysed using GenStat v.13.2 (United Kingdom) at a 95% confidence interval. The student T-test was used to analyse the germination rates of the different treatments used to stimulate spore germination. Restricted Maximum Likelihood (REML) variance component analysis and analysis of variance (ANOVA) were used to analyse the data obtained from visual analysis. Tests on the data were then followed by the Holm-Sidak post-hoc analysis. Regression analysis and REML analysis were also used to analyse the SPAD and chlorophyll fluorescence measurements.

5.3 Results

5.3.1 Germination tests

Germination of *P. melanocephala* spores was significantly (*P*=0.004) stimulated by the addition of 0.001% (v/v) 1-nonanol. Increasing the concentration of 1-nonanol however reduced the rate of spore germination (Fig 5.3.1).

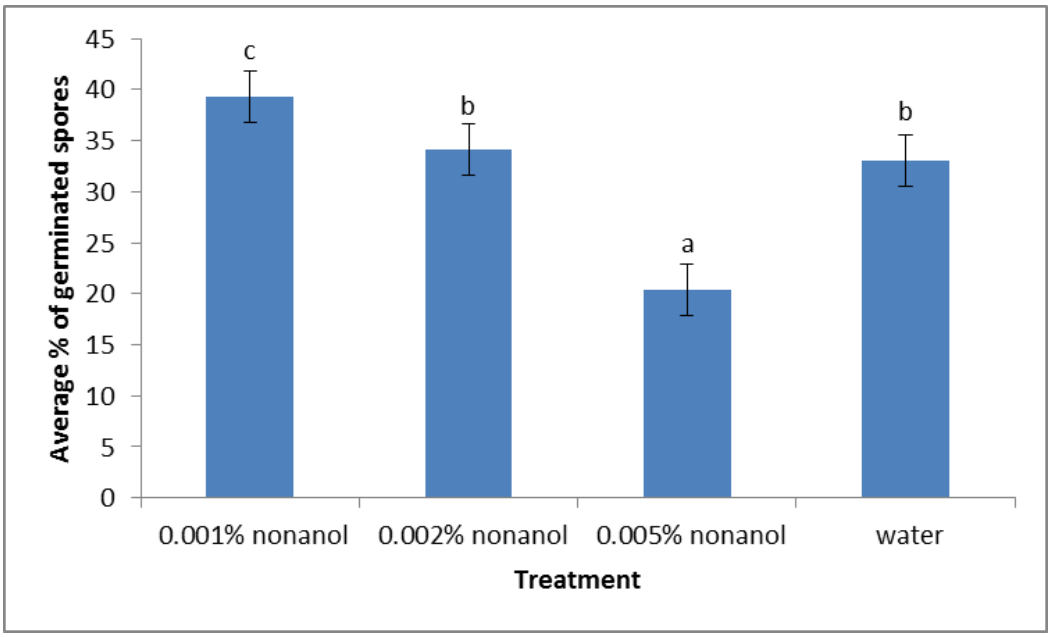

Fig 5.3.1: **Germination rates using different concentrations of 1-nonanol against water.** The average percentage of germinated spores with the same letter is not significantly different.

5.3.2 Pathogenicity tests

There was a significant (*P*=0.004) decrease in the pathogenicity of *P. melanocephala* on N29 when 1-nonanol was added as a germination stimulant when compared to spores suspended solely in distilled water (Table 5.3.1). Addition of 0.001% (v/v) 1-nonanol resulted in fewer lesions developing, coupled with reduced sporulation intensity (Fig 5.3.2). This response was contrary to expected results, as germination of *P. melanocephala* was significantly (*P*= 0.004) higher when 1-nonanol was added (Fig 5.3.2A compared to 5.3.2B). Spores were subsequently suspended in water for all the following DLAs, based on this analysis.

Table 5.3.1: **Infection responses of sugarcane variety N29, when inoculated *with P. melanocephala* spores suspended in water and in 0.001% 1-nonanol.**

Treatment	Average lesion number	Leaves with lesions	Average number of sporulating lesions			
			Heavy	medium	low	nil
Water	29.22	9	29.22	0	0	0
1-nonanol	6.36	10[a]	0	0.36	0.82	5.18

[a]- Ten out of 11 inoculated leaves were used for data collection due to the death of one replicate.

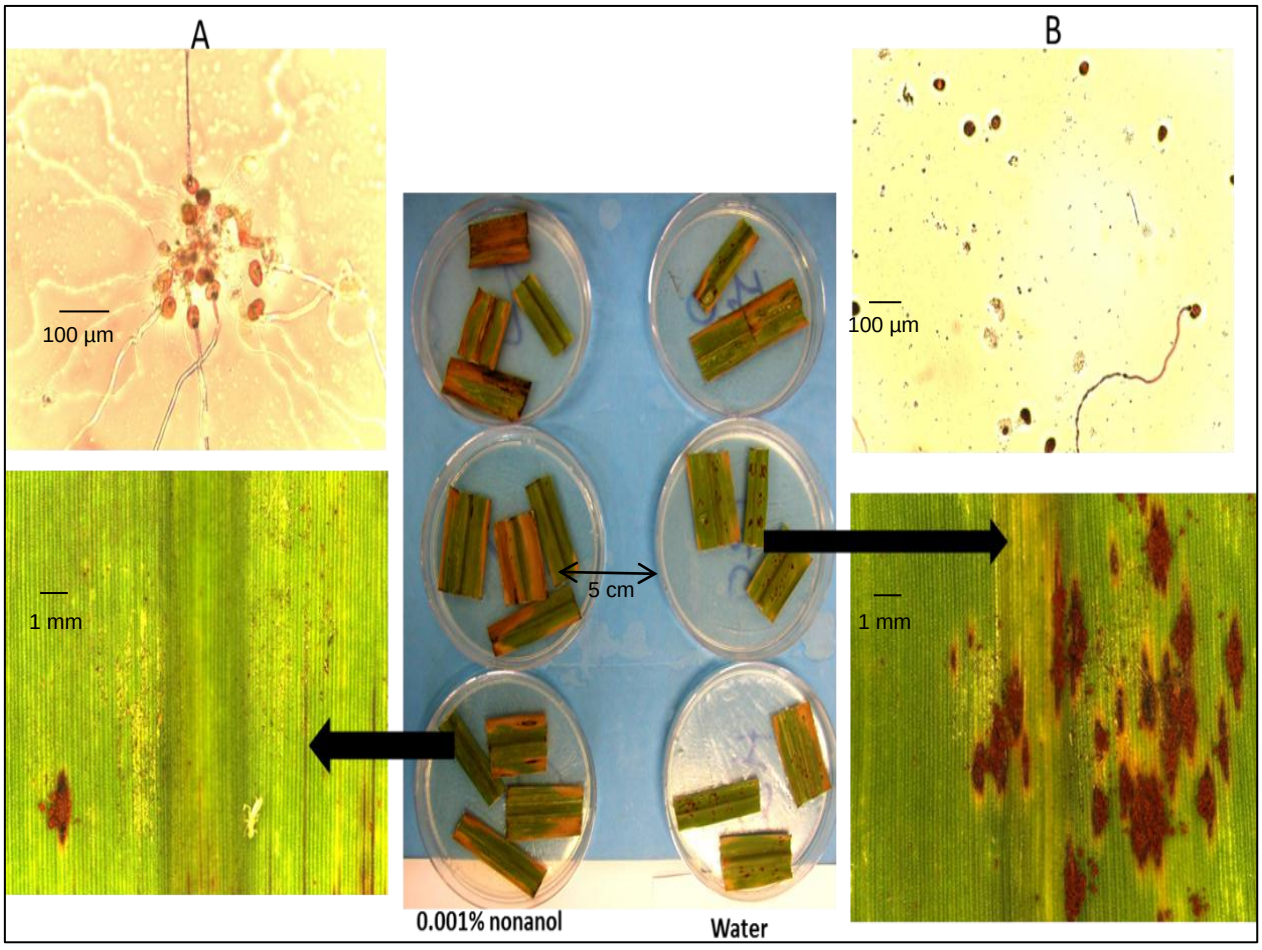

Fig 5.3.2: **Germination and pathogenicity tests on *P. melanocephala* in 0.001% (v/v) 1-nonanol compared to water.** Germination is greater (A) when spores are suspended in 1-nonanol, but reduced pathogenicity is observed when compared to water suspended spores (B), where germination might be low, but infection is more severe, resulting in heavy sporulation. Infection severity is shown by arrows pointing out infected leaves from each treatment at X40 magnification (Pictures by T. T. Mhora).

5.3.3 Visual analysis

The detached leaf assay was optimised using a variety of methods to ascertain a method which would work best for the samples used in this trial. Overall ratings for the DLA were calculated by multiplying the average lesion ratings by the average sporulation ratings for each leaf in a specific genotype. These overall ratings were then correlated to the pot trial phenotypic ratings using the Pearsons product moment correlation coefficient to determine the accuracy of the DLA relative to the pot trials. The method development demonstrated different strengths and weaknesses associated with each method adapted. The method of Braithwaite (2005) allowed easier handling of leaf material after inoculation as compared to the methods using agar as a support medium for the detached leaves (Fig 5.3.4 and 5.3.5). The methods using agar (Jackson *et al*., 2008; Twizeyimana *et al*., 2007) were more difficult to handle and were prone to contamination and secondary infections after continued handling when conducting chlorophyll fluorescence and chlorophyll content measurements (Fig 5.3.4). Contamination in the agar based techniques could have contributed to this method being less correlated to the overall ratings obtained through pot trials. The method of Braithwaite (2005) produced results which were highly correlated to those of corresponding pot trial whorl inoculation results, highlighting its superiority over the agar based methods (fig 5.3.6 and 5.3.7).

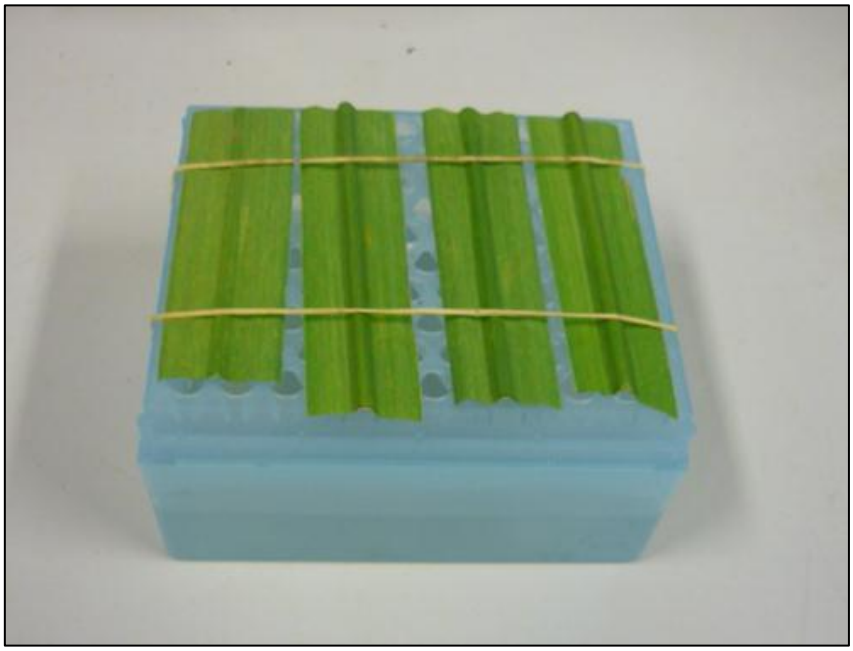

Fig 5.3.3a: **Tip box humidifier containing distilled water.** This apparatus was used for the overnight incubation of inoculated detached leaves as described by Braithwaite (2005) (Picture by T. T. Mhora).

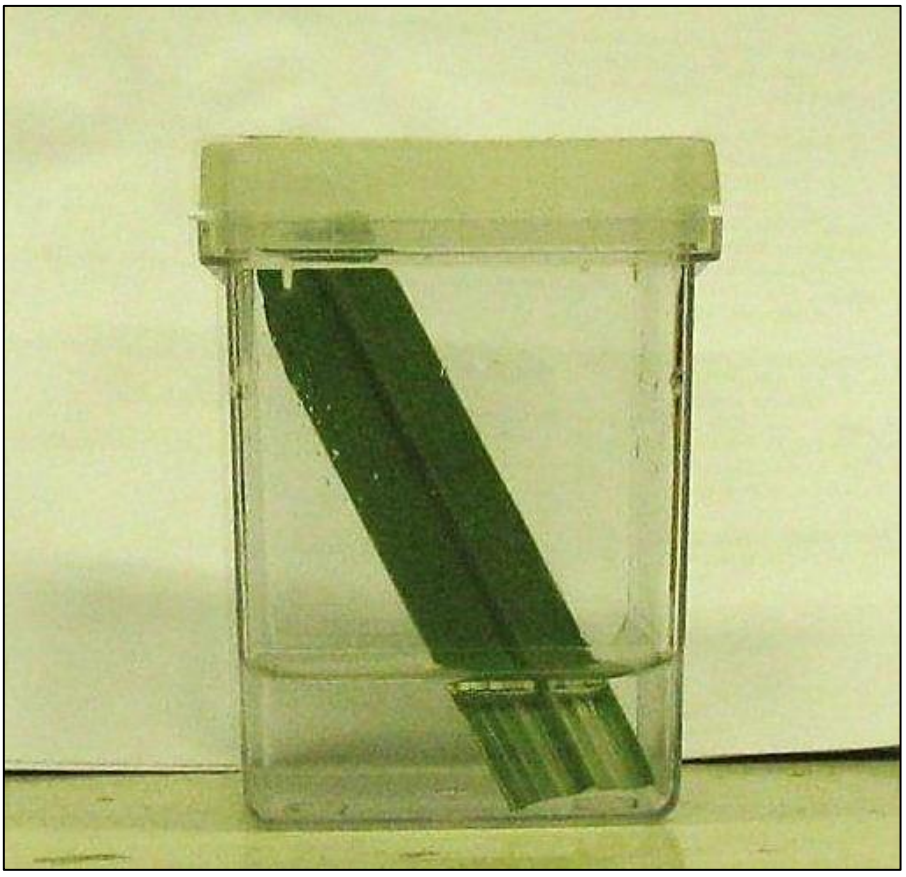

Fig 5.3.3b: A magenta® box containing an inoculated detached leaf. This process was conducted for all the genotypes screened using this method (Picture by T. T. Mhora).

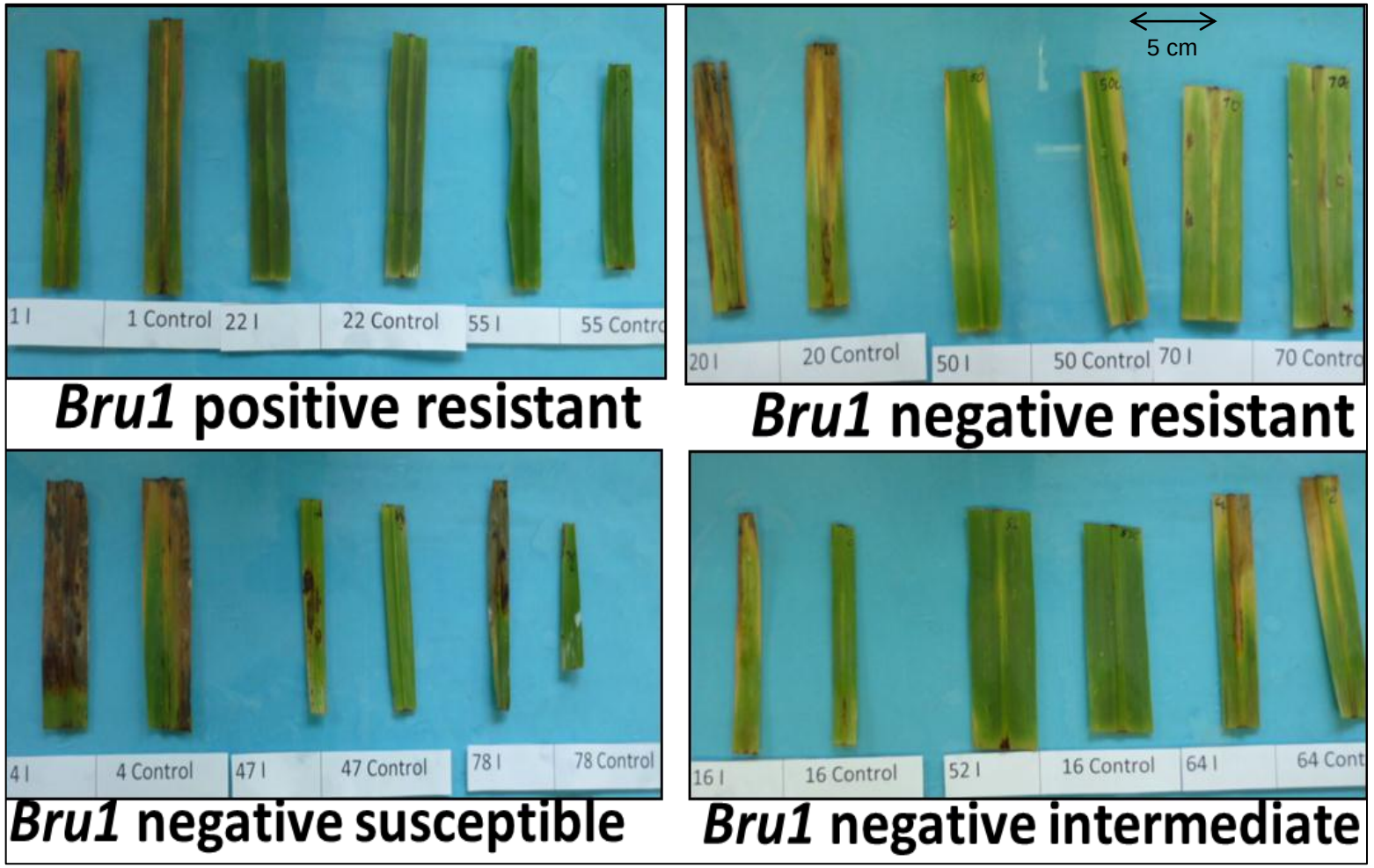

Fig 5.3.4: **Detached leaf assay (DLA) conducted in square plates containing 1% (w/v) agar using the method of Jackson *et al*., (2008).** The numbering on the labels represents the coding each genotype was given prior to planting out into pots. These codes were used to label the leaves used in the DLA, with "1I" representing a leaf from the clone coded one and inoculated, while "1 Control" represents a leaf from clone one without inoculum.

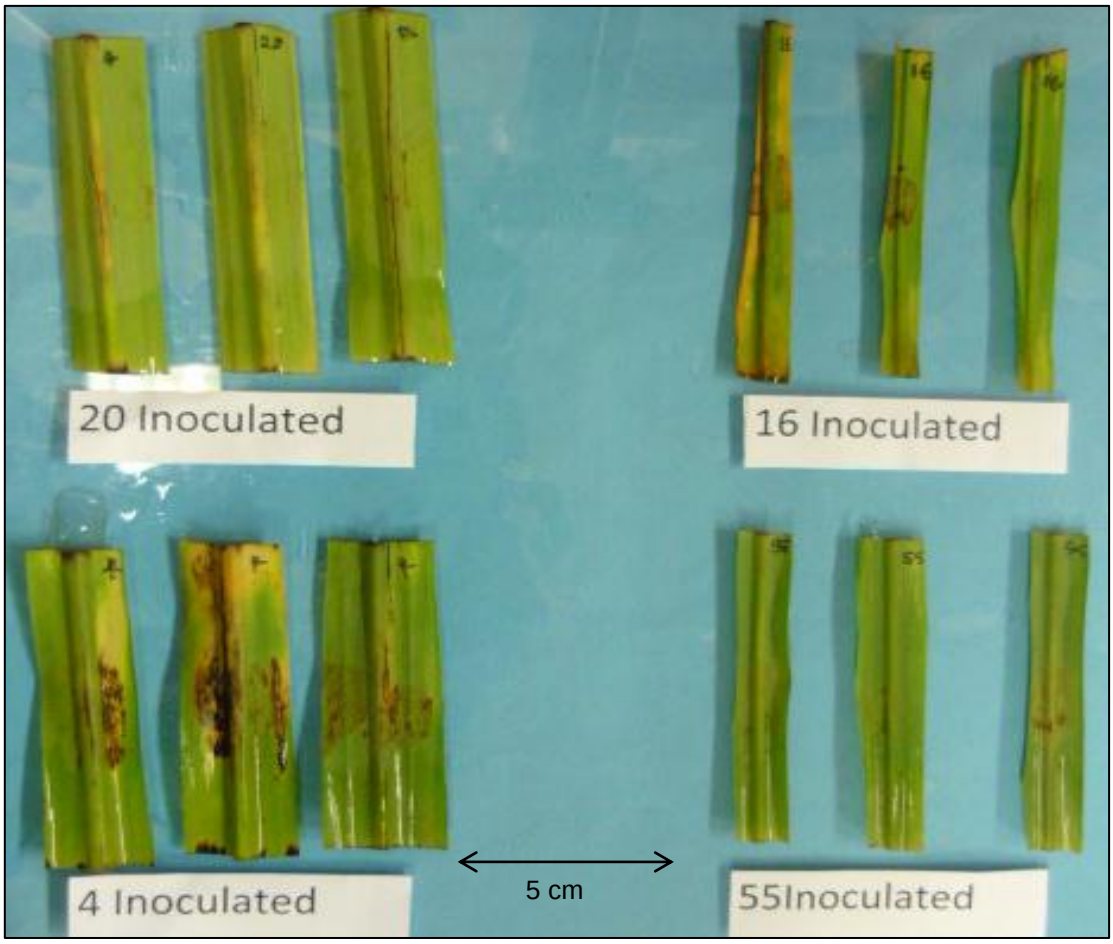

Fig 5.3.5: Detached leaves 15 days post-inoculation, using the method by Braithwaite (2005). *P. melanocephala* spore solution was applied centrally on the abaxial side of detached leaves. Selected genotypes were selected from the LD2 population based on their resistance to *P. melanocephala*. Genotype **93M0004 (20)** was in the resistant *Bru1* negative group, **93E0888 (16)** in the *Bru1* negative Intermediate group, **91M1610 (4)** in the *Bru1* negative susceptible group and **96E0524 (55)** in the resistant *Bru1* positive group.

The detached leaf assay showed it could discriminate between resistant and susceptible genotypes. Results from the selected LD2 genotypes could be related to the controls conducted using cultivars of known rust resistance (N12 and N29), validating the method's ability to classify varieties according to known rust ratings (Table 5.3.2.). The controls demonstrated a trend where lesion formation was significantly (P<0.001) higher in the susceptible cultivars as compared to the resistant ones (Tables 5.3.2 and 5.3.3). REML analysis, followed by the Holm-Sidak post hoc analysis was able to group the genotypes used in the assay. Groups using this method were identical to those based on previous pot trial and molecular characterisation.

Table 5.3.2: Detached leaf assay response of selected LD2 clones compared to two sugarcane varieties with a known rust response in a Petri dish contained experiment.

Sugarcane Clone	Overall resistance rating by whorl inoculation	Average number of lesions formed per leaf [e]	Average lesion rating	Leaves with lesion formation	Average Sporulation rating
N12 (Resistant)[a]		1a (±0.53)	1	3	0
N29 (Susceptible)[a]		17.67bc (±2.27)	4	9	3
***Bru1* –ve Resistant**					
93M0004	1.42	0a	0	0/9	-
96E0212	1.06	1.44a (±0.73)	1	4/9	0
97E0589	1.11	0.67a (±0.33)	1	4/9 [b]	0
***Bru1* +ve Resistant**					
89L0591	0.67	2a (±0.667)	1	6/9 [b]	1
93W0879	1.73	5.89ab (±1.39)	3	8/9 [b]	1
96E0524	1.26	2.78a (±0.98)	2	8/9	0
***Bru1* –ve Intermediate**					
93E0888	1.83	21.11cd (±0.26)	4	9	1
96E0391	3.30	4.33a (±0.60)	3	9	0
96W1340	3.46	3.44a (±1.33)	3	5/9[b]	1
***Bru1* –ve Susceptible**					
91M1610	9.17	31.44e (±3.88)	5	9	2.2
95W1865	6.14	18.36c (±3.34)	4	8/8 [c]	2
97W0568	7.22	29.83de (±3.73)	5	6/6 [d]	3

[a]- Released varieties with known rust ratings used as controls
[b]- Some inoculated leaves had severe chlorosis in inoculated areas, but no lesion formation
[c]- One leaf completely dried out due to severe necrosis. Severe necrosis also occurred around the inoculated parts of three leaves, but did not result in complete leaf death.
[d]- Three leaves completely dried out due to severe necrosis and no results could be taken from these
[e]- Means for all leaves from the replicated assays. Values in brackets are standard errors. Average mean figures followed by the same letter are not significantly different (95% confidence interval) by REML variance components analysis. Lesion numbers were used to calculate the lesion ratings.

Correlation coefficient analysis was conducted between the DLA methods used and the overall rust ratings obtained from whorl inoculation (Figures 5.3.6 and 5.3.7). Both methods showed their ability to reliably match the pot whorl inoculations with significant r^2 values, though the method of Braithwaite (2005) had a higher and more significant r^2 than the Petri dish DLA.

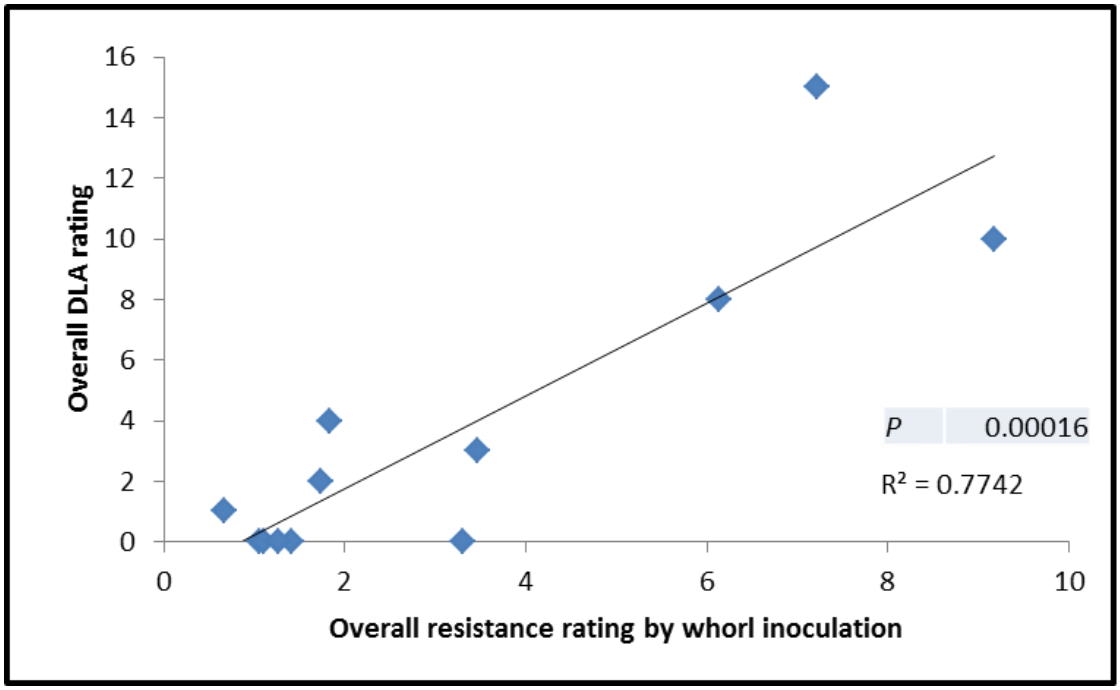

Fig 5.3.6: Correlation coefficient analysis between the overall ratings from the Petri Dish contained detached leaf assay and the Pot whorl inoculation trial (n= 12).

Sporulation also proved to be greater and more intense in the susceptible genotypes. Genotype 97W0568 showed to be highly susceptible, with over 50% of the lesions formed sporulating heavily (Table 5.3.2). Genotype 93W0879, in the *Bru1* positive resistant group showed to be the least resistant of these genotypes, displaying the most severe lesion symptoms along with slight sporulation on one of the nine inoculated leaves.

Results for the method adapted from Braithwaite (2005) gave similar inferences as the method of Twizeyimana *et al.* (2007). In the method of Braithwaite (2005) however, the plants exhibited restricted sporulation in the resistant genotypes, which was present in Twizeyimana *et al.*'s (2007) method, where slight sporulation was observed in the *Bru1* positive resistant genotypes 89L0591 and 93W0879. ANOVA was carried out on the data and the Holm-Sidak post-hoc test was used to group the genotypes. The groupings were able to distinguish among the resistant, intermediate and susceptible groups. The genotype 95W1865 was consistently rated as being susceptible but close to the intermediate group in both the visual rating systems used.

Table 5.3.3: Detached leaf assay response of selected LD2 genotypes using the method of Braithwaite (2005).

Sugarcane Clone	Overall resistance rating by whorl inoculation	Average lesion rating per genotype [a]	Average sporulation rating
***Bru1* –ve Resistant**			
93M0004	1.42	1.25ab (±0.25)	0
96E0212	1.06	1a (0)	0
97E0589	1.11	1.75ab (±0.48)	0
***Bru1* +ve Resistant**			
89L0591	0.67	1.25ab (±0.25)	0
93W0879	1.73	2.5ab (±0.5)	0
96E0524	1.26	1a (±0.41)	0
***Bru1* –ve Intermediate**			
93E0888	1.83	2.25ab (±0.48)	1.25
96E0391	3.30	2.5ab (±0.289)	1
96W1340	3.46	2.25ab (±0.48)	1
***Bru1* –ve Susceptible**			
91M1610	9.17	4.75c (±0.25)	2.5
95W1865	6.14	3bc (±0.41)	2
97W0568	7.22	4.5c (±0.29)	3

[a.] **The average lesion ratings of all leaves from each replicated assay.** Values in brackets are standard errors. Mean figures followed by the same letter are not significantly different (at a 95% confidence interval) by ANOVA.

Overall DLA ratings were calculated by multiplying the average lesion ratings of the detached leaves by the average sporulation ratings in the tables 5.3.2 and 5.3.3. The method of Twizeyimana *et al*. (2007) utilised smaller leaf pieces, and resulted in lesions being counted more easily. The method of Braithwaite (2005) however utilised larger leaves and the lesion ratings described in table 5.2.1 were used to descibe these lesions, as lesions tended to be too numerous to count in the susceptible genotypes.

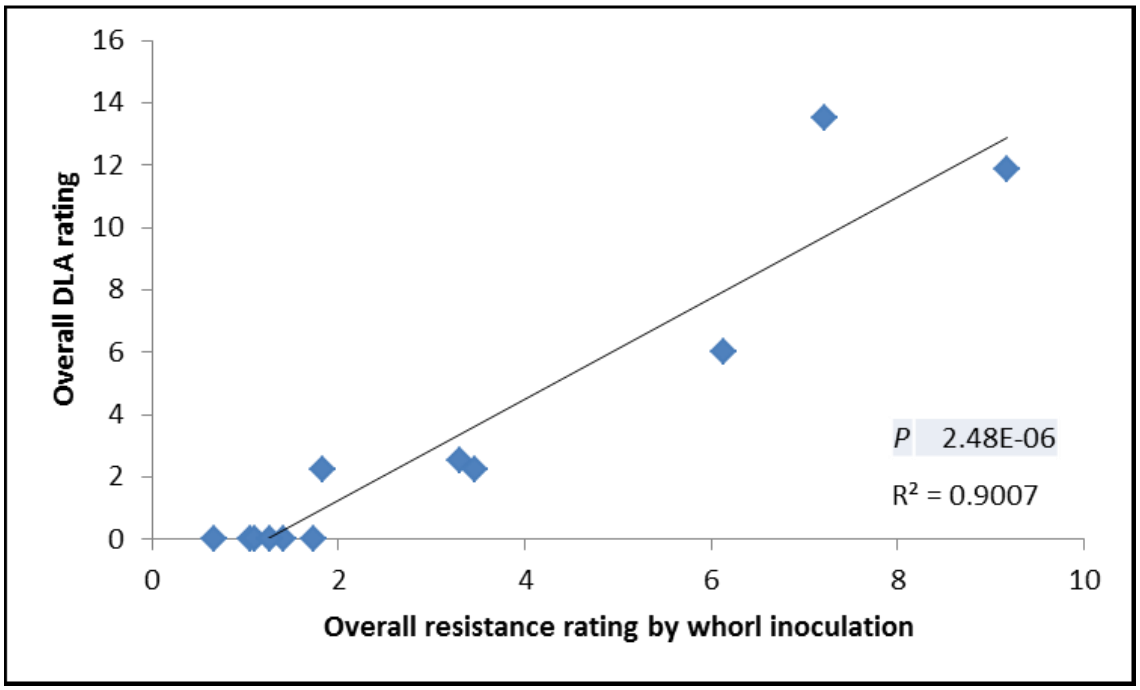

Fig 5.3.7: Correlation coefficient analysis between the overall ratings from the detached leaf assay adopted from Braithwaite (2005) and the Pot whorl inoculation trial (n=12).

5.3.4 Chlorophyll Analyses

ANOVA with repeated measures, followed by the Holm-Sidak post hoc analysis showed that there were significant differences in PI_{ABS} among the genotypes used. The performance index declined steadily over the eight days that measurements were taken (Fig 5.3.8). The decline in PI_{ABS} was more marked in the first three days, but stabilised, as was shown by a less steep gradient in the graphs (Figure 5.3.8). PI_{ABS} measurements for all but two controls in the "*Bru1* negative susceptible" and "*Bru1* negative intermediate" groups showed significant changes in all the resistance groups and treatments at day one compared to day zero. When the inoculated treatments were compared to the un-inoculated treatments, it was found that the *Bru1* negative resistant group was significantly different from the other groups at day one, with a positive difference in $\%\Delta PI_{ABS}$. The *Bru1* positive resistant group showed a highly significant difference (29%) at day four as compared to the other groups (9%-12%). This trend indicated that the *Bru1* positive genotypes can be identified by larger differences between the inoculated leaves and the un-inoculated leaves relative to the other resistance groups (Fig 5.3.9). The general trend showed that the method was able to discriminate between susceptible and resistant genotypes from day two, with the largest difference being observed

at day four (Fig 5.3.9). The *Bru1* negative groups also showed that despite inoculation with *P. melanocephala*, these genotypes maintain significantly lower ΔPI_{ABS} relative to the controls when compared to the other resistance groups. The differences in $\%\Delta PI_{ABS}$ between the inoculated and the un-inoculated regions of inoculated leaves showed that they could discriminate not only between *Bru1* groups, but also between the resistant and the susceptible genotypes in the differently grouped *Bru1* negative genotypes. The *Bru1* negative resistant and intermediate groups were shown to be significantly different from the susceptible group between days two and four.

The differences between $\%\Delta PI_{ABS}$ measurements in inoculated areas and the un-inoculated areas of inoculated leaves showed that there were significant differences between the resistant groups and the susceptible and intermediate groups on the first day (Fig 5.3.10). The $\%\Delta PI_{ABS}$ generally maintained this trend throughout the measurement period, though the *Bru1* negative resistant group significantly rose above the *Bru1* positive resistant group after day five. The sharp decrease observed in the ΔPI_{ABS} of the inoculated regions of the sugarcane leaves relative to the un-inoculated regions of the same leaf showed that the photosynthetic mechanism in the susceptible genotypes is more incapacitated by *P. melanocephala* infection relative to the other resistant groups.

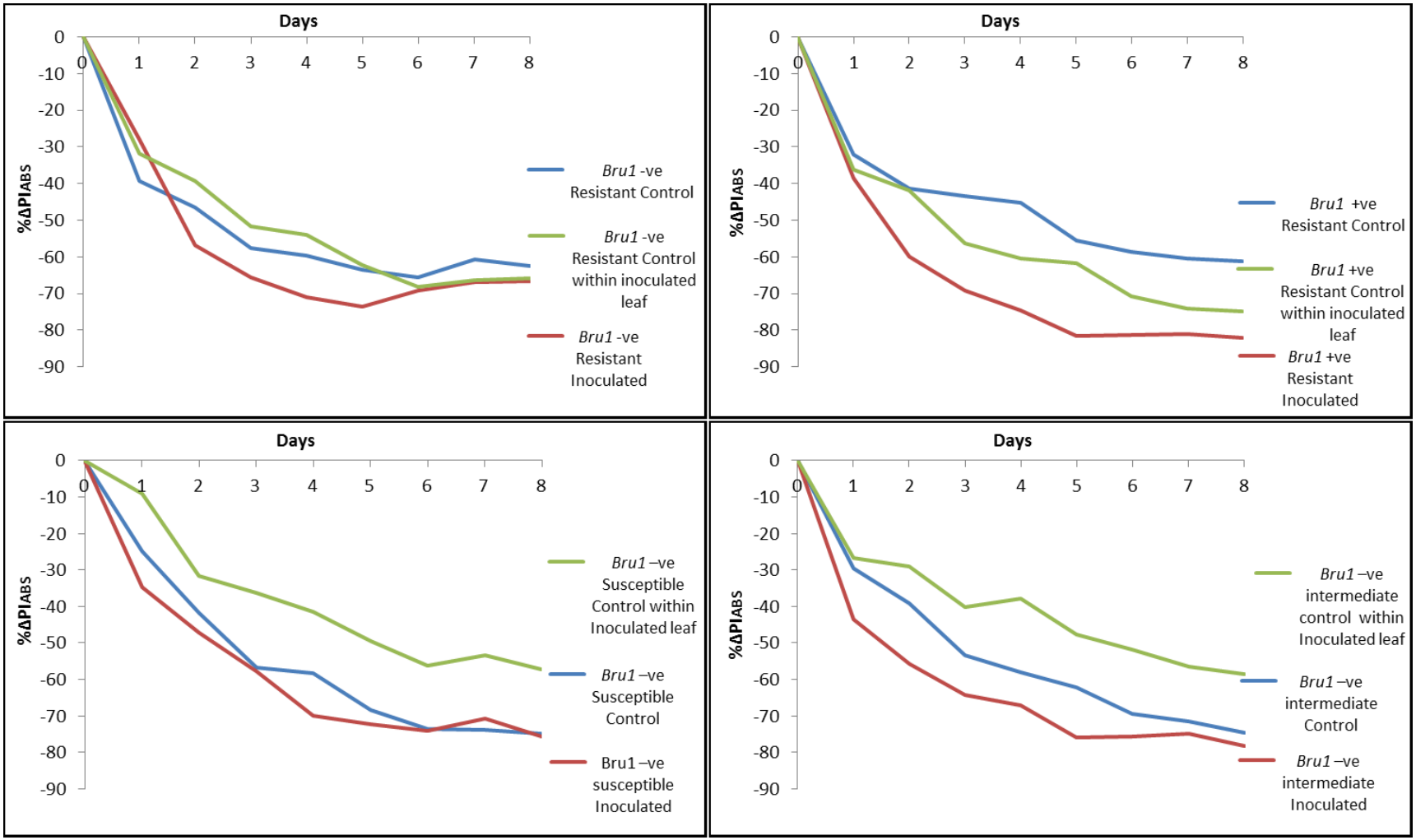

Fig 5.3.8: **Decrease in $\%\Delta PI_{ABS}$ for each treatment among the four classifications** assigned as *Bru1* negative resistant, *Bru1* positive, *Bru1* Susceptible and *Bru1* Intermediate. Chlorophyll fluorescence measurements were taken for each classification from leaves which had been inoculated; un-inoculated control and controls in which measurements were taken from the un-inoculated area of an inoculated leaf. PI_{ABS} was calculated using the JIP test (Strasser *et al*., 2000).

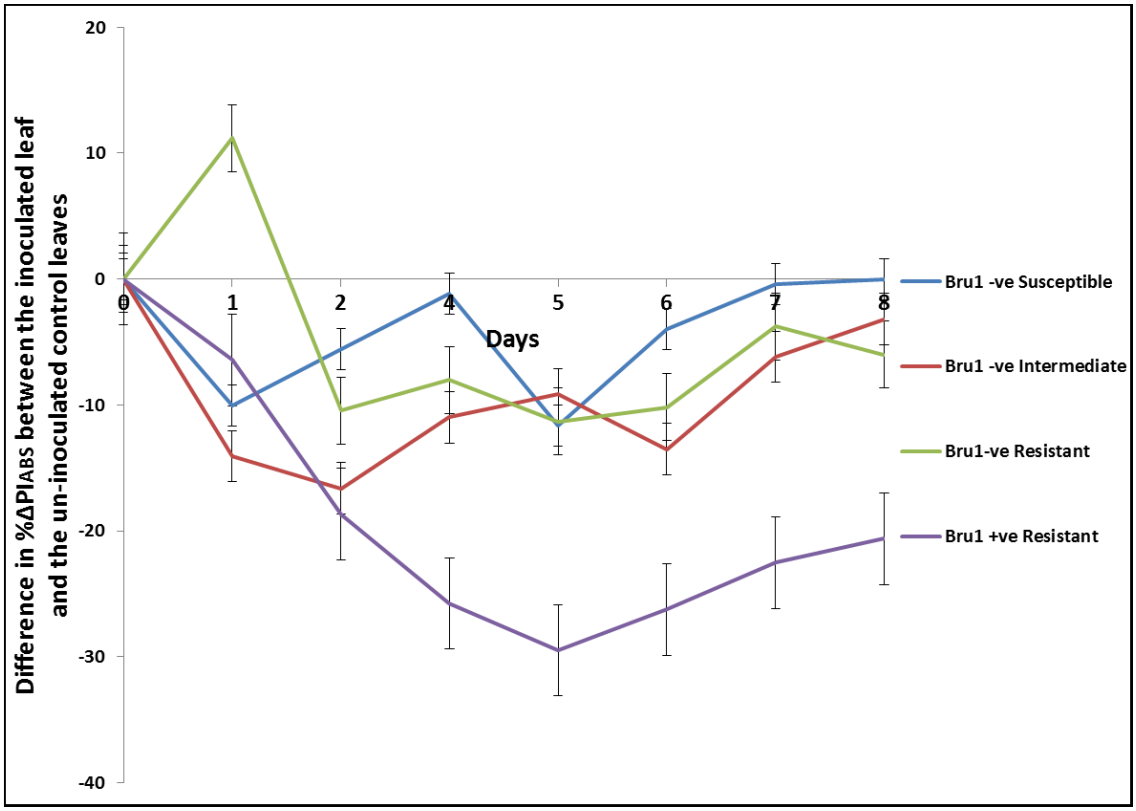

Fig 5.3.9: Differences in %Δ PI_{ABS} when leaves inoculated with *P. melanocephala* spores were compared to un-inoculated leaves over a period of eight days.

An apparent recovery of the inoculated areas to a point where the $\%\Delta PI_{ABS}$ of both the inoculated leaf and the un-inoculated area of the same leaf are almost equal over eight days is observed in the resistant leaves (Fig 5.3.10). This observation is however contrary in the *Bru1* negative susceptible and intermediate leaves as the difference in $\%\Delta PI_{ABS}$ does not decrease significantly. This observation suggests that there is a large compensatory effect characterised by an increase in PI_{ABS} in the uninoculated regions.

The susceptible and intermediate genotypes showed an overall increase in the %Δ PI_{ABS} when the un-inoculated regions of the leaf were compared to the un-inoculated control. This observation suggested that the leaf compensates for rust infection by increasing its photosynthetic capacity in the uninfected areas. The *Bru1* positive resistant group however showed a general decline, suggesting that there is no major difference between the two leaf areas being compared.

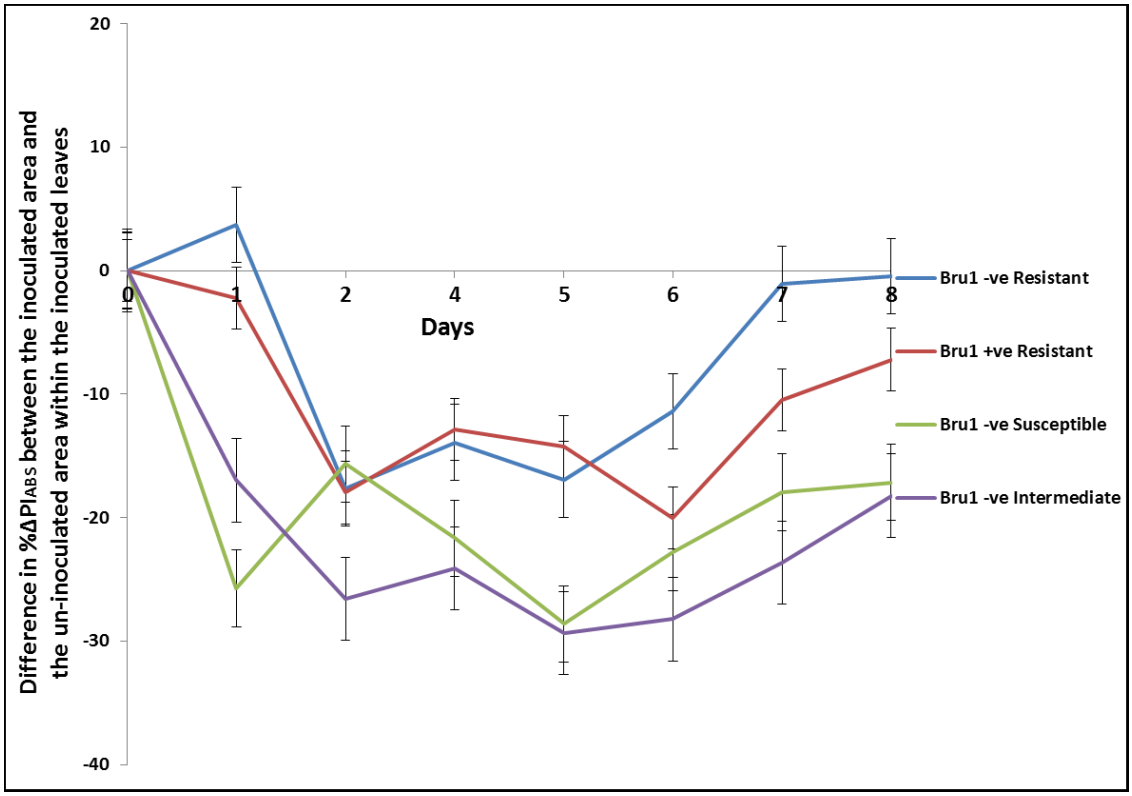

Fig 5.3.10: Differences in %Δ PI_{ABS} when the inoculated areas on leaves were compared to the un-inoculated area on the same leaf over a period of eight days.

The *Bru1* negative resistant genotypes however suggested that the genotypes employ a compensatory method upon infection, but after four days, begins to return to normal as the rust infection is controlled in the affected areas (Figure 5.3.11). This observation would explain why the susceptible and intermediate genotypes maintain their elevated PI_{ABS} in the un-infected areas, as the leaf does not recover or control the infection in the inoculated areas. The steady decline however in the $\%\Delta PI_{ABS}$ difference in the *Bru1* positive genotypes suggests that the diseased leaves are less photosynthetically capable than the healthier controls.

The susceptible and intermediate groups were significantly similar to each other from the second day, suggesting that this method of analysing the data would result in a screening protocol which would eliminate the susceptible and intermediate genotypes as they give similar responses. This characteristic of the screening technique is however not desired as the intermediate genotypes are required to contribute the possible quantitative resistance genes which confer partial resistance to rust. Quantitative genes are important sources of resistance as they are non-host specific, and when stacked, result in a more durable form of resistance (Parlevliet and van Ommeren 1988; Poland *et al*., 2011).

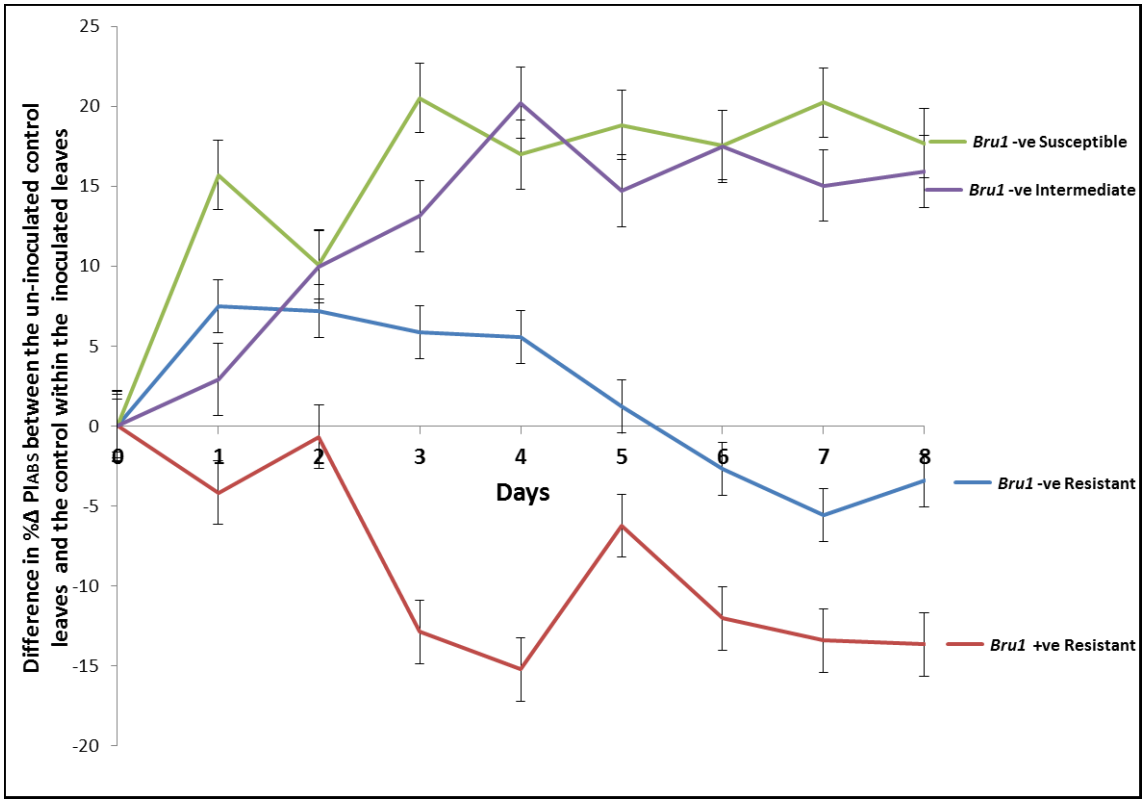

Fig 5.3.11: Differences in %Δ PI_{ABS} when the un-inoculated areas of inoculated leaves were compared to un-inoculated leaves over a period of eight days.

The SPAD index data showed a steady decline in chlorophyll content with time for all the groups (Fig 5.3.12). This observation was synonymous with that made in detached leaves, which tend to have a rapid decrease in chlorophyll content over time (Falqueto *et al*., 2010). There were distinct differences among the groups, as the *Bru1* negative susceptible group showed differences between day three and six; and in the un-inoculated control leaves, which had significantly (P<0.001) higher SPAD indices than both the inoculated leaf and the un-inoculated area within an inoculated leaf. The other groups' treatments were however not significantly different from each other at the different time points at which the measurements were taken. SPAD index measurements were expectedly different for each genotype, and as such, trends within treatments were considered in response to trends within a specific classification or group (Loh *et al*., 2002).

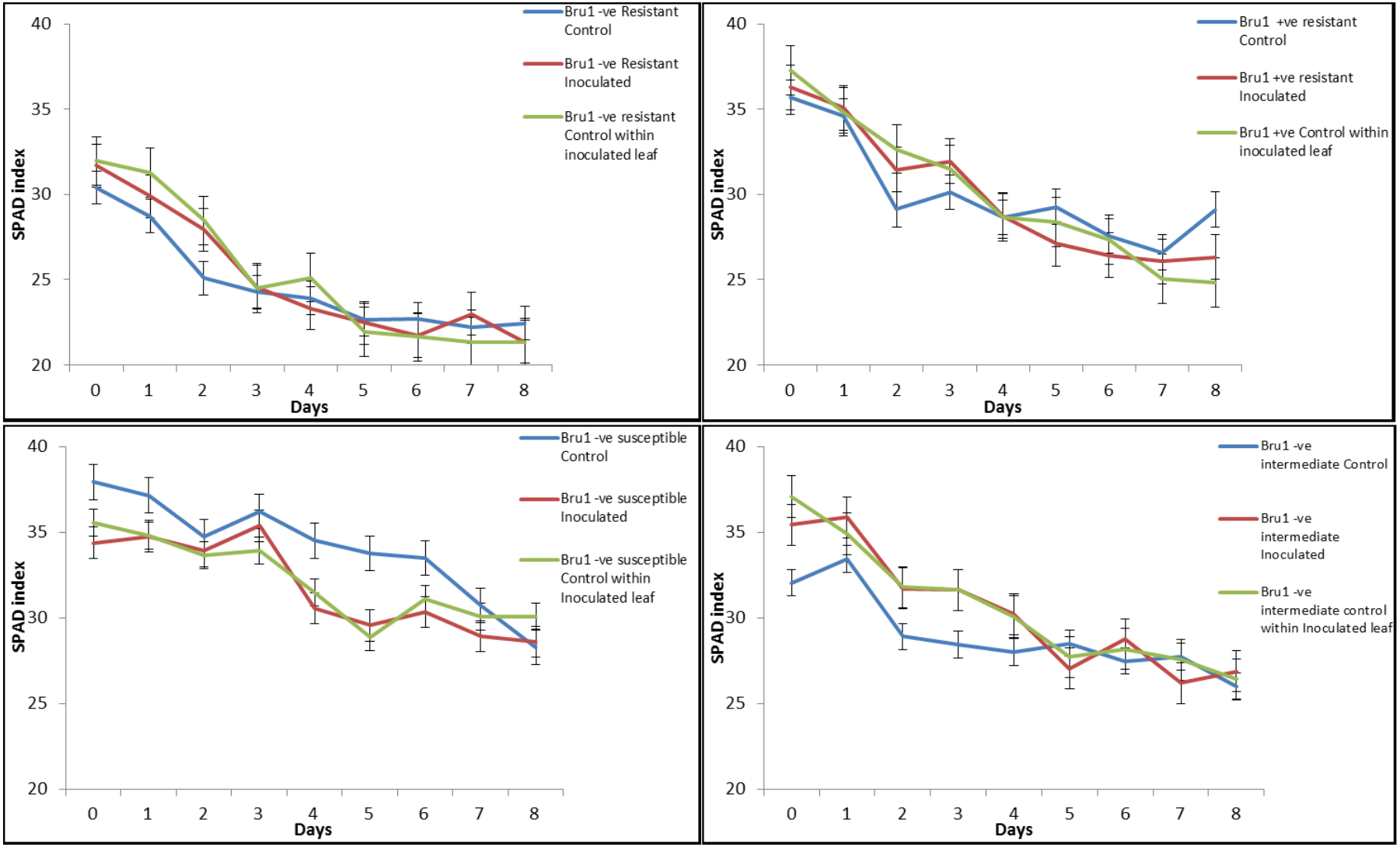

Fig 5.3.12: **Differences in the SPAD Indices among the four classifications** assigned as *Bru1* negative resistant, *Bru1* positive, *Bru1* Susceptible and *Bru1* Intermediate. SPAD measurements were taken for each classification from leaves which had been inoculated, un-inoculated control and controls in which SPAD index measurements were taken from the un-inoculated area of an inoculated leaf.

5.4 Discussion

The effect of 1-nonanol on the germination of *Puccinia melanocephala* spores showed increased germination at 0.001% (v/v), when compared to spores suspended in water. Increased concentrations of 1-nonanol (0.002- 0.005% v/v) decreased the germination of the spores, an effect which was previously reported by French and Gallimore (1972). Previous research had shown 1-nonanol to have its optimal stimulatory effects on spore germination at concentrations of between 0.025- 0.0025% (v/v), with concentrations on either side of this range being relatively lower (French and Gallimore, 1972). The use of 1-nonanol as a spore germination stimulator was found to negatively affect the pathogenicity of *P. melanocephala* spores in the DLA conducted in this research. The optimal concentration of 1-nonanol (0.001% (v/v)) when used to suspend the spores, resulted in noticeably reduced pathogenicity of the spores on the leaves of the rust susceptible sugarcane variety N29. The treated inoculum resulted in only 21% of the lesions observed, when water was used to suspend the rust spores. 1-nonanol treated spores also resulted in less severe sporulation occurring on the leaf surface. These results were contrary to those obtained by Braithwaite (2005) when conducting detached leaf assays using *P. kuehnii* spores. The results were also contrary to those obtained by French and Gallimore (1972) who found that pustule formation was greatly increased in the presence of nonanol in a dew chamber. It is possible that 1-nonanol could have inhibited the growth of *P. melanocephala*, an observation previously made on *Sclerotinia sclerotiorum* whose mycelial growth was completely inhibited by nonanol (Liu *et al*., 2008). The detached leaf assay was subsequently carried out using water suspended spores.

The preliminary methods used to conduct the detached leaf assay were those of Braithwaite (2005), Jackson *et al*. (2008) and Twizeyimana *et al*. (2007). The method of Twizeyimana *et al*. (2007) proved to be useful in providing a wider range of the visual data, as compared to the other methods. This method was also more reliable in terms of cost efficiency, as it facilitated high throughput and allowed large sample sizes to be processed at a minimal cost. This was in comparison to the other methods which required more costly inputs as the square plates and Magenta® plant culture boxes were more costly than the Petri dishes. The method however had the disadvantage of not permitting easy handling of the leaves for the measurement of chlorophyll fluorescence and SPAD indices. Excessive handling in the confined Petri dishes frequently resulted in contamination, which resulted in misdiagnosis and rust unrelated necrosis of the detached leaves. This observation was similar to one made by Asnaghi *et al*. (2001) on sugarcane leaves maintained on agar, also in a detached leaf assay for rust resistance. This method proved that it could be useful as a diagnostic tool and also as a method of propagating pure *P. melanocephala* inoculum.

The production of inoculum would assist with the screening process, which only normally takes place in peak rust periods to be conducted all year round (Purdy *et al*., 1983; Twizeyimana *et al*., 2007). This method could also greatly reduce the time taken to release varieties, as the screening step could then be conducted at any time of the year, compared to the yearly seasonal approach. The ability of the DLA to use only leaf material in rust screening would prevent the use of whole plants which can be subsequently used in bulking up and in other analyses conducted in the sugarcane breeding cycle. This method would also allow saving field space which would have otherwise been used for this analysis to be used for other breeding assessments, reducing cultivar release time by at least one year.

The method adopted from Jackson *et al*. (2008) proved to be the least efficient of the methods attempted for visual analysis, as contamination was prevalent. This was particularly so because agar is prone to the development of microorganisms, more so when the chlorophyll fluorescence and SPAD index measurements were attempted on the detached leaves (Asnaghi *et al*., 2001). This method was also less descriptive when compared to the method by Braithwaite (2005) and Twizeyimana *et al*. (2007) (Figures 5.3.4 and 5.3.5). The method was also more costly and labour intensive as square plates were washed and sterilised, in an attempt to lower costs.

The method of Braithwaite (2005) proved to be the most accommodating, in terms of allowing multiple analyses to be conducted on the leaves. Chlorophyll fluorescence and SPAD indices could be easily measured, as the leaves were maintained upright in water, whose level was at the base of the detached leaf. Visual analysis was simultaneously conducted on the leaves, producing results which discriminated among the 12 test genotypes, similarly to the adopted method used by Twizeyimana *et al*. (2007). Visual analysis of the DLA confirmed previous research, which demonstrated the method's ability to screen plants for disease resistance. This technique has also been found to be suitable for genetic studies on host resistance (Asnaghi *et al*., 2001; Jackson *et al*., 2008). Observations from these assays showed that even resistant varieties developed lesions in response to rust infection. These lesions were however much smaller, did not coalesce and did not sporulate, as was observed in the susceptible genotypes. This observation was consistent with that of Asnaghi *et al*. (2001), who found that sporulation did not occur when various inoculation methods were conducted on the rust resistant variety R570. Sporulation intensity proved to be an important factor when differentiating between susceptible and resistant genotypes. This parameter complemented lesion numbers, and allowed the assay to be more stringent, allowing greater differentiation and separation of the differently grouped genotypes.

The chlorophyll fluorescence data indicated a decrease in the photosynthetic activity of all the leaf treatments over time. These observations were supported by the gradually decreasing SPAD indices, which indicated a decrease in chlorophyll content over time in the detached leaves. Studies have shown that senescence of leaves, a frequent occurrence in detached leaf assays is characterised by discoloration which results in disassembly of the photosynthetic apparatus and subsequent photosynthetic capacity (Falqueto *et al.,* 2010). These results showed that when the leaves were detached, they had a significant decrease in PI_{ABS} in the first day for all treatments and classifications (Fig 5.3.8), as a result of the onset of induced senescence after detachment. The leaves stabilised after day one and the reduction in photosynthetic activity began to decrease in a more gradual manner than observed on the first day. This was thought to be due to the leaves acclimatising to the artificial environment.

Inoculated leaves showed a greater decrease in PI_{ABS} when compared to the other treatments (Figure 5.3.8). This decrease was consistent with findings on tomato plants infected with *Fusarium* (Wagner *et al*., 2006), and also to that observed in rust infected bean leaves, which showed reduced net photosynthetic rates (Lopes and Berger, 2001). The reduced PI_{ABS} in infected leaves was possibly due to *P. melanocephala* competing for nitrogen reserves, which would normally be utilised in the synthesis of ribulose 1, 5- biphosphate carboxylase (Rubisco) (Daley, 1995). The difference between the control and the inoculated leaves was much higher in the *Bru1* positive group, suggesting that the *Bru1* gene results in leaves accumulating resources, which would otherwise be used for photosynthetic activity and instead use them to prevent rust infection. The inoculated leaf showed significant changes on day four as compared to day 1 (day when leaves had acclimatised). These results were synonymous with the observations of Sotomayor *et al*. (1983), who revealed that infection hyphae form 36 hours after infection and that infection terminates before day seven in resistant plants. Non-host interaction studies using *P. melanocephala* showed that substomatal vesicles deteriorate, leading to a termination of the fungal infection in wheat (Sotomayor *et al*., 1983). Infection hyphae however, are subsequently formed in oat leaves, forming haustorial mother cells, after which the infection process is stopped (Sotomayor *et al*., 1983). The response in oats results in chlorosis and a golden fluorescence, synonymous to that observed in the *Bru1* positive genotypes. Chlorophyll fluorescence results also showed that chlorophyll *a* activity in these leaves was drastically reduced at this period (3 - 4 days), which is synonymous with defensive mechanisms terminating rust infection. The *Bru1* negative resistant classification had results which indicated that its defence mechanisms do not result in drastic changes in the photosynthetic activity of the plant, suggesting an alternative resistance mechanism (Rolfe and Scholes, 2010).

The difference between the inoculated and un-inoculated regions on the same leaf showed significant differences in the susceptible and intermediate groups, as compared to the resistant groups (Figure 5.3.10). These significantly (P< 0.001) greater differences in $\%\Delta PI_{ABS}$, compared to the resistant genotypes suggested that the susceptible leaves were compensating for the infection by elevating their photochemical activity in the healthier plant tissues. These results tallied with observations made by Rolfe and Scholes (2010), that high peak fluorescence was observed five days after inoculation. Healthy areas of infected leaves have been shown to exhibit high increases in fluorescence, which quenches rapidly, while infected areas show reduced fluorescence and little quenching, as shown in the leaves of *Nicotiana tabacum* exposed to the Tobacco Mosaic Virus. These observations were consistent for all the sugarcane groups infected with *P. melanocephala*. The resistant groups however began to show signs of recovery which were characterised by reduction in the differences in $\%\Delta PI_{ABS}$ until both regions were almost equal The recovery of the inoculated areas was observed to begin between the fourth and fifth day post inoculation a period synonymous with the termination or control of the pathogen in a resistant sugarcane-rust interaction (Sotomayor *et al*., 1983). This elimination or control would result in the release of resources which were being shared between the host and the pathogen, allowing cellular activity to resume normally (Daley, 1995). This is in contrast with the susceptible leaves where the infection persists and there is only a slight recovery of the leaves.

The almost similar differences in PI_{ABS} between the infected areas and uninfected areas within a sugarcane leaf and the difference between the infected and the uni-inoculated leaves in both resistant groups, suggested a localised response from the sugarcane leaves to *P. melanocephala* infection. These localised infection reactions suggested the presence of resistance mechanisms frequently associated with hypersensitive responses and the restriction of fungal growth (Bürling *et al*., 2011; Sotomayor *et al*., 1983). These responses did not significantly affect the chlorophyll content of the leaves, as compared to the susceptible group, where there was a decrease between day 3-5, which again coincided with the period where infection hyphae and mother haustorial cells are known to freely develop in susceptible varieties, resulting in necrosis and lesion formation of infected tissues (Sotomayor *et al*., 1983). This response was similar to previously obtained results, where *P. kuehnii* resulted in significant changes in chlorophyll content in sugarcane and also to results obtained when *Vigna sesquipedalis* was infected with *Colletotrichum lindemuthianum* and the chlorophyll content of the leaves began falling at the onset of necrosis (Lopez and Berger 2000; Zhao *et al*., 2011). On the contrary, studies on wheat showed no difference in the chlorophyll content of wheat genotypes which were resistant and susceptible to spot blotch (Rosyara *et al*., 2007).

The chlorophyll fluorescence results demonstrated the wide spectrum of host responses expected in the different resistant groups of genotypes, ranging from complete resistance to susceptibility. The observations from this study confirmed that the assessment of photosynthetic metabolism in different disease resistance groups expresses different patterns for various host- pathogen interactions, such as the rust- sugarcane interaction (Bürling *et al*., 2011; Lopes and Berger, 2000; Rolfe and Scholes, 2010). This information is however crucial, in that it can provide important information on host- pathogen interactions, such as the timing and location of pathogen development, while also providing an insight into the underlying mechanisms of host resistance or susceptibility. This technique can assist in developing methods of preventing damaging losses experienced due to rust. This can also be useful as it can show the presence of different forms of resistance mechanisms, aiding in crop improvement programmes as these traits can be added to already existing ones (Rolfe and Scholes, 2010).

The detached leaf assay confirmed its ability to differentiate between resistant and susceptible cultivars consistently, with results comparable to field assessments and resistance ratings assigned by the South African Sugarcane Research Institute (SASRI). The results obtained in this paper demonstrated that chlorophyll fluorescence could be used for more rapid screening for rust resistance when using DLA as compared to visual analysis. Visual analysis takes 12 days compared to 2-4 days using chlorophyll fluorescence and is also prone to error due to excessive leaf loss and damage occurring as a result of contamination. Larger sample sizes are however required to validate the chlorophyll fluorescence technique as previous authors have suggested that the method does not have uniform responses (Bürling *et al*., 2011; Lopes and Berger, 2000; Rolfe and Scholes, 2010).

Near infrared spectroscopy (NIRS) is a potential addition or improvement of this non-invasive method, as it can be used as a more rapid and descriptive alternative to visual analysis (Chaerle *et al*., 2007; Purcell *et al*., 2009). NIRS has shown a large scope in predicting disease ratings and deciphering host-pathogen interaction signatures (Purcell *et al*., 2009), of which there is also scope to use chlorophyll fluorescence, spectral and time-resolved chlorophyll fluorescence imaging as an auxiliary to this method (Berger *et al*., 2010; Chaerle *et al*., 2007). These methods have been shown to discriminate between resistant and susceptible varieties within two days of pathogen inoculation (Bürling *et al*., 2011; Chaerle *et al*., 2004). The DLA demonstrated its differentiating capabilities when known controls (N29 (susceptible) and N12 (resistant)) were sufficiently distinguished and appropriately matched to selected LD2 genotypes of corresponding rust rating using visual analysis (Table 4.3.2). This analysis supported the observations by Asnaghi *et al*. (2001), which described the potential of the DLA to differentiate between resistant and susceptible cultivars. The results also

confirmed the *Bru1* gene's ability to confer resistance to sugarcane genotypes even in a DLA, as shown by the genotypes (N12; 89L0591; 93W0879; 96E0524), which maintained their resistance to rust. Three *Bru1* negative genotypes (93M0004; 96E0212; 97E0589) also proved to be rust resistant. Further genetic analysis needs to be conducted to uncover these genotypes' source of resistance. This resulting knowledge could subsequently be used improve the resistance and durability of rust resistance in future varieties, by stacking these different types of resistance.

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5.6 Appendix

Table A1: Performance Indices (PI_{ABS}) of detached leaves from four sugarcane genotype classifications, each consisting of three genotypes.

[a]- Performance index means followed by the same letter within a banded row are not significantly different (at a 95% confidence interval) using the REML analysis and the Holm-Sidak post-hoc analysis.

Classification	Treatment	Performance index (PI_{ABS}) at 3000µmol m^{-2} s^{-1}								
		Day 0 [d]	Day 1	Day 2	Day 3	Day4	Day5	Day6	Day 7	Day 8
Bru1 negative resistant [a]	Control [b]	9.99 ± 0.7 k	6.07 ± 0.4 hi	5.36 ± 0.4 fghi	4.23 ± 0.2 defg	4.04 ± 0.3 cdef	3.65 ± 0.2 abcde	3.45 ± 0.2 abcde	3.92 ± 0.2 bcdef	3.76 ± 0.2 abcdef
	Inoculated [c]	8.12 ± 0.7 j	5.85 ± 0.6 ghi	3.51 ± 0.3 abcde	2.79 ± 0.3 abcd	2.36 ± 0.2 ab	2.14 ± 0.2 a	2.50 ± 0.2 abc	2.70 ± 0.3 abcd	2.71 ± 0.2 abcd
	Control within inoculated leaf	9.96 ± 0.5 k	6.79 ± 0.4 ij	6.05 ± 0.3 hi	4.80 ± 0.2 efgh	4.58 ± 0.3 efgh	3.76 ± 0.3 abcdef	3.17 ± 0.2 abcde	3.35 ± 0.3 abcde	3.41 ± 0.3 abcde
Bru1 positive resistant	Control	17.26 ± 2.2 g	11.73 ± 0.9 ef	10.14 ± 0.6 de	9.78 ± 0.7 cde	9.45 ± 0.8 bcde	7.70 ± 0.8 abcde	7.13 ± 0.9 abcde	6.81 ± 0.9 abcde	6.71 ± 0.9 abcde
	Inoculated	16.66 ± 3.1 fg	10.25 ± 0.7 de	6.68 ± 0.4 abcde	5.15 ± 0.4 abcd	4.21 ± 0.4 a	3.06 ± 0.2 a	3.13 ± 0.4 a	3.14 ± 0.3 a	2.98 ± 0.4 a
	Control within inoculated leaf	18.06 ± 2.8 g	11.52 ± 0.4 ef	10.48 ± 0.6 ef	7.91 ± 0.6 abcde	7.14 ± 0.7 abcde	6.93 ± 0.8 abcde	5.29 ± 0.6 abcd	4.70 ± 0.5 abc	4.55 ± 0.5 ab
Bru1 negative susceptible	Control	16.41 ± 1.6 j	12.34 ± 0.9 ghi	9.57 ± 0.8 efgh	7.10 ± 0.5 bcde	6.83 ± 0.4 abcde	5.19 ± 0.4 abcd	4.31 ± 0.5 abc	4.30 ± 0.5 abc	4.11 ± 0.5 abc
	Inoculated	13.56 ± 1.2 ij	8.84 ± 0.5 defg	7.15 ± 0.5 bcde	5.71 ± 0.6 abcd	4.06 ± 0.6 abc	3.75 ± 0.5 ab	3.51 ± 0.4 ab	3.97 ± 0.6 abc	3.28 ± 0.4 a
	Control within inoculated leaf	12.79 ± 1.1 hij	11.63 ± 0.9 fghi	8.75 ± 0.9 defg	8.16 ± 1.0 def	7.49 ± 1.2 cde	6.45 ± 0.9 abcde	5.61 ± 0.7 abcd	5.94 ± 0.8 abcde	5.46 ± 0.7 abcd
Bru1 negative intermediate resistant	Control	16.16 ± 1.7 i	11.39 ± 0.9 fg	9.85 ± 0.7 def	7.53 ± 0.6 abcdef	6.78 ± 0.6 abcde	6.09 ± 0.3 abcde	4.94 ± 0.2 abc	4.58 ± 0.3 abc	4.11 ± 0.3 ab
	Inoculated	15.35 ± 1.1 ghi	8.66 ± 0.9 cdef	6.81 ± 1.0 abcde	5.46 ± 0.7 abcd	5.03 ± 0.8 abc	3.71 ± 0.5 a	3.75 ± 0.6 a	3.86 ± 0.6 a	3.35 ± 0.5 a
	Control within inoculated leaf	15.91 ± 2.0 hi	11.68 ± 1.2 fgh	11.28 ± 1.1 fg	9.50 ± 1.0 def	9.89 ± 1.4 ef	8.34 ± 0.1 bcdef	7.65 ± 1.3 abcdef	6.90 ± 0.9 abcde	6.58 ± 1.3 abcde

[b]- Data are means ± standard error of means (*n*=24) for the control measurements for all the classifications

[c]- Data are means ± standard error of means (*n*=18) for the inoculated leaf and the control within inoculated leaf measurements for all the classifications

[d]- Day zero measurements were obtained after 1 hour of dark acclimatisation on the leaves before their respective treatments were conducted

Chapter 6

Analysis of Differential Gene Expression During the Early Stages of Rust Infection in Selected Genotypes of a Sugarcane Population

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Abstract

Resistant cultivars are the most effective way of controlling brown rust of sugarcane caused by *Puccinia melanocephala*. Molecular techniques can be used to identify undocumented rust resistance mechanisms, which can then be utilised in breeding strategies to develop cultivars with increased resistance and durability. Suppression Subtractive Hybridization (SSH) was used to identify differentially expressed genes among sugarcane genotypes with differing resistance profiles. Leaves were sampled from young plants 48h after inoculation with *P. melanocephala*. mRNA from each genotype was purified, quantified and pooled into its respective rust-resistance group. The mRNA was then converted into cDNA using the Clontech SMARTer™ cDNA synthesis kit. Two subtracted cDNA libraries were constructed using a PCR-Select™ cDNA subtraction kit. Library One consisted of Resistant *Bru1* positive subtracted from Resistant *Bru1* negative. Library Two consisted of pooled samples of Susceptible and Intermediate genotypes subtracted from Resistant *Bru1* negative genotypes. Subtracted cDNA libraries were transformed into *Escherichia coli*. cDNA was inserted into individual bacterial colonies using the pGEM®-T easy vector and was amplified by PCR. Successfully transformed clonescontained cDNA inserts ranging from 200-1300bp. DNA Sequence analysis of inserts and BLAST searches for both libraries revealed the presence of sequences homologous to various disease and drought stress related gene sequences and protein homologies. Library One revealed enrichment for protein-kinases, reticulon-like proteins and RNA recognition motif domains found in proteins involved in post-transcriptional gene expression processes. Sequences homologous to oxidative stress proteins responsible for triggering hypersensitive responses were also identified in Library One. Library Two showed the presence of sequences homologous to proteins responsible for transcriptional control and in the regulation of the salicylic acid pathway, which results in systemic acquired resistance to pathogens in plant systems. cDNA sequences homologous to leucine-rich repeat proteins were found to be in the majority of clones in both libraries. These

sequences contained a conserved domain homologous to a resistance protein in *Arabidopsis thaliana,* which was unusual due to the presence of stop codons, suggesting the transcription of pseudogenes. Pseudogenes have been associated with mRNA stabilisation of their parent genes and the translation of truncated proteins among other functions. These results gave an insight into the possible mechanisms of rust resistance contained in both libraries. They also showed the usefulness of SSH in providing information on gene expression in response to brown rust infection. This information could be used to develop markers for resistance, having potential application in the SASRI breeding and selection programme.

6.1 Introduction

Suppression Subtraction Hybridization (SSH) is a technique which allows the distinguishing of differentially expressed DNA between two closely related samples (Diatchenko *et al*., 1996; Rebrikov *et al*., 2004; Roelofs *et al*., 2007). This technique has been widely used in studies conducted to isolate differentially expressed genes in both compatible and incompatible plant-pathogen interactions (Shi *et al*., 2005; Zhang *et al*., 2003). SSH has been successfully used in elucidating gene expression in interactions between cereals and rust fungi. Examples of cereals that have been analysed in this manner include barley, wheat, and sugarcane (Neu *et al*., 2003; Watt 2003; Yan *et al*., 2009; Zhang *et al*., 2003)

Previous research on differential gene expression using SSH has been conducted on a variety of crops, including wheat challenged with *Puccinia triticina* and *P. recondita* interactions (Huang, 2008; Yan *et al*., 2009; Zhang *et al*., 2003). These results have indicated the expression of genes related to signal transduction, transcription regulation and hypersensitive response when wheat was inoculated with *P. recondita* (Yan *et al*., 2009). Such observations corresponded with previous research conducted using cDNA- AFLP, which indicated that response genes expressed when wheat was challenged with a pathogen included hydrolytic enzymes such as chitinase and β-1, 3-glucanase, antifungal proteins and enzymes involved in antimicrobial biosynthesis (Zhang *et al*., 2003).

Most SSH analyses are carried out in the initial stages of infection, as that is the period when defence mechanisms are initiated. The analysis of gene expression is critical at both the pre- and post-haustorial stages of fungal infection, as it explains the mode and degree of resistance in the genotypes concerned (Neu *et al*., 2003). Studies have shown that the best form of defence is those that occur in the very early stages of infection and result in the absence of leaf damage, which is a major cause of yield losses. These early defence mechanisms have been demonstrated by the use of

SSH and have revealed the presence of nonspecific wall- associated defence responses and the production of antifungal saponins (Neu *et al*., 2003). Most of these mechanisms have been found to be mainly non-host specific, presenting a potential source of durable resistance (Neu *et al*., 2003).

Brown rust of sugarcane is a disease whose severity can be effectively controlled by the use of resistant cultivars. The high complexity of the sugarcane genome has however resulted in only two major rust resistance genes having been identified in recent times (Le Cunff *et al*., 2008; Raboin *et al*., 2006). The complexity of the genome makes it difficult to use modern techniques to identify alternative sources of resistance. This has left the production of rust resistant varieties to traditional methods which are not only land and labour intensive, but take a very long time before results can be achieved. Utilisation of SSH in analysing rust-sugarcane interactions and identifying alternative sources of resistance will greatly aid in producing more resistant sugarcane varieties. This can be achieved by identifying markers for these genes and using this technology to stack the genes, in turn producing varieties that have strong and durable resistance (Parlevliet and van Ommeren, 1988). These developments can subsequently reduce the pressure on the major rust resistance gene (*Bru1*), and cater for the possibility of this gene being overcome by *Puccinia melanocephala,* the causal agent of sugarcane rust.

6.2 Materials and Methods

6.2.1 Experimental design

In order to conduct subtractions which would elucidate the resistance mechanisms present in the LD2 population, four resistance groups of sugarcane were created. These groups were created based on the genotypes AFLP data, resistance ratings after whorl inoculation and *Bru1* status as shown in Chapter Four. Twelve genotypes in total were selected, three to each group as shown below:

- *Bru1* positive resistant (89L0591; 93W0879; 96E0524)
- *Bru1* negative resistant (93M0004; 96E0212; 97E0589)
- *Bru1* negative intermediate (93E0888; 96E0391; 96W1340)
- *Bru1* negative susceptible (91M1610; 95W1865; 97W0568)

From these groups, the following subtractions were conducted:

1- Resistant *Bru1* positive taken away from resistant *Bru1* negative

2- Susceptible *Bru1* negative + Intermediate *Bru1* negative taken away from resistant *Bru1* negative.

6.2.2 Host material preparation

Five single budded setts of each of the selected genotypes were grown in 20 litre pots containing potting soil and fertigated weekly for one month. The plants were inoculated using the whorl inoculation technique with *P. melanocephala* inoculum as described in previous chapters. Plants were then dark acclimatised overnight at 20°C in a temperature controlled glasshouse before being exposed to normal light and temperature conditions. Leaves from the inoculated plants were harvested 48 hours post-inoculation. The leaves were immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

6.2.3 Poly (A) mRNA purification

Total RNA was extracted from the inoculated leaves using the Qiagen RNeasy® Plant Mini kit (Hilden, Germany) according to manufacturer's instructions. The RNA was quantified using the NanoDrop® ND-1000 Spectrophotometer (Thermo Fischer Scientific Inc.). The RNA was then assessed for its quality by denaturing gel electrophoresis. One microgram of RNA was mixed with 10 µl deionised formamide, 4.5 µl formaldehyde, 5 µl 5X 3-(N-morpholino) propanesulphonic acid (MOPS) buffer, 4 µl 6X orange loading dye (Fermentas) and made up to 40 µl with diethylpyrocarbonate (DEPC) treated water. The resulting samples were heated to 65°C and chilled on ice before being fractionated via gel electrophoresis on a 1.2% (w/v) agarose gel in 1% sterile 1XTBE at 60V for one hour. After electrophoresis, the gel was stained in 1mg/ml ethidium bromide for 30 minutes before viewing under the Alpha Imager™ 2200 (Alpha Innotech Corporation) at 302nm.

The total RNA was bulked according to its respective resistance group and aliquoted into 200 µg containing volumes. Poly (A) mRNA was then isolated from one 200 µg total RNA aliquot using the

Macherey-Nagel (MN) NucleoTrap® midi kit (Düren, Germany) according to manufacturer's instructions.

6.2.4 Construction of subtraction cDNA libraries

The mRNA was reverse transcribed into cDNA using the SMARTer™ cDNA synthesis kit. cDNA SSH was then conducted using the PCR-Select™ cDNA subtraction kit (Clontech Laboratories Inc.) according to the manufacturer's instructions. Optimisation of the LD-PCR stage was carried out and 24 cycles were used to synthesise the cDNA.

6.2.5 Cloning of subtracted cDNA libraries

Subtracted cDNA products from the SSH process were inserted into the pGEM®T vector's T-overhangs using the Promega pGEM® T-easy kit (Promega Corpration). Insertion of the SSH products was conducted by adding cDNA (2µl) from each subtracted library to pGEM® T-easy ligation mix and incubating it overnight at 4°C to allow the cDNA to ligate to the vector. A negative control was made using 2 µl of water instead of cDNA.

The overnight ligated mix (2 µl) was mixed with 50 µl *Escherichia coli* electro-competent cells and incubated on ice in a micropulser cuvette for ten minutes. Electroporation was carried out in the BioRad Micropulser™ (Bio-Rad Laboratories) using the bacterial Ec2 time and settings, after which 450µl of Luria Bertani (LB) broth was immediately added to the cuvette, mixed and incubated in a shaking incubator for one hour at 37°C. Successfully transformed colonies were isolated using blue-white colony selection on LB agar plates containing 100mg/l ampicillin, 20mg/l X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and 0.5mM isopropyl-beta-D-thiogalactopyranoside (IPTG). Plates were incubated overnight at 37°C and inspected for transformed colonies containing inserted cDNA (white), which were then picked using a sterile toothpick and stabbed into a Nunclon™ 96 well cell culture plate (Kamstrupvej, Denmark) containing LB media, to maintain individual colonies. A PCR reaction was then conducted using DNA from each selected colony as a template using SP6 and T7 primers as described in the pGEM® T-easy manual. This was done to analyse the insert size and to ensure that only a single cDNA insert had been transferred to each well in the Nunclon™ 96 well cell culture plate (Kamstrupvej, Denmark). The PCR products were loaded into an agarose gel, electrophoresed and observed as previously described (Section 6.2.3).

6.2.6 cDNA sequence analysis

Plasmid extraction and purification was conducted using the MN Nucleic Acid and Protein Purification Kit (Düren, Germany). Overnight cultures of the clones transformed in section 6.2.5 were prepared for plasmid extraction by inoculating 1 ml LB broth in cell culture plates provided in the MN Nucleic Acid Purification kit. Plasmid extraction was conducted as instructed by the manufacturer. Inserts were amplified using the colony PCR protocol and purified using the Celtic PCR clean up kit (Cape Town, South Africa). PCR product (40 ng) was then sequenced with the BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems) using the stepped elongation time cycle sequencing protocol (St_eP) (Platt *et al*., 2007). Sequencing was conducted in both directions using the SP6 and T7 primers in an Applied Biosystems 3500 Genetic analyser.

6.2.7 Sequence data analysis

The cDNA sequences obtained were trimmed to remove vector sequences and edited for ambiguities using Geneious (version 5.3.4). cDNA sequences were compared to the NCBI GenBank database using the BLASTN (nucleotide database), BLASTX (nr protein databases) and TBLASTX (translated nucleotide database) algorithms. BLASTX is a program which translates the cDNA into all possible reading frames and compares the resultant protein sequence against a protein sequence database (Altschul *et al*., 1997; Anderson and Brass, 1998).

6.3 Results

Plants inoculated with *P. melanocephala* and retained to confirm the success of whorl inoculation displayed symptoms of rust ranging from chlorotic flecks to severe lesion formation and necrosis in the third week post inoculation. The observations from these inoculations were all expected as the inoculated genotypes maintained their previously observed resistance ratings.

6.3.1 cDNA insert analysis

mRNA was successfully purified and converted into cDNA (fig 6.3.1). cDNA inserted using the pGEM®-T easy vector was amplified using a crude PCR on transformed cells from each individual colony. Gel electrophoresis of the PCR amplicons showed successfully transformed clones with cDNA inserts ranging from 200-1300bp in both libraries (Fig 6.3.2). Some of the sequences had similar sizes (kb) when observed on the agarose gel, suggesting that these similar sized cDNA amplicons could be the same. This assumption was confirmed as sequencing showed identical or near identical nucleotide sequences in most of the same size cDNA fragments. The higher levels of certain amplicons suggested elevated expression levels.

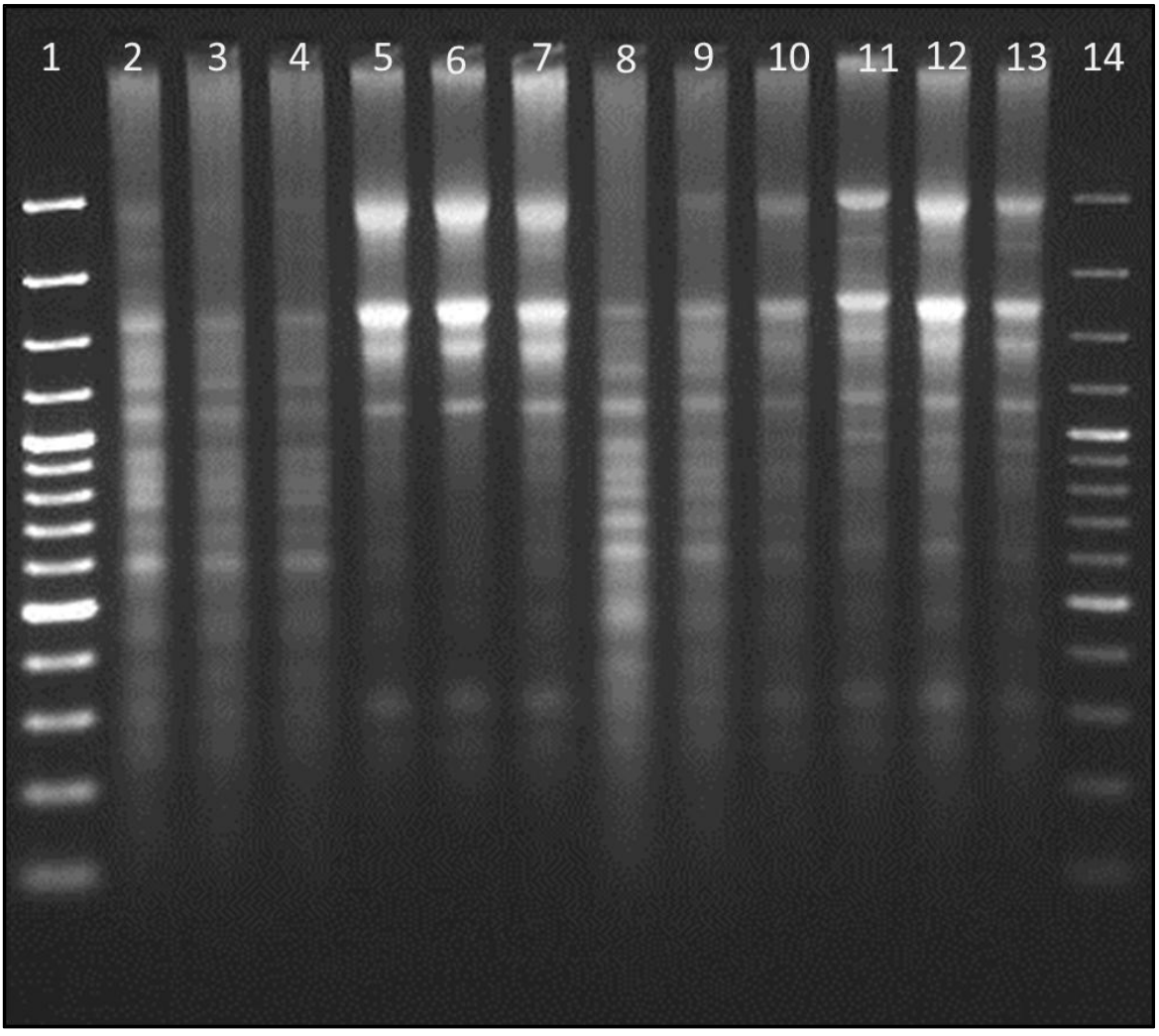

Fig 6.3.1: Gel electrophoresis image showing the extracted total RNA from the 12 genotypes analysed. Lanes 1 and 14 are a 500 bp DNA ladder.

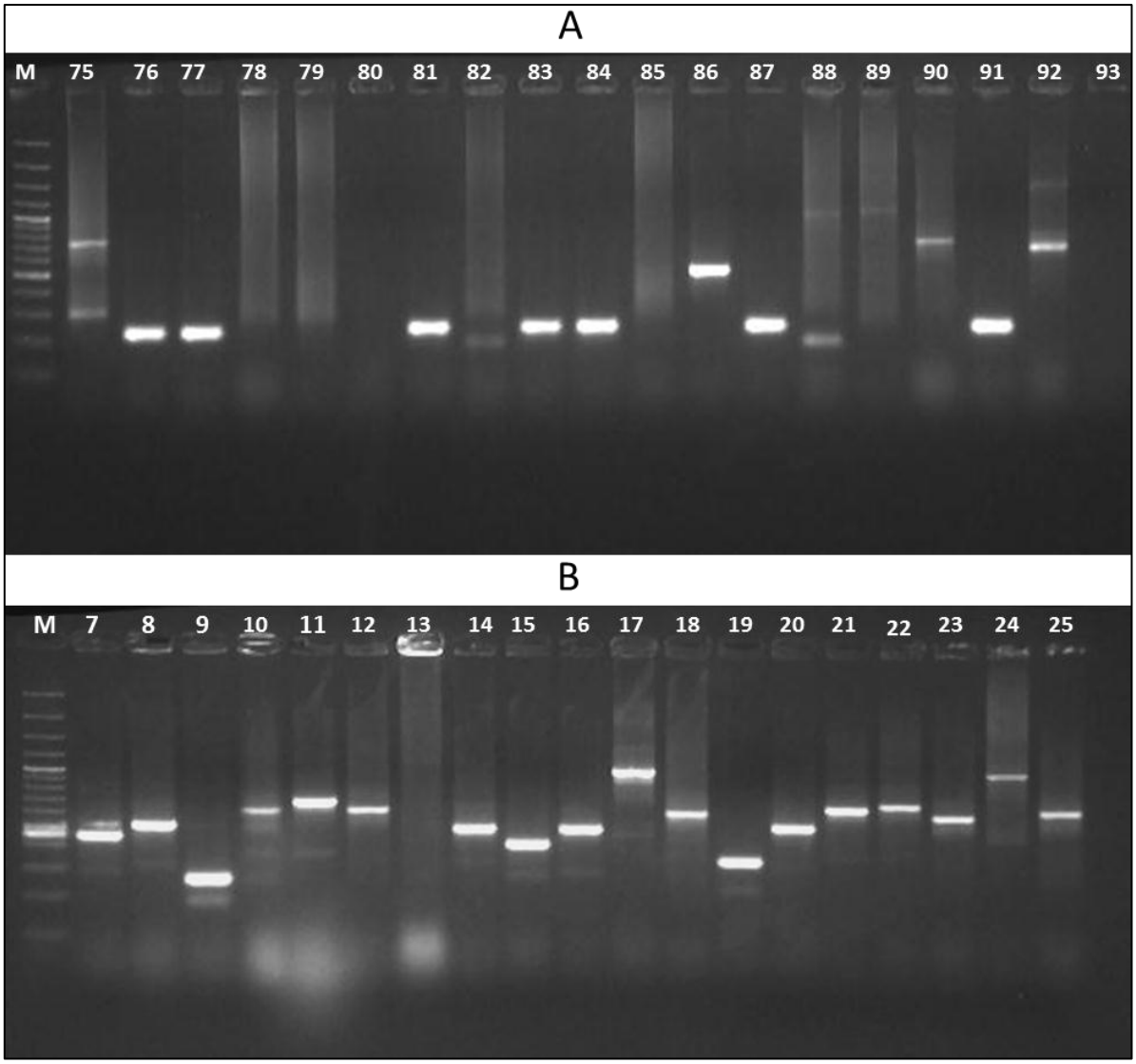

Fig 6.3.2: **Gel electrophoresis image showing the size of cDNA inserts after transforming into *E. coli* competent cells.** Image **A** shows subtraction library one, in which Resistant *Bru1* positive genotypes were taken away from resistant *Bru1* negative genotypes. Image **B** shows subtraction library two, in which Susceptible *Bru1* negative + Intermediate *Bru1* negative genotypes were taken away from resistant *Bru1* negative genotypes.

6.3.2 Putative identification of differentially expressed genes

6.3.2.1 BLASTN analysis of Library One and Library Two

BLASTN analysis was conducted on the sequences obtained from the cDNA subtraction libraries and the sequences grouped according to the description given to each respective BLASTN search (Tables 6.3.1 and 6.3.2). Approximately 18.6% of the sequences from subtraction Library One and 24% from Library Two were best described as *Saccharum* hybrid cultivar and subsequently grouped in the

category Sugarcane EST. The sequences in both libraries were found to be mainly homologous to GeneBank sequences obtained from drought and disease stressed plants. Drought stressed sequences made up 36% of these sequences for both groups, making up the highest contributions to both libraries.

Library One had an additional group described as “maturation associated sequences”. One of the sequences (clone ssh4_49_A07) had a conserved domain which was homologous to a pentatricopeptide repeat (PPR), which is about 35 amino acids long and found in up to 18 copies in some proteins. This family appears to be greatly expanded in plants and may be involved in RNA stabilisation. This domain is also known to occur in crp1 that is involved in RNA processing (Marchler-Bauer *et al*., 2011).

Two clones from Library One (ssh4_1_A01) and Library Two (ssh2_34.1_B05) were found to be homologous to the same nucleotide sequence (gb|EY275297.1). The sequence obtained from the BLASTN database contained a conserved domain that was homologous to the multiprotein bridging factor 1 (MBF1) which has been shown to make direct contact with the TATA-box binding protein. This domain is part of a transcriptional regulator.

Table 6.3.1a: Putative sequence homology from subtraction Library One as identified using BLASTN analysis

Clone No.	Accession No.	Source of matching sequence	e-value ([a] BLASTN)	No. of identical clones
Sugarcane EST				
ssh4_10 _B02	gb|CA093276.1|	*Saccharum hybrid* cultivar cDNA clone	0	5
ssh4_13_2 _E02	gb|CA099097.1|	*Saccharum hybrid* cultivar cDNA clone	0	2
ssh4_2 _B01	gb|CA224795.1|	*Saccharum hybrid* cultivar cDNA clone	0	1
ssh4_23 _G03	gb|CA165921.1|	*Saccharum hybrid* cultivar cDNA clone	1E-61	1
ssh4_7 _G01	gb|CA128742.1|	*Saccharum hybrid* cultivar cDNA clone	2E-54	1
ssh4_2_B01	gb|CA267461.1|	*Saccharum* hybrid cultivar cDNA clone	8E-46	1
ssh4_10_B02	gb|CA236611.1|	*Saccharum officinarum* hybrid cultivar (mixed) cDNA clone	8E-44	1
ssh4_3.3 _C01	gb|CA130550.1|	*Saccharum hybrid* cultivar cDNA clone	1E-43	2
Water stress associated sequences				
ssh4_17 _A03	gb|DN743506.1|	Full Length Mixed Tuber *Solanum tuberosum* cDNA clone	0	1
ssh4_54 _F07	gb|HO065556.1|	SSH library *Cicer arietinum* cDNA	0	1
ssh4_27 _C04	gb|HO066630.1|	SSH library *Cicer arietinum* cDNA.	0	4
ssh4_18 _B03	emb|AJ770915.1|	*Populus euphratica* leaf cDNA clone	0	9
ssh4_14 _F02	gb|GH734256.1|	*Camellia sinensis* var. *assamica* cDNA clone	0	1
ssh4_13 _E03	gb|GH734756.1|	*Camellia sinensis* var. *assamica* cDNA clone	0	5
ssh4_37 _E06	gb|GH738521.1|	*Camellia sinensis var. assamica* cDNA clone	0	1
ssh4_10_B04	gb|GT969111.1|	*Camellia sinensis* var. *assamica* cDNA clone	0	1
ssh4_25 _2_B02	gb|GH734512.1|	*Camellia sinensis* var. *assamica* cDNA clone	9E-98	1
ssh4_4 _D01	gb|GW348341.1|	*Cajanus cajan* cDNA library.	3E-95	1
ssh4_10_B03	emb|CU223764.1|	*Populus* EST from leave	2E-73	1
ssh4_50 _3_C03	gb|HO066319.1|	SSH library *Cicer arietinum* cDNA	2E-20	1

[a.] Putative identification indicates the best match to a sequence in the NCBI EST nucleotide database.

Table 6.3.1b: Putative sequence homology from subtraction Library One as identified using BLASTN analysis

Clone No.	Accession No.	Description	e-value (BLASTN)	No. of identical clones
Disease associated sequences				
ssh4_25_3 _C02	emb|FN822468.2|	*Fagus sylvatica* cDNA clone.	1E-67	2
ssh4_50_2 _B03	gb|JG293147.1|	SSH library *Cicer arietinum* cDNA.	2E-32	1
ssh4_1 _A02	gb|JG292508.1|	SSH library *Cicer arietinum* cDNA.	8E-25	1
ssh4_50_4 _D03	emb|FN822322.2|	*Fagus sylvatica* cDNA clone.	2E-18	1
Maturation associated sequences				
ssh4_49 _A07	gb|FL302236.1|	*Zea mays* cDNA clone.	0	1
ssh4_14_F02	gb|FL814960.1|	*Panicum virgatum* cDNA clone.	0	1
ssh4_15 _6_F01	gb|GO897975.1|	SSH library *Cucumis sativus* cDNA.	0.000001	1
Miscellaneous				
ssh4_5 _E01	gb|EY275296.1|	*Hordeum vulgare* subsp. vulgare cDNA	0	1
ssh4_1_A01	gb|EY275297.1|	*Hordeum vulgare* subsp. vulgare cDNA	0	1
ssh4_12 _D02	gb|EY275298.1|	*Hordeum vulgare* subsp. vulgare cDNA	0	9
ssh4_17_A03	gb|EY275299.1|	*Hordeum vulgare* subsp. vulgare cDNA	0	2
ssh4_12_D02	gb|GO313654.1|	SSH library of *Pettunia* x hybrida cDNA.	0	2
ssh4_19 _C03	gb|HS389735.1|	*Camellia sinensis* var. *assamica* cDNA clone.	0	2
ssh4_25_A04	gb|DT629876.1|	SSH library *Pinus taeda* cDNA clone.	2E-150	1
ssh4_26 _B04	gb|DV103654.1|	SSH library *Solanum lycopersicum* cDNA clone.	2E-105	3
ssh4_9 _A03	gb|GW355581.1|	*Cajanus cajan* cDNA library.	6E-80	1
ssh4_9_A02	gb|JK036689.1|	*Chlorophytum borivilianum* cDNA.	3E-54	1
ssh4_15_1_A01	gb|GH738599.1|	*Camellia sinensis* var. *assamica* cDNA clone.	7E-33	1
ssh4_25_1_A02	gb|EX465249.1|	SSH library *Nicotiana tabacum* cDNA clone.	2E-19	1
ssh4_15_2_B01	gb|JK492888.1|	*Chlorophytum borivilianum* cDNA.	4E-17	1

Table 6.3.2: Putative sequence homology from subtraction Library Two as identified using BLASTN analysis

Clone No.	Accession No.	Description	e-value (BLASTN)	No. of identical clones
Sugarcane EST				
ssh2 34 _B05	gb|CA274231.1|	*Saccharum* hybrid cultivar (mixed) cDNA clone	2E-159	1
ssh2 85 _E11	gb|CA227446.1|	*Saccharum officinarum* cDNA	2E-138	1
shh2 22 _D03	gb|CA065892.1|	*Saccharum* hybrid cultivar cDNA clone	4E-82	1
shh2 21 _D03	gb|CA218499.1|	*Saccharum* hybrid cultivar cDNA clone	4E-82	4
ssh2 34.2 _B05	gb|CA261641.1|	*Saccharum* hybrid cultivar cDNA clone	1E-14	1
Water stress associated sequences				
ssh2 38 _F05	gb|GT969092.1|	*Camellia sinensis* var. *assamica* cDNA clone	0	1
ssh2 19 _C03	gb|GH734640.1|	*Camellia sinensis* var. *assamica* cDNA clone	4E-163	1
ssh2 35 _C05	gb|FE840563.1|	SSH library *Saccharum* hybrid cultivar cDNA clone	3E-162	3
ssh2 19.2 _C03	gb|HO066472.1|	SSH library *Cicer arietinum* cDNA	2E-157	3
ssh2 46 _F06	gb|GH709899.1|	*Camellia sinensis* var. *assamica* cDNA clone	4E-100	2
ssh2 86 _F11	gb|CF093497.1|	*Helianthus argophyllus* cDNA clone	7E-99	1
ssh2 65 _A09	gb|FF682691.1|	SSH library *Saccharum* hybrid cultivar cDNA clone	0.000006	1
Disease associated sequences				
ssh2 60 _D08	gb|GW787732.1|	SSH library *Camellia sinensis* cDNA clone	0	1
ssh2 59 _C08	gb|JG293012.1|	SSH library *Cicer arietinum* cDNA	3E-162	1
ssh2 14.2 _F02	gb|JG292871.1|	SSH library *Cicer arietinum* cDNA	1E-44	1
Miscellaneous				
[a] ssh2_34.1_B05	gb|EY275297.1|	*Hordeum vulgare* subsp. *vulgare* cDNA	0	1
ssh2_43_C06	gb|FG745096.1|	*Anolis carolinensis* cDNA library	0	3
ssh2_84_D11	gb|EY275299.1|	*Hordeum vulgare* subsp. *vulgare* cDNA	9E-142	1
ssh2_36 _D05	dbj|FS750605.1|	*Bombyx mori* cDNA clone	6E-138	1
ssh2_16_H02	gb|DV103804.1|	*Solanum lycopersicum* cDNA clone	9E-97	2
ssh2 45_E06	gb|DT630116.1|	SSH library *Pinus taeda* cDNA clone	9E-69	1
ssh2 62_F06	gb|DT630556.1|	SSH library *Pinus taeda* cDNA clone	1E-67	1

Table 6.3.3a: Putative differentially expressed protein sequences from subtraction Library One as identified using BLASTX analysis

Clone No.	Accession No.	Source of matching sequence	No. of identical clones	e-value ([b] BLASTX)	Putative protein name/ functions
Transcriptional regulation					
ssh4_10_B02	XP_002463097.1	SORBIDRAFT_02g037800 [*Sorghum bicolor*]	1	1E-87	RRM (RNA recognition motif)
ssh4_50_3_C03	CAD59768.1	*Cicer arietinum*	1	9E-15	Putative Reverse transcriptase
ssh4_25_2_B02	ACL54966.1	*Cichorium intybus*	1	1E-08	MEF2-like/Type II (transcriptional regulator)
Oxidative stress					
ssh4_15.1_A01	Q59296.1	*Campylobacter jejuni*	1	8E-57	Catalase
ssh4_15_B01	XM_002271718.1	*Vitis vinifera*	1	1E-53	Cytochrome C oxidase subunit II
ssh4_2_B01	ref|XP_002457769.1	SORBIDRAFT_03g013290 [*Sorghum bicolor*]	1	2E-36	[a] Dihydrolipoamide dehydrogenase
ssh4_9_A02	emb|CAD42938.2|	*Taiwanofungus camphoratus*	3	1E-07	Manganese superoxide dismutase
Protein processing/ signal transduction					
ssh4_10_B01	XP_002438444.1	SORBIDRAFT_10g019720 [*Sorghum bicolor]*	11	3E-119	Reticulon like protein
ssh4_15.6_F01	XP_002521235.1	*Ricinus communis*	1	4E-30	Catalytic domain of Protein Tyrosine Kinases
ssh4_9_A02	CAD98809.1	*Spodoptera frugiperda*	1	0.0001	Cell signalling Proteins
Disease resistance					
ssh4_50.4_D03	XP_003541206.1	*Glycine max*	1	2E-48	Putative senescence-associated protein
Metabolic proteins					
ssh4_22_F03	DN743506.1	*Solanum tuberosum*	1	5E-102	S-adenosyl-L-homocysteine hydrolase
ssh4_14_F02	ref|XP_002466662.1	SORBIDRAFT_01g011810 [*Sorghum bicolor]*	1	2E-84	Magnesium transporter MRS2-A
ssh4_25.1_A02	P49037.1	*Solanum lycopersicum*	2	8E-57	GT1 family of glycosyltransferases

a. This sequence was found to have a conserved domain homologous to the pyridine nucleotide-disulphide oxidoreductase, dimerisation domain; This family includes both class I and class II oxidoreductases and also NADH oxidases and peroxidases (an Expect value of 1.11E-47).

b. Putative identification indicates the best match to a sequence in the NCBI protein database.

Table 6.3.3b: Putative differentially expressed protein sequences from subtraction Library One as identified using BLASTX analysis

Clone No.	Accession No.	Source of matching sequence	No. of identical clones	e-value (BLASTX)	Putative Protein function/ similarities
Miscellaneous					
ssh4_23_G03	XP_002457020.1	*Sorghum bicolor*	2	1E-38	hypothetical protein
ssh4_49_A07	NP_001143372.1	*Zea mays*	1	4E-11	hypothetical protein
ssh4_12_D02	emb|CCD21012.1	*Trypanosoma vivax*	41	3E-17	[a] Leucine-rich repeat protein
ssh4_1_A01	XP_002455846.1|	*Sorghum bicolor*	4	0.000001	hypothetical protein

[a.] This Leucine-rich repeat was found to contain a conserved domain homologous to the resistance protein found in *Arabidopsis thaliana* resistance to *Pseudomonas syringae* (an Expect value of 2.12E-15)

6.3.2.2 BLASTX analysis of Library One and Library Two

The NCBI BLASTX program was used to determine the putative functions of the cDNA sequences in both subtraction libraries (Tables 6.3.3 and 6.3.4). Both libraries contained a large number of clones (55% for Library One and 33% for Library Two) homologous to protein sequences coding for a leucine-rich repeat protein (LRRP). This LRRP contained a conserved domain homologous to that of the resistance protein found in *Arabidopsis thaliana* and conferring resistance to *Pseudomonas syringae* (Expect value of 2.12E-15) (Marchler-Bauer *et al*., 2011). There was a difference between libraries, as Library One contained sequences homologous to oxidative stress related proteins such as manganese superoxide dismutase, catalase and cytochrome oxidase. Another major difference between both libraries was that of the disease resistance associated proteins found in Library One (putative senescence-associated protein found in *Glycine max*). This sequence is unique as it is the difference between the two sets of brown rust resistant sugarcane groups, but is absent in Library Two, suggesting that it is also found in the susceptible/ intermediate groups. Library Two contained more sequences homologous to disease resistance proteins, showing the difference between the *Bru1* negative resistant and susceptible/intermediate groups (Table 6.3.4). Library One was also unique in that it possessed metabolic proteins, one of which was the magnesium transporter MRS2-A, which is responsible for the transport of magnesium ions which are necessary for the action of dihydrolipoamide dehydrogenase, which was among the sequence homologies for antioxidant enzymes found in Library One. A large number of sequences in both libraries were also found to be homologous to proteins responsible for transcriptional regulation, suggesting the potential source of alternative resistance between the groups of sugarcane analysis.

Table 6.3.4: Putative differentially expressed protein sequences from subtraction Library Two as identified using BLASTX analysis

Clone name	Accession No.	Source of matching sequence	No. of identical clones	e-value (BLASTX)	Putative Protein/ Function
Transcriptional regulation					
ssh2_27 _C04	XP_003225516.1	LOC100561123 [*Anolis carolinensis*]	1	6E-19	Exonuclease/endonuclease/phosphatase domains
ssh2_34 _B05	XP_002466155.1	*Zea mays*	1	2E-12	Global transcription factor
ssh2_34.2 _B05	Q8H6B1.1	*Zea mays*	1	2E-11	Chromatin transcription complex subunit SPT16
Disease resistance					
ssh2_34 _B04	XP_003589199.1	*Medicago truncatula*	1	0	Disease resistance protein
ssh2_20 _D03	NP_001152232.1	*Zea mays*	4	5E-29	Auxin repressed; Dormancy/ auxin associated protein
ssh2_65 _A09	XP_002169446.1	*Hydra magnipapillata*	1	1E-26	Baseplate hub subunit and tail lysozyme.
ssh2_26_B04	XP_001019401.1	*Tetrahymena thermophila*	2	0.74	Ras family protein. Small GTPase Rab11D
ssh2_14.2 _F02	O23758.1	*Cicer arietinum*	1	8E-69	Non-specific lipid-transfer protein
Protein Processing					
ssh2_85 _E11	XP_002458745.1	SORBIDRAFT_03g039500 [*Sorghum bicolor*]	1	1E-74	Ribonuclease catalytic domain
Miscellaneous					
ssh2_14_ F02	XP_001627639.1	*Nematostella vectensis* >gb|EDO35539.1|	6	2E-16	Hypothetical protein found at transcript level
ssh2_46 _F06	XP_001767263.1	*Physcomitrella patens* subsp. Patens	1	1E-12	Hypothetical protein found at transcript level
ssh2_6 _F01	YP_173374.1	*Nicotiana tabacum*	1	9E-37	Hypothetical protein similar to mitochondrial protein
ssh2_38 _F05	CCD21012.1	*Trypanosoma vivax* Y486	11	3E-17	[a] Leucine-rich repeat protein
ssh2_48 _H06	XP_002455846.1	SORBIDRAFT_03g026090 [*Sorghum bicolor*]	1	0.000001	Hypothetical protein

[a.] This Leucine-rich repeat was found to contain a conserved domain homologous to the resistance protein found in Arabidopsis thaliana resistance to Pseudomonas syringae (an Expect value of 2.12E-15)

Library One contained 17% sequence homologies which were associated with protein processing and signal transduction. The majority of these sequences were those homologous to the reticulon like protein which is associated with the rough endoplasmic reticulum and is responsible for protein modification. The remaining sequence homologies in this group were similar to proteins responsible for cell signalling, an expected result as the subtractions were conducted 48 hours post inoculation.

6.4 Discussion

The identification of disease resistance mechanisms is important in the breeding process of sugarcane as it would allow the production of commercial sugarcane varieties with good disease resistance among other desirable agronomic properties (Le Cunff *et al*., 2008). Suppression subtraction hybridization (SSH) is such a method which could allow the rapid identification of genes responsible for these characteristics (Degenhardt *et al*., 2005; Huang, 2008). The identification of these genes could allow for the development of markers which can then be used in a breeding program for the selection of sugarcane genotypes with desirable characteristics. Such markers have the advantage in that they could significantly reduce the production time of a sugarcane cultivar which is currently 12-15 years. The use of markers could also see the production of cultivars with stacked resistance, a condition in which a genotype contains multiple sources of resistance which ensures more stable and complete resistance.

The SSH method has been successfully used for for identifying differentially expressed genes in sugarcane varieties exposed to smut (*Ustilago scitaminea* H&P Sydow) and *P. melanocephala* among other diseases (Butterfield *et al*., 2004; Heinze *et al*., 2001; Oloriz *et al*., 2012). SSH has also been used on other plants and has resulted in the elucidation of the transcription profiles in cultivars such as *Malus domestica* that respond differently to apple scab (Degenhardt *et al*., 2005). The subtraction libraries constructed in this study were able to show differences between the two libraries, and subsequently among the different sugarcane resistance groups. These changes were visible within 48 hours of inoculation, demonstrating the immediate changes resistant sugarcane varieties make when challenged with the rust pathogen, relative to intermediate and susceptible varieties. The presence of sequences homologous to transcription factors confirmed that there were changes in gene expression due to the rust

challenge. Bray (2002) noted that several different classes of transcription factors are induced by stress and result in the up-regulation of genes, many of which are signalled through abscisic acid (ABA). ABA has been demonstrated to act together with auxins and subsequent ethylene production in transcription and post transcriptional control (Hansen and Grossmann, 2000; Stafstrom *et al*., 1998). Among the sequences found to be homologous to mRNA coding for transcription factors, was the auxin repressed/ auxin associated protein coding mRNA sequence. This protein has been found associated with disease resistance in maize and in *Arabidopsis thaliana*, where the repression of auxin signalling by the salicylic acid (SA) pathway has been shown to induce antibacterial resistance (Llorente *et al*., 2008). The SA molecule is vital in plant defence systems and is known to elicit both the local and systemic-acquired resistance by inducing the accumulation of pathogenesis related proteins (Loake and Grant, 2007; Shah, 2003).

The enzyme adenosyl-l-homocysteine hydrolase is known to prevent feedback inhibition of transmethylation reactions by impeding the build-up of S-adenosyl-l-homocysteine (Malanovic *et al*., 2008; Wu *et al*., 2005). Methylation has been shown to play a role in the rust-sugarcane interaction as enzymes involved in methylation have displayed altered expression levels during infection (Oloriz *et al*., 2012). S-adenosylmethionine (SAM) is a precursor in the biosynthesis of the polyamines spermine and spermidine and also in ethylene biosynthesis (Marini *et al*., 2001). The enzyme S-adenosylmethionine decarboxylase (SAMDC) catalyses the conversion of SAM to S-adenosylmethioninamine, a substrate involved in the biosynthesis of these polyamines from putrescine and is also known to influence the rate of ethylene biosynthesis (Marini *et al*., 2001; Oloriz *et al*., 2012). The *samdc* gene was shown to be up-regulated in the incompatible *P. melanocephala*- sugarcane reaction, leading to the biosynthesis of the polyamines, which are known to accumulate and play a role in hypersensitive responses exhibited by this type of reaction (Marini *et al*., 2001; Oloriz *et al*., 2012; Torrigiani *et al*., 1997; Yoda *et al*., 2003; Yoda *et al*., 2009).

Library two contained a sequence homologous to the Facilitates Chromatin Transcription (FACT) complex, a transcription factor involved in multiple processes that require DNA as a template such as mRNA elongation, DNA replication and DNA repair (Duroux *et al*., 2004; Marchler-Bauer *et al*., 2011). The FACT complex also acts in establishing transcription initiation complexes and promotes SPT15/TBP-binding to a TATA box (Marchler-Bauer *et al*., 2011). The compacting of DNA in chromatin plays an important role in gene regulation as it represses the

transcription of genes by restricting the access of DNA binding regulatory factors to their DNA target sites with the subsequent effect of inhibiting initiation and elongation of transcription by RNA polymerase II (Duroux *et al*., 2004; Lolas *et al*., 2010). Biochemical analysis has revealed that the FACT complex in maize, partly consisting of the SSRP1 protein can specifically recognize certain DNA structures and structurally flexible regions in linear DNA (Lolas *et al*., 2010). The FACT complex has also been found to be enriched over the entire transcribed region of RNA Polymerase II-transcribed genes in *Arabidopsis* and also in areas where there is less compacted chromatin in maize (Lolas *et al*., 2010). This observation suggests that the FACT complex is responsible for the transcription of specific genes, which in this case, could be those responsible for rust resistance in the genotypes analysed.

Library One showed an interesting difference between the sugarcane genotypes resistant to rust, as the *Bru1* containing genotypes did not seem to contain the oxidative stress coding sequences shown in Table 6.3.3a. This observation could be an indicator of the alternative source of resistance contained by the *Bru1* negative genotypes. The early stages of a rust infection are normally characterised by an oxidative burst generating reactive oxygen intermediates (ROS), which is synonymous with the hypersensitive response (Carmona *et al*., 2004). Proteins predicted from sugarcane ESTs and involved in this form of defence response mechanism include superoxide dismutase and catalases, which have been found in the subtraction Library One (Carmona *et al*., 2004). Protein kinase involvement in the recognition of pathogens is useful in triggering disease resistance mechanisms such as these oxidative bursts and the activation of signal transduction cascades, which are features of gene for gene interactions found in host-pathogen interactions (Carmona *et al*., 2004; Hu *et al*., 1996). The clone ssh4_15.6_F01 in subtraction library one contained a sequence homologous to a tyrosine protein kinase, confirming the possibility of a protein kinase mediated hypersensitive response to rust in the *Bru1* negative genotypes.

Reactive oxygen species and redox signalling has also been shown to undergo synergistic and antagonistic interactions with phytohormones and in turn regulating the protective responses of plants against biotic and abiotic stress (Tognetti *et al*., 2010). Recent studies have shown that hydrogen peroxide-responsive UDP-glucosyltransferase is involved in the modulation of plant architecture and water stress response through its activity towards the auxin indole-3-butyric acid (IBA) (Tognetti *et al*., 2010). The presence of two clones containing a sequence homologous to the GT1 family of glycosyltransferases suggests that this is another possible

mechanism of rust resistance in the *Bru1* negative rust resistant genotypes, which is open to further study.

The great majority of the sequences in both libraries were homologous to Leucine-rich repeat proteins (LRRP). LRRPs are synonymous with disease resistance proteins, and this was shown by the LRRP's obtained in both libraries containing conserved domains significantly homologous (an Expect value of 2.12E-15) to the resistance protein found in *A. thaliana* to *P. syringae* (Kim *et al*., 2009; Zhang *et al.,* 2000). However, the majority of these sequences contained stop codons, an indication of their possibly being pseudogenes (Ameline-Torregrosa *et al*., 2008; Zou *et al*., 2009). Pseudogenes are defined as non-functional genomic sequences with significant sequence similarity to functional RNA or protein-coding genes (Hirotsune *et al*., 2003; Zou *et al*., 2009). Protein-coding sequences are defined as pseudogenes if degenerative features such as premature stops, frameshift mutations, and truncations of the full-length gene are present (Pink *et al*., 2011; Zou *et al*., 2009). These duplicated genes normally lose their protein-coding potential due to the loss of promoters or enhancers or crippling mutations (Pink *et al*., 2011). Other common features of pseudogenes include their poly-A tracts and direct repeats at either end of the gene (Pink *et al*., 2011). The use of a translation tool showed that in all three reading frames, the resulting amino acid sequence from BLASTx searches was essentially identical for many of these putative pseudogenes (Figure 6.4.1 A) (Zhang *et al*., 2000).

Pseudogenes have been shown to control the epigenetic regulation of gene function in cells with their regulatory capabilities varying among different situations (Pink *et al*., 2011; Zhang *et al*., 2006). Pseudogenes have been shown to increase or decrease the expression of their parent genes by regulating mRNA stability (Hirotsune *et al*., 2003). They have also been shown to be able to be processed into small interfering RNA (siRNA) molecules whose effect can be positive or negative on the expression levels of the parent genes. The effects of separate pseudogenes however needs to be studied individually, as the genes have also been found to be capable of undergoing transcription and coding for truncated proteins (Pink *et al*., 2011; Zhang *et al*., 2006).

A: 5'3' Frame 1

T L R **Stop** **Y H C L L C V D T T A Y S A L I P L L T L R** **Stop** **Y H C L L C V D T T A Y S A L I P L L T L R** **Stop** **Y H C L L C V D T T A Y S A L** **Met** **P L L T L R C Y H C L L C V D T T A Y S A L I P L L T L R** **Stop** **Y H C L L C V D T T V Y S A L I P V L T L R** **Stop** Y Q C L L C V D T T A H S A L I P L L T L R **Stop** **C H C L L C V V T T A H S A L I P L L T L R** **Stop** Y H C L L C V D T T A W Q S

5'3' Frame 2

L C V D T T A Y S A L T P L L T L R **Stop** **Y H C L L C V D T T A Y S A L I P L L T L R** **Stop** **Y H C L L C V D T T A Y S A L I P L L T L R** **Stop** **C H C L L C V V T T A Y S A L I P L L T L R** **Stop** **Y H C L L C V D T T A Y S V L I P L F T L R** **Stop** Y Q C L L C V D T S A Y S A L I P L L T L R **Stop** **Y H C L L C V N A T A Y S A L L P L L T L R** **Stop** Y H C L L C V N T T A Y S A L I P L L G R

5'3' Frame 3

S A L I P L L T L R **Stop** H H C L L C V D T T A Y S A L I P L L T L R **Stop** Y H C L L C V D T T A Y S A L I P L L T L R **Stop** Y H C L L C V N A T A Y S A L L P L L T L R **Stop** Y H C L L C V D T T A Y S A L I P L L T L C **Stop** Y H C L L C V D T S A Y S A L I P V L T L R **Stop** Y H C S L C V D T T A Y S A L **Met** **P L L T L R C Y H C S L C V D T T A Y S A L I P L L T L R** **Stop** Y H C L A E

B:

http://systemsbiology.liv.ac.uk/legr/troutbase/troutbase_2_0//subgroup_html_priority/118-123.html

BLASTX 2.2.5 [Nov-16-2002]

>ref|XP_344805.1| similar to putative protein (5I806) [Rattus norvegicus] Length = 509

Score = 83.6 bits (205), Expect = 3e-16 Identities = 57/142 (40%), Positives = 64/142 (45%) Frame = -2

```
Query: 528
YHCLLCVDTTAYSALIPLLTLR*YHCLLCVDTTAYSALIPLLTLR*YHCLLCVDTTAYSA 349
YH L  V+TTA    +P      YH L  V TTAY   +P L    YH L  + TTA
Sbjct: 99
YHSLSTVNTTACQLSVPQPVNCQYHSLSTVSTTAYQLSVPQLVNCQYHSLSIISTTACQL 158

Query: 348
LIPLLTLR*YHCLLCVDTTAYSALIPLLTLR*YHCLLCVDTTAYSALIPLLTLR*YHCLL 169
 +P L    YH L  V TTA    +P      YH L  V TTAY   IP      YH L

Sbjct: 159
SVPQLVNCQYHSLTTVSTTACQLSVPQPVNCQYHSLSTVSTTAYQLSIPQPVNCQYHSLS 218

Query: 168 CVDTTAYSALIPLLTLR*YHCL 103
           V TTAY   +P L    YH L
Sbjct: 219 TVSTTAYQLSVPQLINCQYHSL 240
```

Fig 6.4.1: A- Translated putative LRR cDNA sequence in three reading frames for the cDNA clone ssh4_12_D02; B- Google search using resultant amino acid sequence.

ssh2_19_C03

ACTCTGCGTTGATACCACTGCTTACTCTGCGTTGATACCACTGCTTACTCTGCGTTGATACCACTGCTT
ACTCTGCGTTGATACCACTGCTTCGCATGTACTCTGCGTTGATACCACTGCTTACTCTGCGTTGATAC
CACTGCTTACTCTGCATTGATACCACTGCTTACTCTGCATTGATACCACTGCTTACTCTGCGTTGATAC
CACTGCTTACTCTGCGTTGATACCACTGCTTACTCTGCGTTGATACCACTGCTTACTCTGCGTTGATAC
CACTGCTTACTCTGCGTTGATACCACTGCTTTCTCTGCGTTGATACCACTGCTTCCCGTGTACTCTGC
GT 345

Blastx

Accession	Description	E value	Max ident
XP_001623329.1	predicted protein [Nematostella vectensis] >gb|EDO31229.1| predicted protein [Nematostella	9e-06	39%
CAJ17107.1	hypothetical protein Tb11.1280 [Trypanosoma brucei brucei strain 927/4 GUTat10.1]	4e-05	27%

```
>    gb|EDO31229.1|  predicted protein [Nematostella vectensis]
Expect = 9e-06  Frame = +1

Query  19   LLTLR*YHCLLCVDTTAYSALIPLLRMYSALIPLLTLR*YHCLLCIDTTAYSALIPLLTL  198
            L TL   H  LC   T + AL  LL ++ AL  LLT+   H  LC   T + AL  LLT+
Sbjct  6    LCTLLMIHHALCTLLTIHHALCTLLMIHHALCTLLTI---HHALCTLLTIHHALCTLLTI  62

Query  199  R*YHCLLCVDTTAYSALIPLLTLR*YHCLLCVDTTAFSALIPLLPVYSA  345
               H  LC     + AL  LLT+   H  LC   T   AL  LL ++ A
Sbjct  63   ---HHALCTLLMIHHALCTLLTI---HHALCTLLTIHHALCTLLMIHHA  105
```

Blastn vrs est database

Accession	Description	E value	Max ident
HO066472.1	Chickpea drought stressed cDNA SSH library	2e-157	99%
DV103804.1	Tomato root subtractive cDNA library for heat-shock down-regulated genes Solanum lycopersicum	2e-157	100%
GH734439.1	Camellia sinensis var. assamica cDNA	6e-157	98%

Blastx using first est

```
>    gb|EDO31229.1|  predicted protein [Nematostella vectensis]
Expect = 3e-08 Frame = -2

Query  511  TLR*YHCLLCVDTTAYSALIPLLTLR*YHCLLCVDTTAYSASIPLLTLR*YHCLLCVDTT  332
            TL   H  LC   T + AL  LLT+   H  LC     + A   LLT+   H  LC   T
Sbjct  148  TLLTIHHALCTLLTIHHALCTLLTI---HHALCTLLMIHHALCTLLTI---HHALCTLLT  201

Query  331  AYSALTPLLTLR*YHCLLCVDTTAYSALIPLL-------TLR*YHCLLCVDTTAYSALIP  173
              + AL  LL +   H  LC   T + AL  LL       TL   H  LC   T + AL
Sbjct  202  IHHALCTLLMI---HHALCTLLTIHHALCTLLMIHHALCTLLMIHHALCTLLTIHHALCT  258

Query  172  LLPAYSALIPLLTLR*YHCLLCVDTTAYSSLIPLLTLR*YHCLLC  38
            LL  + AL  LLT+   H  LC   T + +L  LLT+   H  LC
Sbjct  259  LLMIHHALCTLLTI---HHALCTLLTIHHALCTLLTI---HHALC  297
```

Fig 6.4.2: Results of BLAST searches for one of the putative LRR transcripts.

Performing a Google search using this amino acid sequence also revealed that other researchers have encountered this same phenomenon, for example in trout (Fig 6.4.1B). The BLAST results for the one of these putative LRRPs are shown in Fig 6.4.2 and again it appears that this repetitive sequence has been encountered in EST libraries from chickpea, tomato and green tea. These sequences could therefore be artifacts of the SSH methodology. Given their high frequency in this study, the SSH experiments will need to be repeated and complemented with techniques such as Northern Blot analysis and qPCR. Pseudogene sequences coding for these LRRPs have previously been isolated from separate studies using different organisms exposed to a variety of conditions. Expression analysis of genes identified through this method can then be evaluated using real time PCR (Scholtz and Visser, 2012).

This differential gene expression study can also be improved by the additional use of a reverse SSH. Reverse SSH will provide information on the enriched population of the up-regulated transcripts resulting from successful infection by *P. melanocephala* in the resistance groups used as drivers in this study (Huang, 2008; Sahu and Shaw, 2009). Additional libraries can also be constructed to discriminate between the various individual resistance groups or genotypes in order to gain additional information on variety specific modes of resistance or susceptibility. The use of Northern blots can also be employed to ensure the quality control and effectiveness of the SSH procedure. Data obtained this way would be useful in developing a marker based system for selecting genotypes with stacked rust resistance mechanisms in the SASRI breeding programs.

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Chapter 7

Concluding Remarks and Future Work

Resistance to brown rust of sugarcane is conferred by a major gene called *Bru1*, among other poorly documented/ undocumented resistance mechanisms (Raboin *et al*., 2006). A study to improve the existing rust screening procedures and to subsequently uncover alternative resistance mechanisms using the SASRI Linkage Disequilibrium mapping population (LD2) was undertaken. Of the screening techniques employed, it was confirmed that whorl inoculation produced more or less consistent results (Sood *et al*., 2009). Two pot whorl inoculation trials were conducted in different weather conditions and showed differences in the severity of rust infection between both trials after four weeks. These differences underlined the effect of GxE interactions on disease development, despite both trials being exposed to optimal rust conditions on the first day. The second pot whorl inoculation showed less severe rust infection as it was conducted in sub optimal conditions relative to pot trial one. Despite these differences, the trials were still significantly correlated to each other at a 95% confidence interval.

A detached leaf assay was also tested as a possible rust resistance screening method for the SASRI breeding and selection program. Methods of conducting detached leaf assays (DLA) were obtained from literature and compared to each other using sugarcane leaves from cultivars of known rust resistance inoculated with *Puccinia melanocephala* (Braithwaite, 2005; Jackson *et al*., 2008; Twizeyimana *et al*., 2007; Zhao *et al*., 2011). The use of the chlorophyll fluorescence derived performance index (PI_{ABS}) proved to be a more time saving method of screening for rust resistant genotypes as compared to visual observations. The observations made in detached leaves corresponded with the infection stages described by Sotomayer *et al*. (1983) with regards to the timing of the onset of infection and infection termination in resistant plants. These observations also allowed inferences to be made regarding the routes taken by differently rated sugarcane genotypes to ward off or survive rust infection. Though visual observations have been proved to be a reliable and consistent method of analysis, they take a longer time when compared to the use of PI_{ABS} and are also prone to secondary infection due to prolonged stay in artificial media during symptom development. The use of PI_{ABS} also has the advantage of obtaining readings within 2-5 days post inoculation, reducing the chances of the deterioration of chlorophyll which has a direct impact on the PI_{ABS} (Falqueto

et al., 2010). A gradual decrease in chlorophyll content and PI_{ABS} on all the detached leaves regardless of treatment was observed, confirming Falqueto *et al.'s* (2010) observations. A wide spectrum of host responses ranging from complete resistance to susceptibility have in the past dictated that no single pattern of response will emerge from assessing photosynthetic metabolism (Bürling *et al*., 2011; Lopes and Berger, 2001; Rolfe and Scholes, 2010). The results obtained however suggested that PI_{ABS} can be used and increasing the sample size could validate this method. Percentage differences between the PI_{ABS} of inoculated treatments and their controls were compared and indicated trends that had potential diagnostic implications among the different resistance groups. These trends were maintained in some LD2 genotypes selected on the basis of their resistance ratings obtained from the pot whorl inoculation trials, showing the efficiency of the DLA approach. The trends obtained demonstrated that the use of ΔPI_{ABS} readings taken from different treatments and leaf locations could be used to clearly distinguish between susceptible and resistant genotypes by the second day post inoculation. Differences between inoculated leaves and un-inoculated regions of the same leaf showed significant differences between the resistant groups and the susceptible and intermediate groups on the first and second days of comparing the ΔPI_{ABS}. The observations made in the rust resistance screening experiments all showed the potential of these methods to significantly improve and shorten the current rust screening stage in the breeding and selection cycle. Developments such as these could significantly impact on the improved release of rust resistant cultivars, curbing resultant losses and related expenses arising from rust outbreaks (Asnaghi *et al*., 2004; Bailey, 2004; Purdy *et al*., 1983). The DLA was successfully used to confirm the resistance ratings obtained in the pot whorl trials using twelve genotypes selected from LD2.

The use of Near Infrared (NIR) Spectroscopy could also be employed for the rapid screening of rust resistant genotypes and compared to that of chlorophyll fluorescence for efficiency and reproduceability. NIR is a technology which utilises the ability of different compounds in differing compositions to be analysed and characterised using their unique absorption capacity of specific wavelengths of NIR light energy (Unity Scientific Brochure, 2010). Specifically, NIR light affects the C-H, N-H, and O-H molecular bonds which are directly related to proteins, lipids and carbohydrates among other metabolites that could be produced in plants in response to disease infection (Unity Scientific Brochure, 2010). This quality of NIR spectroscopy makes it a potential tool in the rapid and non-invasive detection/ screening of disease resistance in commercial crops. The speed at which NIR can give accurate predictions has been

observed in the analysis of soya bean resistance to mosaic virus, where an analysis has been made as early as the latent symptomless stages of infection (Jinendra *et al*., 2010). NIR has been successfully used in the detection of disease resistance in many other crops, with subsequent reductions in analysis times coupled with high degrees of accurate disease predictions of over 90% in most reported cases (Draganova *et al*., 2010; Jinendra *et al*., 2010; Sankaran *et al*., 2010). NIR spectroscopy has also been used in sugarcane breeding programs where screening of smut and *Eldana saccharina* Walker resistance has been successfully conducted (Churchill *et al*., 2006; Purcell *et al*., 2010; Rutherford, 1998). There is potential for NIR to be used in the current study to screen for rust resistant genotypes from the LD2 population. This method is useful in detecting genotypes containing either innate and pathogen induced resistance, allowing the screening exercise to be even more descriptive. To explain whether the resistance is innate or induced, the screening process can be conducted on non-challenged genotypes and on challenged genotypes, an exercise which will allow the type of resistance available to each particular genotype to be revealed. The use and calibration of NIR for use in a rust screening protocol could result in the ability to screen larger genotype populations at earlier stages within the SASRI breeding and selection program, subsequently achieving gains in efficiency and a significant retention of genetic variability (Purcell *et al*., 2010). The use of high performance liquid chromatography (HPLC) could further elucidate the mechanisms involved in rust resistance, as specific metabolites responsible or found in response to rust resistance could be identified (Rutherford, 1998). Such a metabolomics based approach could also allow a cheaper biochemical assay to be used as a screening tool.

Marker analysis was conducted on the LD2 and LD1 mapping populations using two flanking marker sets in order to distinguish between genotypes containing *Bru1* and those containing other unknown resistance mechanisms towards brown rust of sugarcane. Approximately 33% (26 of 80) of LD2 did not contain *Bru1*, and of these, 18 of these genotypes were resistant based on the overall rating scale, five were intermediate and three were susceptible. The *Bru1* positive genotypes all had overall rust ratings in the resistant or more resistant genotypes of the intermediate range, showing the efficacy of *Bru1* in conferring resistance to rust.

LD2 was subsequently grouped based on *Bru1* presence and then ranked according to the phenotypic data obtained from pot whorl inoculation trials. Correlation between the phenotypic ratings and the AFLP marker data were then calculated for the genotypes and the best markers were selected. Two models were created based on the overall rust ratings and

the other on the lesion ratings. The selected markers were then further reduced using stepwise multiple linear multiple regression analysis to come up with the best markers and an equation to predict rust resistant genotypes using their respective AFLP marker profiles. The AFLP marker analysis showed the potential to provide useful markers for use in selecting markers for non-*Bru1* rust resistance traits. The AFLP analysis resulted in more informative data about LD2 being obtained and in the subsequent selection of 12 genotypes for use in suppression subtractive hybridization (SSH). These genotypes were divided into groups which were *Bru1* positive resistant, *Bru1* negative resistant, *Bru1* negative intermediate and *Bru1* negative susceptible.

The selected genotypes were planted and whorl inoculated after two months and mRNA extracted from the leaves 48 hours post inoculation. The SSH procedure was then carried out and two subtraction libraries were constructed. These libraries revealed possible rust resistance mechanisms available in the *Bru1* negative genotypes, with the identification of possible resistance mechanisms in sequences homologous to proteins involved in the jasmonate pathway and to leucine-rich-repeat (LRR) proteins. SSH showed it can also be useful not only in decoding the alternative rust resistance mechanisms used by the *Bru1* negative genotypes in LD2, but also in the development of markers for the selection of varieties containing these qualities. This study resulted in more questions being raised, as a large number of clones were homologous to LRR pseudogenes, resulting in the need to repeat the analyses and confirm whether or not LRR pseudogene homologues are artifacts of SSH.

Overall, the possibilities of improving the analyses conducted and incorporating more probing assays toward the development of screening techniques and marker systems for selecting rust resistant genotypes were displayed in this research. These improvements could result in a better understanding of the rust resistance mechanisms within this mapping population and in sugarcane in general, resulting in an improvement of the commercial varieties produced for the South African Sugarcane industry. In improving this study, the SSH profiling of the different genotypes can be exhaustively conducted, as only a limited number of clones were analysed due to time constraints. In the non-exhaustive SSH profiling exercise conducted, only forward subtractions were conducted, giving only an indication of the up-regulated genes in resistant genotypes after rust inoculation. SSH could also have been conducted with the *Bru1* negative susceptible genotypes being the tester in order to determine if there are genes that could be resulting in the susceptibility of these genotypes (Huang, 2008). Differential screening can then

be conducted on clones obtained through SSH using Northern blots, together with the analysis of the expression levels of these genes using Real-time PCR (qPCR) (Huang, 2008; Krishnaraj *et al*., 2011; Scholtz and Visser, 2012; Zhang *et al*., 2012).

The majority of isolated resistance (*R*) genes are grouped in a class of *R* genes called the Nucleotide-Binding-Site-Leucine-Rich-Repeat (NBS-LRR) genes (du Preez, 2005). This class of *R* genes is divided into subclasses based on their N-terminal domain which are the Toll-Interleukin receptor homology NBS-LRR (TIR-NBS-LRR) genes, leucine-zipper homology NBS-LRR (LZ-NBS-LRR) genes and the coiled-coil homology NBS-LRR (CC-NBS-LRR) genes (du Preez, 2005; Martin *et al*., 2003). NBS profiling is a method designed to probe functional diversity in and near disease resistance genes of the NBS-LRR type (van der Linden *et al*., 2004). NBS profiling can be used to produce markers tightly linked to *R*-genes and *R*-gene clusters for genomic mapping and positional cloning, and to mine for new alleles and new sources of disease resistance in available germplasm (Gu *et al*., 2008; Mantovani *et al*., 2006; van der Linden *et al*., 2004). NBS profiling will be used in the continuation of this study to aid in the identification of *R* genes or resistance gene analogues (RGAs) present in the *Bru1* negative resistant genotypes. The markers generated from this technique will also be correlated to the rust phenotypic responses of the genotypes analysed in order to isolate the NBS-LRR genes directly responsible for rust resistance (Gu *et al*., 2008). The use of Northern blots or microarray technology can also be considered for further use in the rapid identification of genotypes containing these resistance mechanisms to aid in the selection of future genotypes in the breeding and selection cycles (Alsop *et al*., 2011). The *R* genes identified through NBS profiling can also be directly compared to subtracted mRNA pools isolated from rust challenged genotypes through hybridisation in order to confirm their involvement in rust resistance and that the isolated *R* genes or RGAs have a direct impact on rust resistance.

Genetic mapping using AFLP markers has been successfully used in the discovery of two rust resistance genes which include *Bru1* (Asnaghi *et al*., 2004; Hoarau *et al*., 2001; Le Cunff *et al*., 2008; Raboin *et al*., 2006). The current work conducted can be further improved by increasing the number of genotypes incorporated into the model (Butterfield, 2007; Raboin *et al*., 2001). Existing AFLP data together with that which will be obtained from the additional genotypes can then be used to generate markers for this genetic mapping exercise. Markers generated from SSH and NBS profiling can also be used to increase the coverage and resolution of this proposed genetic map, making it more informative and increasing the chances of isolating

major resistance genes or Mendelian factors involved in QTL (Alsop *et al*., 2011; Gu *et al*., 2008; Hoarau *et al*., 2001; Le Cunff *et al*., 2008; Raboin *et al*., 2006). Previous studies conducted on genetic mapping exercises have utilised large progeny numbers coming from bi-parental crosses and subsequent phenotypic analyses arising from field trials and greenhouse experiments (Raboin *et al*., 2001). The use of linkage disequilibrium (LD) and association methods is however a method that has recently become preferable to that of using bi-parental crosses, as it has immense benefits which include;

- The sampling of the full allelic variation within the breeding population, as opposed to that occurring only within a bi-parental progeny population;
- Detecting QTL effects within a diverse genetic background representing elite varieties means that markers detected are less likely to be background-specific, and more widely applicable in breeding (Butterfield, 2007).

As the *Bru1* negative genotypes from the LD1 and LD2 populations have already been identified, additional genotypes lacking the *Bru1* gene could be selected for further use in improving the prediction model (Raboin *et al*., 2006). A balanced set with equal numbers of resistant, intermediate and susceptible genotypes could be assembled. The phenotypic data for this balanced set could be assessed using the optimised pot whorl inoculation technique and the DLA screening methods combined with chlorophyll fluorescence or NIR for detecting reaction phenotypes. Field analyses using either whorl inoculation or natural infection can also be conducted to ensure that as much descriptive information about the genotypes is obtained (Raboin *et al*., 2001; Sood *et al*., 2009).

The techniques and strategies outlined above are all methods which can be undertaken towards the aim of better understanding the mechanisms responsible for rust resistance. Once these mechanisms are identified and understood, marker systems can be developed which will allow the rapid and more efficient selection of genotypes for use in the breeding and selection process for rust resistant varieties. These rapid selection techniques will significantly aid in the reduction of the time used in the current techniques and aid in producing more rust resistant genotypes for the industry. Improving the screening techniques will also greatly assist in the initial stages of identifying which markers tag specific traits. These methods will also be invaluable in confirming the resistance of the genotypes selected through MAS. The methods of NBS profiling and AFLP analyses could produce markers which can form the basis of analyses for resistance to other diseases within these and other sugarcane genotypes.

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Conference Papers and Abstracts

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Analysis of differential gene expression during the early stages of rust infection in a sugarcane breeding population.

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Resistant cultivars are the most effective way of controlling brown rust of sugarcane caused by *Puccinia melanocephala*. Molecular techniques can be used to identify undocumented rust resistance mechanisms, which can then be utilised in breeding strategies to develop cultivars with increased resistance and durability. Suppression Subtractive Hybridization (SSH) was used to identify genes differentially expressed between sugarcane genotypes that are resistant (two groups with different modes of resistance, each group having three genotypes), intermediately resistant and susceptible to rust. Leaves were sampled 48h after inoculation with *P. melanocephala*. mRNA for each genotype was purified, pooled into its respective groups and converted into cDNA using the Clontech SMARTer™ cDNA synthesis kit. Two subtracted cDNA libraries were constructed using a PCR-Select™ cDNA subtraction kit. For Library 1pooled samples of Susceptible and Intermediate resistant genotypes were subtracted from Resistant Group One. For Library 2, Resistant Group Two was subtracted from Resistant Group One. Subtracted cDNA libraries were transformed into *Escherichia coli*. The cDNA inserted using the pGEM®-T easy vector in individual bacterial colonies was amplified by PCR and showed successfully transformed clones with cDNA inserts ranging from 200-1300bp. DNA Sequence analysis of inserts and BLAST searches for both libraries revealed that over 70% of the sequences were identified as various disease and drought stress related gene sequences. Library 1 revealed enrichment for serine/ threonine kinases, reticulon-like proteins and RNA recognition motifs, domains found in proteins involved in post-transcriptional gene expression processes. The *Mob1* gene was also identified in Library 1. *Mob1* is known to play a role in hypersensitive responses and in cytokinesis, critical processes in host resistance to pathogens. Processing of Library 2 results is still in progress. These results show the usefulness of SSH in providing information on gene expression in response to brown rust infection. This information could be used to develop markers for resistance, having potential application in the breeding and selection programme.

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POSTER SUMMARY

A RAPID *IN VITRO* METHOD OF DETECTING RESISTANCE TO *PUCCINIA MELANOCEPHALA* IN SUGARCANE

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Abstract

Puccinia melanocephala is the causal agent of brown rust in sugarcane. Brown rust can result in over 50% yield loss. The most reliable and eco-friendly way of combating rust is through development and use of resistant varieties. A rapid screening method in the form of a detached leaf assay (DLA) is required to speed up the varietal selection process. Media compositions were formulated in 1% agar using water and 10 mg/L of kinetin, benzylaminopurine (BAP) and benzimidazole as the base of the media. Lactic acid, benomyl and pentachloronitrobenzene (PCNB) were also used to make up varying media combinations. Leaves of varieties N12, N29 and N39 exposed to *P. melanocephala* spores were cut and sandwiched between media inside square plates. The leaves were dark-acclimatised at 25°C before incubation in a growth room at 25°C and 18 h photoperiod. Analysis of the effects of rust on the leaves was conducted using a chlorophyll fluorescence meter at a light intensity of 3000 µmol m_2/s and by visual observation of lesion and spore formation. The Performance Index (PI_{ABS}) showed water was a better medium for maintaining photosynthetic activity and that the resistant variety N12 had the best _PI_{ABS} when exposed to spores after the fifth day as compared to the susceptible and intermediate varieties N29 and N39. Sporulation was observed on the 12th day on N29 maintained on Kinetin. The leaves on water, BAP and benzimidazole agar sporulated after 13-14 days. These results show that with refinements, a DLA could be reliably used to rapidly diagnose resistance to rust in sugarcane.

Keywords: Puccinia melanocephala, sugarcane, brown rust, Detached Leaf Assay, Performance Index

Introduction

Brown rust of sugarcane is caused by the basidiomycete *Puccinia melanocephela* H. & P. Sydow (Hoy and Hollier, 2009). Brown rust presents as reddish-brown pustules that erupt on the abaxial side of sugarcane leaves (Dixon, 2010; Bailey, 2004). The earliest symptoms are small, elongated yellowish spots that are visible on both leaf surfaces (Purdy *et al*., 1983). The spots increase in length, become red-brown in colour, mature and sporulate within 10-14 days. Lesions typically range from 2-10 mm in length, but occasionally reach 30 mm (Raid and Comstock, 2000). Rust is an economically important disease that results in severe reductions in yield, with losses as much as 40-50% having been experienced in susceptible varieties (Bailey, 2004). In recent years, resurgence in the incidence of brown rust has been observed, with the variety N29 registering losses of up to 26% (McFarlane *et al*., 2006).

The most effective and economically feasible method of long term control of rust is through the use of resistant cultivars and cultivar diversification (Asnaghi *et al*., 2004; Bailey, 2004). Breeding programmes now incorporate a rust screening step to mitigate the effects of rust. Natural infection has been the primary means of assessing rust resistance in sugarcane cultivars. This method is, however, not always efficient as it is dependent on environmental conditions (Sood *et al*., 2009). A more reliable and robust screening method is consequently required to effectively select resistant genotypes.

An *in vitro* method using detached leaf assays has been formulated, and has shown potential to overcome the current limitations associated with resistance screening. This makes it a potentially better method of screening for disease resistance, as it is cost effective, space saving and a faster method of conducting screening tests on large sample sizes (Jackson *et al*., 2008). This method also has the advantage of having control over environmental conditions which have been a common source of errors in past analyses (Purdy *et al*., 1983).

Materials and Methods

Three varieties (N12 (resistant), N29 (susceptible) and N39 (intermediate)) were selected from a rust challenged environment and the leaves from the middle section of the top visible dewlap (TVD) cut into 6 cm pieces. The mid rib was removed and the leaf cuttings rinsed in sterile water. A 1% agar media was prepared based on combinations of plant growth regulators (Kinetin (10 mg/L); BAP (10 mg/L); benzimidazole (10 mg/L)), two fungicides (benomyl (0.05%) and PCNB (0.33 g/L)) and lactic acid (3 ml/L). The 16 combinations used in this experiment are outlined below:

Treatment 1 water and PCNB
Treatment 2 water, benomyl and PCNB
Treatment 3 water, lactic acid and PCNB
Treatment 4 water, lactic acid, benomyl and PCNB
Treatment 5 BAP and PCNB
Treatment 6 BAP, benomyl and PCNB
Treatment 7 BAP, lactic acid and PCNB
Treatment 8 BAP, lactic acid, benomyl and PCNB
Treatment 9 kinetin and PCNB
Treatment 10 kinetin, benomyl and PCNB
Treatment 11 kinetin, lactic acid and PCNB
Treatment 12 kinetin, lactic acid, benomyl and PCNB
Treatment 13 Benzimidazole and PCNB
Treatment 14 Benzimidazole ,benomyl and PCNB
Treatment 15 Benzimidazole, lactic acid and PCNB
Treatment 16 Benzimidazole, lactic acid, benomyl and PCNB.

The media was autoclaved, set in square plates and cut into slabs to make a sandwich. The sugarcane leaf ends were embedded within the agar slabs, adaxial sides exposed. The agar amending solution was added in between the agar layers. Four leaf cuttings were used in each plate (Jackson *et al*., 2008).

Two chlorophyll *a* fluorescence measurements were taken from each dark-acclimatised leaf cutting using a Hansatech Handy Pea fluorescence meter at a light intensity of 3000 μmol m_2/s. The leaves were then embedded into their respective agar treatments, incubated

overnight in the dark at 25°C, and then transferred to an 18-hour photoperiod growth room. Chlorophyll *a* fluorescence measurements were carried out every 24 hours for five days, and then every two days thereafter until the ninth day. The leaves were constantly monitored visually to observe any visual rust symptoms (lesions and sporulation) and to monitor how long they maintained their colour.

The chlorophyll fluorescence data was processed using the JIP test (Strasser *et al*., 2000), which lead to the calculation of the Performance Index (PI_{ABS}), which is regarded as a reliable indicator of electron transport efficiency and photosynthetic capacity during stress (Strauss *et al*., 2007). $\_PI_{ABS}$ was calculated using the formula: $\_PI_{ABS} = PI_{ABS}$ (day 0) - PI_{ABS} (day x).

Results and Discussion

Treatment 1 (water and PCNB) was the best media combination in maintaining leaf pigmentation and rust development. Lesion formation began after the first week, whereas sporulation was observed on the 12th day of incubation in N29. The leaves however tended to dry out from the middle section and spread towards the ends within the third week. Benzimidazole treatments were the best in retaining leaf pigmentation, but inhibited maximal presentation of rust symptoms, possibly due to its fungicidal properties. All the treatments induced declines in $\_PI_{ABS}$ in the initial three days, after which values stabilised and remained relatively constant until day nine. This was thought to be as a result of the leaves being detached, entering and then acclimatising to foreign environments. The $\_PI_{ABS}$ of the leaves was further reduced when components besides water were added to media. This trend confirmed the visual observations where treatment 1 had the best overall response in terms of pigment retention and symptom development. Variety N29 had the largest negative $\_PI_{ABS}$ due to it developing lesions and spores. Rust is known to reduce the photosynthetic activity of sugarcane and this was confirmed by the lower $\_PI_{ABS}$ shown in N29 as compared to N12 and N39 (Rolfe and Scholes, 2010). N12 had the least change in PI_{ABS}, and this was attributed to the leaves being asymptomatic, with consequently better photosynthetic activity. Further trials will determine whether chlorophyll *a* fluorescence measurements can be used in a diagnostic way. Tubes have also been acquired in an attempt to develop a method in which handling and contamination of test material is reduced.

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