



**STUDIES ON BROWN RUST (*PUCCINIA MELANOCEPHALA*) OF
SUGARCANE IN SOUTH AFRICA**

by

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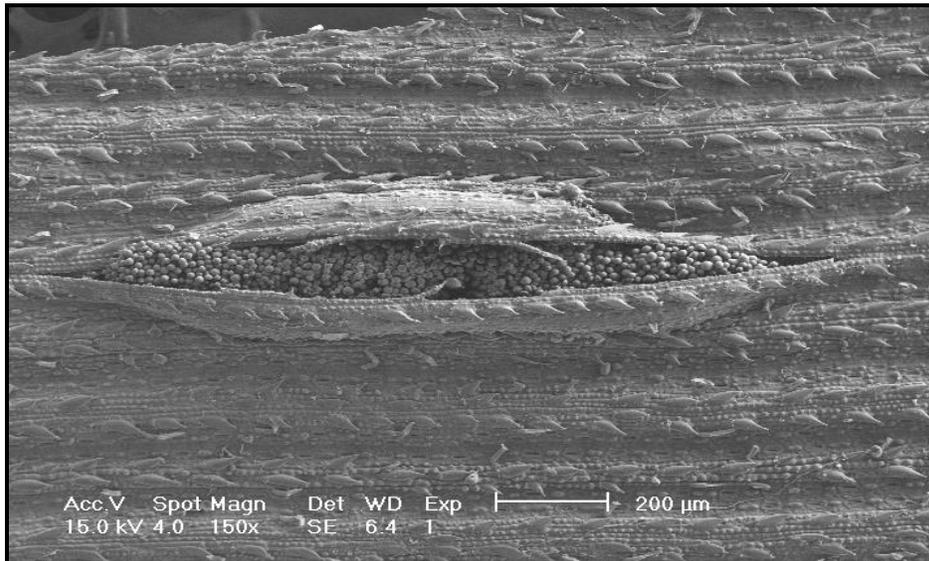
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FRONTISPIECE



Brown rust lesions on a sugarcane leaf with urediniospores emerging from the abaxial surface



Electron micrograph of *Puccinia melanocephala* urediniospores within a lesion on a sugarcane leaf

ABSTRACT

The first serious outbreak of brown rust of sugarcane caused by *Puccinia melanocephala* Syd. & P. Syd. was reported in India in 1907. It was first reported in South Africa (SA) in 1941 on the variety Co301 and is now present in almost all the sugarcane growing areas of the world. In SA, it is now described as an important disease of sugarcane, causing yield losses of up to 26% in susceptible varieties. Within the SA sugar industry, rust is controlled through the use of resistant varieties as it is the most economical method of control. However, most of the newer varieties that are being released have an intermediate resistance rating for rust. An integrated management approach for the control of rust is therefore being investigated.

Aspects investigated in this study included environmental conditions required for development of the disease i.e. epidemiology, the use of silicon (Si) as a cultural control method against brown rust and identification of gene sequences expressed in response to brown rust infection.

For the epidemiology study, inoculated plants were incubated in a dew chamber at different temperatures and leaf wetness periods. The choice of leaf wetness duration and temperature was based on urediniospore germination studies. The optimum temperature for urediniospore germination and disease development at > 98% relative humidity was found to be between 20 and 25°C with nine hours of leaf wetness. Silicon has been shown to reduce the incidence of diseases and pests in a number of crops. The ability of sugarcane to accumulate Si and the location of Si deposition was established using two uptake and deposition trials. Different concentrations of Si were applied to the plant and accumulation in the roots, stalks, old leaves and young leaves was determined using inductively coupled plasma optical emission spectrometry, with accumulation found to be roots > old leaves > stalks > young leaves. Silicon deposition in the leaves was determined using energy dispersive X-ray mapping on freeze dried specimens and significant differences were found between the upper epidermis, lower epidermis and mesophyll with the most Si being deposited in the lower epidermis. For

disease severity, plants were naturally infected with rust and rated weekly. A significant decrease in disease severity and area under disease progress curve was noted when the Si concentration increased, indicating that Si has potential in reducing rust incidence. Currently, the most reliable and economical method of managing brown rust is with the use of resistant varieties. Identification of resistance within breeding lines is therefore important. For this part of the study, suppression subtractive hybridization was used as a tool to identify differentially expressed genes between a susceptible and resistant variety and a susceptible and intermediate variety, in response to brown rust infection. Two efficient subtracted cDNA libraries were generated and differentially expressed sequences were identified within each library. The results of this study show potential for the development of molecular markers which could be used for the early identification of brown rust resistance during the breeding process.

This study forms a firm basis on which an integrated management strategy, for the management of brown rust in the SA sugar industry, could be designed. The cDNA sequences identified could be further investigated and used to develop molecular markers to select for rust resistant varieties, the epidemiology results together with further field data could be used to develop a disease prediction model and Si has potential in the field to reduce brown rust severity.

DECLARATION

I, Prabashnie Vengetsamy Ramouthar, declare that the research reported in this thesis, except where otherwise indicated, is my own original research. This thesis has not been submitted for any degree or examination at any other university.

.....

P.V. Ramouthar (Mrs)

.....

Dr. P.M. Caldwell (Supervisor)

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DEDICATION

To the two most important people in my life:
my husband Rounak Ramouthar and my mum, Irene Naidoo.
Without either of you, none of this would have been possible.

FOREWORD

The research presented in this thesis was undertaken at the University of KwaZulu-Natal, Discipline of Plant Pathology under the supervision of Dr P.M. Caldwell and the South African Sugarcane Research Institute, Crop Biology Resource Centre under the supervision of Mrs S.A. McFarlane.

Brown rust, caused by *Puccinia melanocephala*, was first reported in South Africa (SA) in 1941 on the sugarcane variety Co301. It later reappeared on N55/805 in the mid 1970s and was then still regarded as an economically unimportant pathogen. However, a second resurgence of this disease in 2000 increased the importance of brown rust within the SA sugar industry. Yield losses are estimated at 26% in the rust susceptible variety N29. Sugarcane growers in SA have limited options available to control rust, the most economical being the use of resistant varieties. In addition, most of the newer sugarcane varieties being released into the industry only have intermediate resistance to brown rust. There was therefore a need to move towards an integrated method of control requiring research on the pathogen itself, as well as investigating alternative methods of control.

This research study looked at various aspects of brown rust development with the overall objective being to use this information in the future to develop control methods for the disease. The study consists of six chapters: Chapter One - a review of the current literature available worldwide on brown rust, covering the geographical distribution, taxonomy, morphology, host range, infection process, epidemiology, symptoms, economic importance and control options presently available; Chapter Two - development of the disease under controlled environmental conditions i.e. temperature, relative humidity and leaf wetness duration; Chapter Three - uptake and accumulation of silicon (Si) in the different sugarcane plant parts in response to Si application and its deposition in the different tissues of the leaf; Chapter Four - effect of different levels of Si application on the severity of brown rust; Chapter Five - suppression subtractive hybridization to identify genes associated with brown rust resistance and Chapter Six -

review of the experimental results of the entire study with suggestions for future research.

Information from research conducted in Chapter Two, together with results from other studies, will aid in the development of a disease prediction model for brown rust for use in the SA sugar industry. Chapters Three and Four demonstrate the possibility of using Si as an alternative control measure for control of brown rust and information from Chapter Five could be used to develop molecular markers associated with brown rust resistance, enabling sugarcane Breeders to use marker assisted selection during the breeding and selection process.

CHAPTER ONE

LITERATURE REVIEW

1.1. Introduction

Sugarcane, which includes cultivated and wild species, varieties and forms of the genus *Saccharum*, is native to the tropical and sub-tropical regions of the Orient (Edgerton, 1955). It belongs to the Kingdom: Plantae, Phylum: Magnoliophyta, Order: Poales, Family: Poaceae and Genus: *Saccharum* (L.) (Anonymous, 2009a). It is one of the most important sources of sucrose and currently, 78% of the world's sugar comes from sugarcane (Anonymous, 2009b) which is predominantly grown in the tropical and sub-tropical regions of the southern hemisphere. The remainder comes from sugarbeet, which is grown in the temperate regions of the northern hemisphere (Coote, 1987).

The most commonly planted sugarcane is *Saccharum officinarum* L. and it was this species that allowed the sugar industry to spread throughout the tropics and sub-tropics (Blackburn, 1984; Raid and Comstock, 2000). In 2005, Brazil was the leading sugar-producing region in the world, producing just over 28 million tonnes of sugar, followed by the European Union with approximately 21.5 million tonnes and India with almost 14 million tonnes per season (Anonymous, 2007).

The first sugarcane plants in South Africa (SA) were planted on the KwaZulu-Natal North Coast in 1848 (Anonymous, 2007). Currently SA is the tenth largest producer of sugarcane in the world, producing an average of 2.5 million tonnes of sugar per season. About 50% of this sugar is marketed within the Southern African Customs Union while the remainder is exported to markets in Africa, Asia, the Middle East and the United States of America (USA). The SA sugar industry generates an average direct income of R6 billion annually (Anonymous, 2007; 2008).

Stevenson and Rands (1938) listed more than 440 bacteria and fungi that affect sugarcane and with the increase in production of the crop, an increase in the number of pathogens affecting the crop was noted. In SA, there are over 50 different pathogens of sugarcane (Rott *et al.*, 2000).

Brown rust (*Puccinia melanocephala* Syd. & P. Syd.), previously known as common rust, is currently an important disease in the SA sugar industry. Yield losses of up to 26% have been reported on the susceptible variety N29 (McFarlane *et al.*, 2006).

1.2. Background information

1.2.1. History of brown rust

Puccinia melanocephala Syd. & P. Syd., the causal agent of brown rust, was first described in India in 1907 on *Erianthus ravennae* (Cummins, 1971; Sathe, 1971). It was later observed on sugarcane (*Saccharum* spp.) in 1949 (Patel *et al.*, 1950) but was identified as *P. erianthi* Padw. and Khan (Egan, 1979). The two fungi were later recognised to be the same and it was accepted that the two names were synonymous, with *melanocephala* having priority over *erianthi* (Cummins, 1971; Egan, 1979).

This pathogen received much attention in the late 1970s, particularly after its rapid spread into the New World. This sparked a number of papers being published on its movement across the globe as well as a description of the causal agent. Its importance decreased with the removal of susceptible varieties but the fungus has reappeared in recent years, particularly due to the breakdown of resistant varieties (Raid and Comstock, 2000).

1.2.2. Geographical distribution

The first serious outbreak of brown rust on sugarcane was reported from India (Sivanesan and Waller, 1986; Fig. 1.1). It is proposed that the pathogen was then accidentally introduced into SA from there in 1941 (Fig. 1.1) where it caused the first ever rust outbreak on variety Co301 (Bailey, 1995; Sauntally and Autrey, 1999). It

probably spread from SA through southern and eastern Africa from 1950 to the mid-1960s and thereafter into Angola and Cameroon (Saumtally and Autrey, 1999; Fig. 1.1). It later reappeared in SA in the mid-1970s on variety N55/805 which exhibited severe symptoms (Bailey, 1995). Up until 1978, this fungus was present in scattered locations in Asia and Africa and manifested itself as localized epidemics that were of minor significance (Purdy *et al.*, 1985).

In July 1978, *P. melanocephala* was observed in the Dominican Republic (Fig. 1.1) and marked the beginning of an epidemic in the Americas (Purdy *et al.*, 1983). It was proposed by Purdy *et al.* (1985) that brown rust had spread from Cameroon, its nearest source, to the Americas via cyclonic winds. In one year it had spread to almost all the sugarcane growing areas in America except Columbia and Venezuela (Purdy *et al.*, 1985). In late 1978, the pathogen was detected in Queensland, Australia (Fig. 1.1) and spread quickly throughout the cane-growing areas of the country, severely affecting their major varieties (Taylor, 1992). The spread into Australia may have occurred from the Americas via wind or by the monsoonal winds from the Indian Ocean (Whittle and Holder, 1980).

By 1981, sugarcane rust was present in 34 countries including Australia, China, India, Mexico, SA and the USA, some of the major sugar producing countries in the world (Anonymous, 1981). By 2000, the disease had spread to 22 more countries including Argentina, Brazil, Pakistan, Philippines and Turkey and is now present in almost all the sugarcane growing areas of the world (Raid and Comstock, 2000). Most recently, brown rust was reported in Ethiopia for the first time in March, 2007 (Kelly *et al.*, 2009).

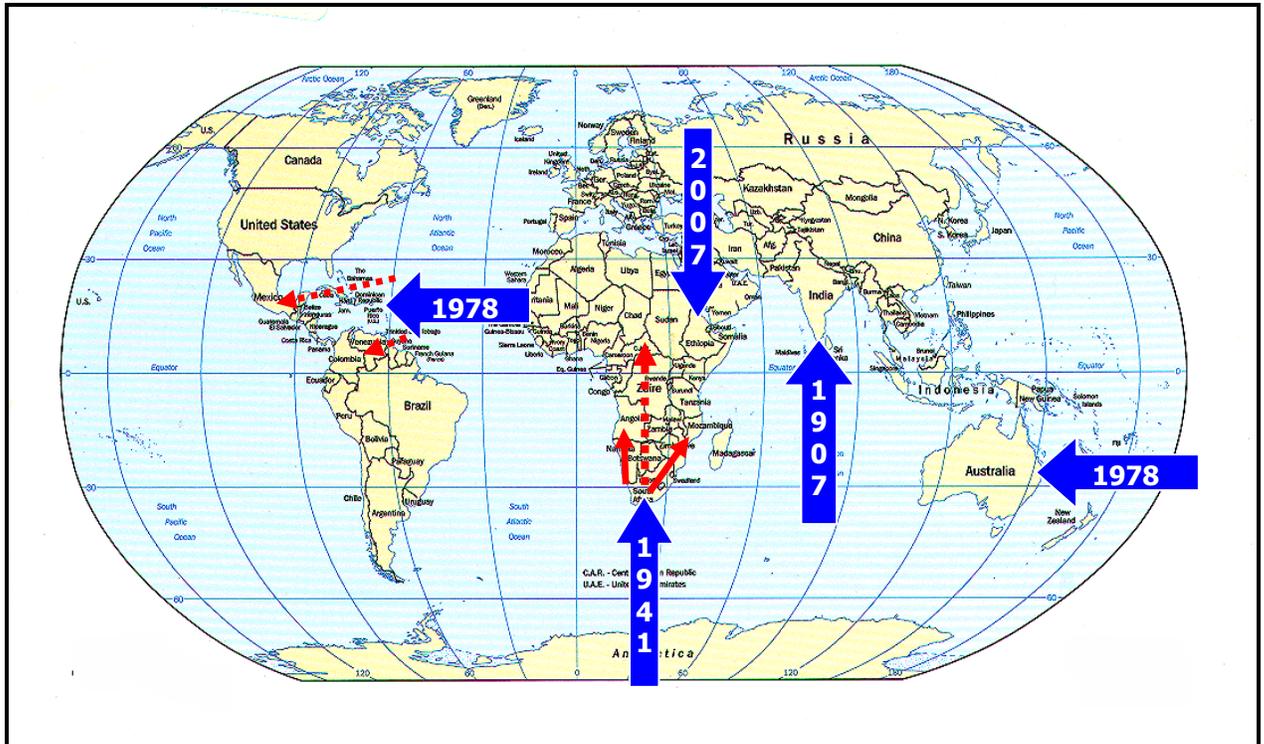


Figure 1.1. Worldwide distribution of sugarcane brown rust. Blue arrows indicate the year in which brown rust first appeared in that country. Red arrows indicate the spread of the disease; dashed lines indicate gradual spread and solid lines indicate direct entrance into a country bypassing countries before it (Prabashnie Ramouthar).

1.2.3. Economic importance

As with any other rust of Poaceae spp., brown rust may kill young plants but more often affects foliage, root growth and yield by reducing photosynthetic rate, increasing the rate of respiration, decreasing the translocation of photosynthates from the infected tissue and instead diverting materials to the infected tissue (Agrios, 2005). A reduction in cane length, cane diameter and even number of stalks per stool has been noted in severe brown rust infections. This loss in stalk number as well as biomass causes a reduction in cane tonnage (Purdy *et al.*, 1983; Raid and Comstock, 2006). Carmona *et al.* (2004) stated that one of the most important factors responsible for low yields in sugarcane is brown rust. It was, however, initially regarded as an economically unimportant disease and it was only with its spread into the Western Hemisphere in 1978 that its importance increased. This was due to the fact that it affected the variety B4362 severely, which at

that point was the principle commercial variety in most sugarcane-growing countries in that area (Liu, 1980). Yield losses of 33% were reported on this variety when the disease first appeared in the Caribbean. Due to its high rust susceptibility, this variety was removed from production in various Caribbean countries (Burgess, 1979).

In Mexico, losses due to sugarcane rust ranged from 1.7-21.7 tonnes of cane per hectare with an average loss of about 12.6 tonnes. This was an estimated 50% of the total yield lost due to brown rust between 1981 and 1982 (Bernard, 1980). By 1978, over 5000 hectares of sugarcane were affected by brown rust in Jamaica (Burgess, 1979). Yield losses recorded in replicated pot trials conducted by Comstock *et al.* (1992a) were consistent with losses observed in the field. The results of their studies showed a 53.5% loss in the highly susceptible variety B4362, 6.9% in the moderately susceptible variety CP78-1247 and 2.3% on the resistant variety CP70-1133. In the 1980/1981 season, Cuban president Fidel Castro, predicted that sugarcane yield losses in Cuba would be in the range of 57 to 71% in variety B4362 already affected in the previous season (Anonymous, 1980). In Hawaii, the commercial clone H54-0775 was phased out of production earlier than scheduled due to its susceptibility to rust which caused a 25-50% reduction in yield (Comstock and Ferreira, 1986).

In the Philippines, infection of up to 90% was noted on some sugarcane fields planted with the country's major commercial variety Phil 56-226 (Gargantiel and Barredo, 1980). About 2000 hectares of sugarcane were infected with brown rust in Taiwan from 1981-1982 (Jiang, 1985). Taylor *et al.* (1986) reported a significant reduction in shoot number in the susceptible Australian varieties 70S77, 71A123 and Q105. A 26% reduction in tonnes of cane as well as sugar per hectare and a 10% decrease in stalk height in the most susceptible variety 70S77 were noted. The variety Q90 showed a 16% reduction in tonnes of cane and a 6% reduction in tonnes of sugar per hectare. However, these results were not statistically different from the fungicide-treated control, possibly due to the fact that the fungicide programme did not give complete control of the pathogen.

It was believed that sugarcane plants could compensate for early rust infection if conditions became unfavourable for rust development. However, trials conducted by Comstock *et al.* (1992b) contradicted this. It was found that growth losses due to early rust infection persisted through to harvest. Loss of biomass did decrease as the plants matured but higher yields would have been obtained if the plants were not exposed to rust.

In SA, brown rust was initially regarded as an economically unimportant disease. Mean yield losses in the variety N55/805 were estimated to be about 5% when the disease first reappeared in SA in 1979. This variety constituted about 10% of the total cane crop and yield losses were estimated at about 100 000 tonnes of cane annually (Bailey, 1979b). It was proposed that brown rust would not increase in severity largely due to the fact that it was restricted to one variety, which was likely to diminish in popularity in the future (Bailey, 1979a). However, brown rust reappeared in SA and has been prevalent since 2000 due to environmental conditions being ideal for rust development (Cadet *et al.*, 2003) and a possible change in the pathogen (Pillay *et al.*, 2005). Of the current varieties, N29 and N33 are the most susceptible. Losses due to brown rust in the absence of control measures were estimated at 26% of the total yield in a susceptible variety such as N29 (McFarlane *et al.*, 2006). Although this variety has many other good traits, growers were dissuaded from planting it further (Cadet *et al.*, 2003). Brown rust occurs in all areas of the SA sugar industry, but is particularly prevalent in the southern parts of the industry. A number of varieties released in recent years have intermediate or partial resistance to brown rust and some losses due to the disease are likely each year (Anonymous, 2009c).

1.3. The pathogen

1.3.1. Taxonomy and morphology

Puccinia melanocephala belongs to the Phylum Basidiomycota, Class Pucciniomycetes, Order Pucciniales, Family Pucciniaceae and the Genus *Puccinia* (Agrios, 2005, Anonymous, 2009d). Other rust fungi that infect sugarcane are *P. kuehnii* (W. Krüger)

E.J. Butler 1914, the causal agent of orange rust, *Puccinia pugiensis* (Tai) and *Puccinia miscanthi* (Miura). However, only two are of economic importance; *P. melanocephala* and *P. kuehnii* (Sivanesan and Waller, 1986). Virtudazo *et al.* (2001) suggest that major rust fungi infecting sugarcane can be classified into just two species i.e. *P. melanocephala* and *P. kuehnii*.

Puccinia melanocephala and *P. kuehnii* are easily distinguished from each other by the size and morphology of the urediniospores (Purdy *et al.*, 1983; Raid and Comstock, 2000). Orange rust lesions are also never dark brown (Raid and Comstock, 2000). *Puccinia kuehnii* has been identified and distinguished from *P. melanocephala* in Florida using primers based on highly conserved regions of the 18S and 28S genes (Glynn and Comstock, 2007). Virtudazo *et al.* (2001) found that differences in the D1/D2 regions of the LSU rDNA clearly delineate *P. melanocephala* and *P. kuehnii*.

Pustules of *P. melanocephala* appear on the abaxial surface of the leaf and are cinnamon-brown containing capitate, hyaline to golden-brown, club-shaped paraphyses (Fig. 1.2). Urediniospores are orange-brown in colour, oval to pear-shaped and spiny. They are variable in size but usually have dimensions of 21-40 × 17-27 µm (Hsieh *et al.*, 1977; Liu, 1979; Sivanesan and Waller, 1986; Saumtally and Autrey, 1999). They are echinulate (Fig. 1.3a) and contain 4-5 equatorial germ pores (Fig. 1.3b) and no thickened apical wall (Liu, 1979; Purdy *et al.*, 1983; Sivanesan and Waller, 1986). The spines on the urediniospores occur in regular spaces of 1-1.5µm (Raid and Comstock, 2000). Globose to ellipsoidal appresoria form from germinated urediniospores (Sotomayor *et al.*, 1983).

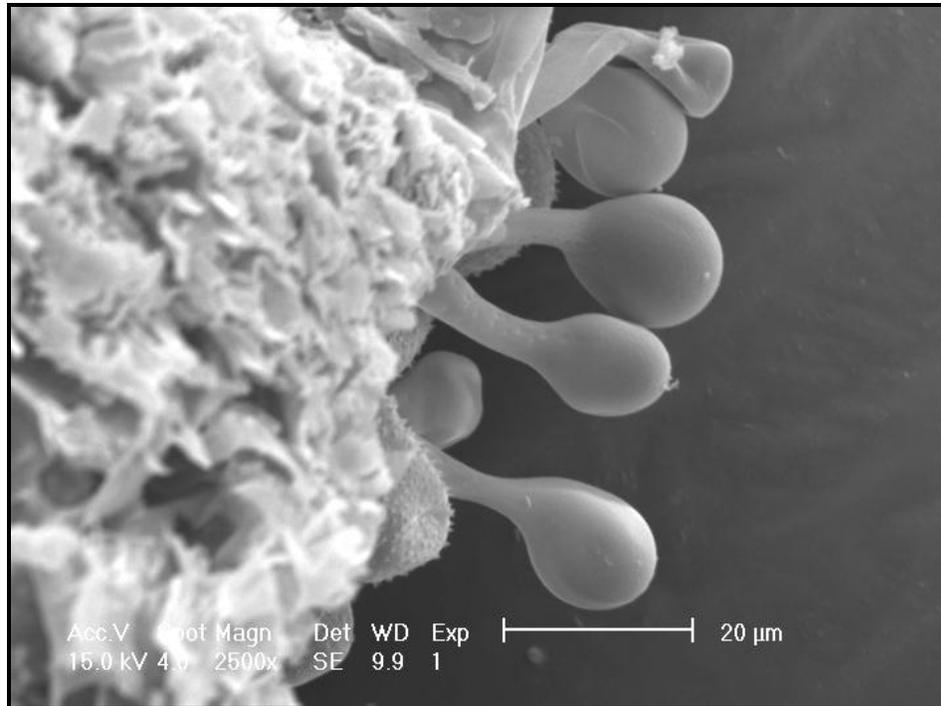


Figure 1.2. Club-shaped paraphyses contained within uredinia of *Puccinia melanocephala* (Prabashnie Ramouthar).

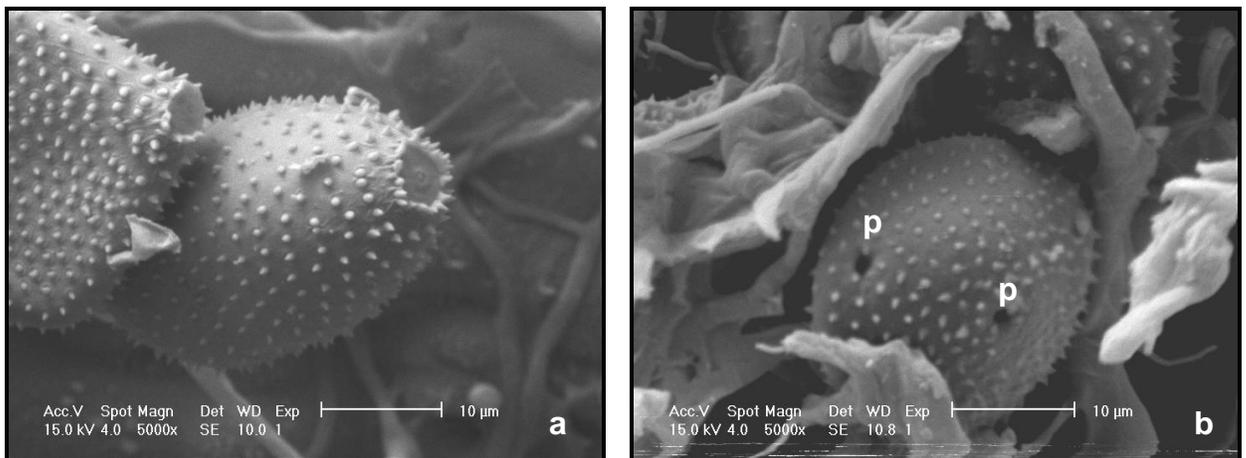


Figure 1.3. Oval shaped urediniospores with spines, (a) echinulations present at tip and (b) visible equatorial germ pores (p) (Prabashnie Ramouthar).

Telia are abaxial, dark brown, smooth and exposed. Teliospores are smooth, oblong to club-shaped, mostly two-celled but occasionally three-celled. There is a rounded apical cell with an elongated lower cell and slight constriction at the septum (Hsieh *et al.*, 1977; Purdy *et al.*, 1983, Virtudazo *et al.*, 2001). They are 32.0-58.8 µm long and 18.2-19.6 µm wide. The apical wall is 3-5 µm thick while the lower cell is 1.4-1.9 µm. Pedicels are thin-walled and up to 11 µm long (Liu, 1979). The aecial stage is unknown (Sivanesan and Waller, 1986). Teliospores (T) and urediniospores (U) (Fig. 1.4) can be found on the same plant indicating that brown rust has a short life cycle.

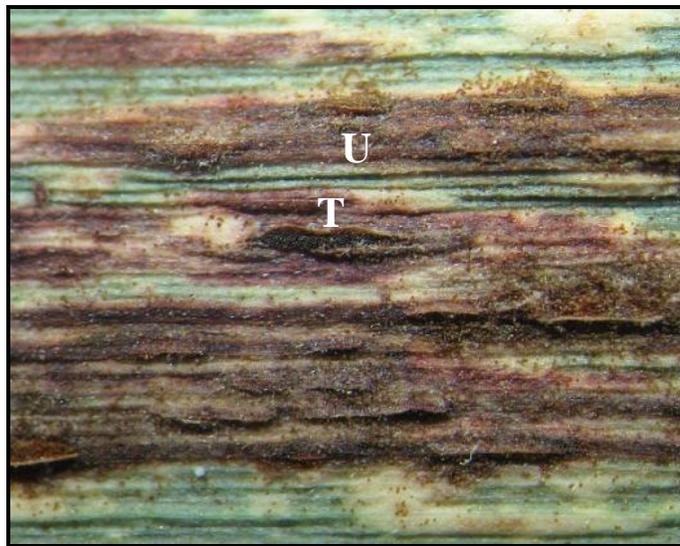


Figure 1.4. Orange-brown urediniospores (U) and black teliospores (T) on the abaxial surface of a sugarcane leaf (Kelly *et al.*, 2009).

1.3.2. Symptoms

The earliest symptoms of brown rust appear as small, elongated yellowish spots or flecks visible on both sides of the leaf (Gargantiel and Barredo, 1980) (Fig. 1.5). Although brown rust is primarily a foliar disease, uredinia have been reported on leaf sheaths and occasionally on stalks of some *S. spontaneum* L. plants (Purdy *et al.*, 1983; Raid and Comstock, 2006). The spots increase in length, turn brown to orange-brown or red-brown in colour and develop a slight but definite chlorotic halo (Fig. 1.6). Lesions usually range from 2-10 mm in length but occasionally may reach 30 mm. They are

however, never more than 1-3 mm in width and usually lie parallel to the leaf venation (Purdy *et al.*, 1983; Raid and Comstock, 2006). Up to two thirds of the leaf may be partially or completely covered with lesions (Burgess, 1979). Lesions are usually more abundant closer to the leaf tip and decrease in number toward the base (Raid and Comstock, 2006).

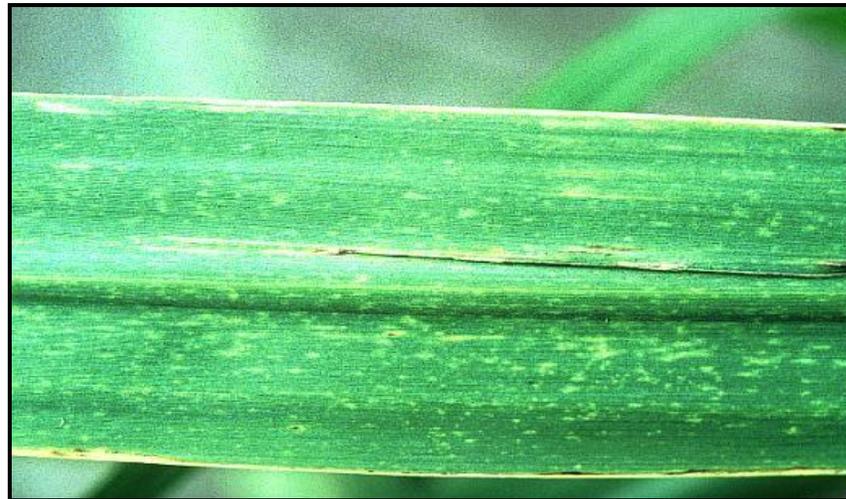


Figure 1.5. Early rust symptoms present on a sugarcane leaf (Anonymous, 1996).

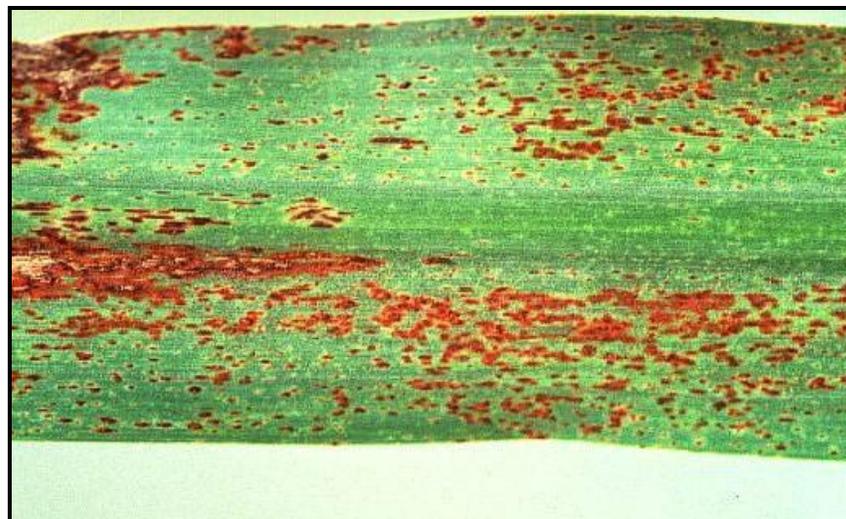


Figure 1.6. Advanced rust lesions present on a sugarcane leaf (Anonymous, 1996).

At maturity pustules erupt, burst through the epidermis and expose orange-brown urediniospores (Raid and Comstock, 2000). In highly susceptible varieties, lesions may coalesce to form large, irregular, necrotic areas. In severely infected plants, young leaves may die prematurely (Raid and Comstock, 2006). Pustules usually remain active for a long period of time. Development of urediniospores is highly dependent on climatic conditions. Lesions eventually darken, teliospores may develop and the surrounding leaf tissue becomes necrotic. In some varieties teliospores may develop without a uredinial stage (Purdy *et al.*, 1983; Raid and Comstock, 2006).

1.3.3. Host range

Puccinia melanocephala was originally found and identified on *Erianthus ravennae* (L.) Beauv. and later on sugarcane. The pathogen now predominantly occurs on species of *Saccharum*, the main hosts of the pathogen. Sporulating pustules have also been observed on *E. fulvus* Nees ex Hack., *E. rufipilus* (Steud.) Griseb. and *Narenga porphyrocoma* L. Resistance type-symptoms have been observed on *Bambusa* spp. as well as other *Erianthus* spp. (Purdy *et al.*, 1983; Autrey *et al.*, 1996; Raid and Comstock, 2000).

1.4. Infection process and epidemiology

1.4.1. Life cycle and infection process

The development of brown rust is dependent on a sequence of events starting with the germination of urediniospores and culminating in the establishment of the fungus within a susceptible plant. Every step has certain requirements in terms of temperature, humidity and even nutrient status of the plant.

Both urediniospores as well as teliospores of *P. melanocephala* have been identified and described (Purdy *et al.*, 1983). Urediniospores are more common and are generally present throughout the season while teliospores are usually found towards the end of the season as lesions darken. Basidiospores have been found but do not initiate infection on sugarcane (Purdy *et al.*, 1983).

The life cycle starts with the germination of a urediniospore to produce a germ tube of variable length. Formation of an appresorium follows after the germ tube comes into contact with a stoma. Sotomayor *et al.* (1983) found that no additional nutrients were required for the formation of appresoria or other structures, indicating that the formation of these structures is initiated by topographical stimuli. The germ tube of *P. melanocephala* can distinguish stomata from almost all other surface features (Sotomayor *et al.*, 1983).

Following formation of an appresorium over the stoma, a short penetration peg, which enters through the stomatal aperture, is formed. Direct penetration by this fungus has not been noted. After passing through the guard cells, the penetration peg enlarges and forms a vesicle that can occupy almost the entire substomatal cavity. The substomatal-vesicle is lobed and two or more (usually four) infection hyphae develop. Infection hyphae then colonize the leaf and grow intercellularly (Purdy *et al.*, 1983).

Upon contact with a mesophyll cell, the tip of the infection hypha is cut off by a septum and a haustorial mother cell is formed. The mesophyll cell wall is penetrated before the development of the haustorium. The haustoria of *P. melanocephala* differ from haustoria of other *Puccinia* spp. in that they are lobed with several lobes arranged like fingers on a hand. Haustoria develop about 36h after infection (Sotomayor *et al.*, 1983).

Puccinia melanocephala grows parallel to the leaf venation and advances in both directions from the point of penetration. Seven days after initial infection, urediniospores and paraphyses develop from sporogenous hyphae and the epidermis is subsequently ruptured by a mass of urediniospores (Purdy *et al.*, 1983) (Fig. 1.7). Brown rust is said to have a short reproductive cycle. A urediniospore can land on a leaf, infect and develop into a sporulating pustule in as little as 14 days (Raid and Comstock, 2000).

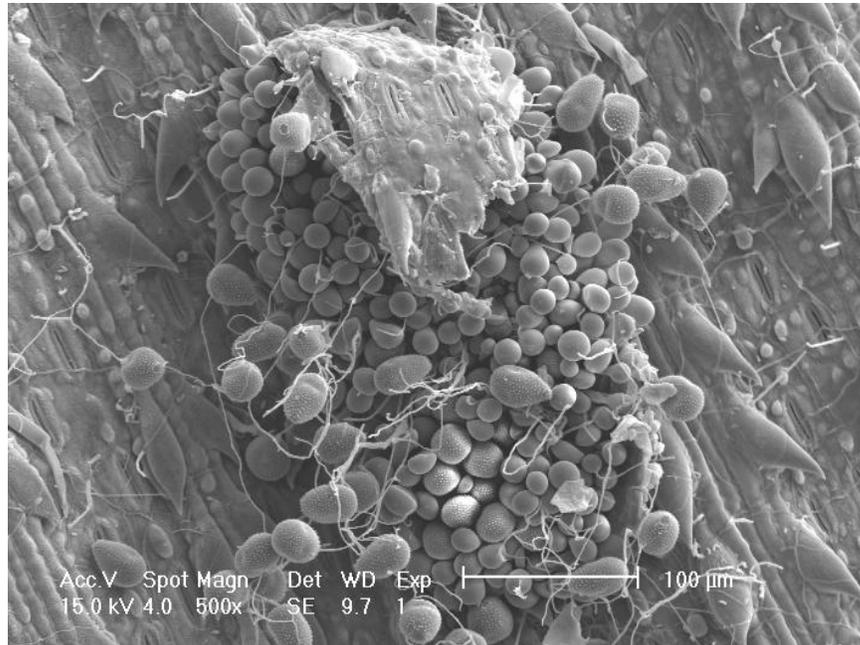


Figure 1.7. Urediniospore mass rupturing the epidermis of a sugarcane leaf (Prabashnie Ramouthar).

1.4.2. Epidemiology of brown rust

There are many factors that influence the development of brown rust on a susceptible host. The most important factors are leaf wetness and temperature (Comstock and Raid, 1994). Six-eight hours of free moisture on the leaf surface at a favourable temperature are necessary for spore germination, infection and subsequent spread of the disease. Rainfall is not always favourable for rust development as, even though it may promote leaf wetness, heavy rains tend to remove spores from the atmosphere rendering them ineffective if they land on the soil.

Urediniospore germination and appressorium formation usually occur over a fairly wide temperature range from 5-34°C requiring at least 6-8h hours of continuous leaf wetness (Purdy *et al.*, 1983; Raid and Comstock, 2000). The optimum temperature was found to be 25°C (Purdy *et al.*, 1983). Hseih *et al.* (1977) found that urediniospores germinated over the same range with optimum germination occurring at 22-26°C. Research by Sotomayor *et al.* (1983) and Raid and Comstock (2000) supported these findings. As

with urediniospore germination, appresorium formation occurs over a range of 5-30°C but with an optimum of 15-30°C. Appresorium development is reduced at temperatures of 5-10°C (Purdy *et al.*, 1983; Sotomayor *et al.*, 1983). In addition, Sotomayor *et al.* (1983) found that urediniospores began to germinate within one hour of inoculation at 25°C and 30°C and that spores germinated over a temperature range of 5-30°C in four hours of leaf wetness but appresorial formation required at least six hours of leaf wetness.

Other factors affecting development include variety, plant age and soil factors such as soil pH and nutrient status (Anderson *et al.*, 1990). Large differences in resistance have been observed among different varieties contributing to varying disease incidence in different countries as well as different areas within the same country planting different varieties. The disease is more severe in younger plants with plants between two and six months being most susceptible (Rott *et al.*, 2000).

1.5. Disease modeling

Models are often used to improve the understanding of plant disease epidemics (Jeger, 2004). They can also be used to compare epidemics which is crucial to understanding diseases, and can help in establishing and implementing management practices (Kranz, 1974). Models can also be used in disease management as a means of evaluating disease management practices or as part of an integrated disease management programme (Jeger, 2004). This is usually in the form of a complex simulation model that is used in disease forecasting (Putter 1982; Jeger, 1986), warning farmers to spray fungicides on a specific day when the risk or probability of a disease outbreak is high (Putter, 1982).

The use of disease prediction models as management tools has been researched extensively over a number of years. These models cover various crops as well as diseases including grey leaf spot on corn, Fusarium head blight and leaf and stripe rust on wheat (De Wolf and Isard, 2007). A model developed for potato late blight was used

to reduce the number of fungicide sprays by predicting periods of low disease probability and consequently enabling the farmer to either cancel or delay sprays (Henshall *et al.*, 2006).

Currently, the SA sugar industry aims to release varieties with more durable partial resistance rather than focusing on complete resistance. Brown rust may therefore still develop on the crop warranting fungicide use. However, most fungicides being tested for the control of this pathogen are protectants and application of the fungicide is required before the disease is too advanced in order for them to be effective (McFarlane, 2007). De Wolf and Isard (2007) stated that disease prediction models created and evaluated thus far have focused on fungal pathogens affecting aerial parts of the plant. Using this knowledge and the current status of brown rust in SA, the SA sugar industry is well poised to implement a disease prediction model as part of an integrated management strategy against brown rust.

1.6. Suppression subtractive hybridization and marker assisted selection

Identification of cDNA sequences differentially expressed between resistant and susceptible genotypes can be a useful tool to identify markers or genes associated with resistance. Subtractive hybridization is a successful method of isolating and identifying differentially expressed sequences (Hendrick *et al.*, 1984; Duguin and Dinauer, 1990; Hara *et al.*, 1991). However, rare sequences or those expressed at very low levels are often not detected using these methods. A refinement to traditional subtractive hybridization methods has been developed that improves the efficiency of detecting rare sequences. This method, termed Suppression Subtractive Hybridisation (SSH), combines normalization and subtraction in one step. The normalization equalizes the abundance of cDNAs within the target population and the subtraction step eliminates common sequences between the two populations. This makes it possible to detect differentially expressed sequences of both high and low abundance. It is a PCR-based technique that allows only differentially expressed sequences to amplify. Highly

expressed genes based on the PCR banding pattern are quickly identified (Diatchenko *et al.*, 1996). Suppression subtractive hybridization has been used extensively in identifying resistance genes associated with various different pathogens including *P. triticina* on barley (Neu *et al.*, 2003), hyperosmotic shock in *Dunaliella salina* (Xiong *et al.*, 2001) and tobacco blue mould (Borrás *et al.*, 2007).

Marker assisted selection is a useful tool when selecting for traits that are difficult to screen for i.e. resistance to many diseases (McIntyre *et al.*, 2005). Genotypes with resistance to a particular pathogen can be identified early in the breeding process using this methodology, saving both time and money. It has, therefore, been used extensively in the development of genotypes resistant to various pathogens (Melchinger, 1990; Kelly, 1995; Penner *et al.*, 1995; Miklas *et al.*, 1996, Sanchez *et al.*, 2000). Studies by McIntyre *et al.* (2005) suggest that markers identified on the sugarcane genome could be broadly effective in selecting brown rust and parenchyma root rot resistant genotypes within breeding programmes.

1.7. Disease management

Control of brown rust of sugarcane is largely achieved through the use of resistant varieties. Intensive fungicide programmes could be used to control the disease but are usually not economically worthwhile (Bailey, 1979a). Releasing varieties with some resistance to brown rust is therefore the most economically feasible method of control (Raid, 1992). Liu (1980) stated that fungicide spraying alone does not control the pathogen. When taking into consideration other factors that affect disease development e.g. age of cane, temperature, relative humidity, nutrient status of the soil and soil water holding capacity, an integrated disease management programme using agricultural practices as well as fungicide sprays could be implemented.

1.7.1. Resistant varieties

To date, the best way to control brown rust is to grow resistant varieties. Many resistant varieties have been identified but resistance has not been durable on some varieties,

usually due to mutation of the fungus (Raid and Comstock, 2000). Planting a range of resistant varieties is considered key to successfully controlling rust as this will reduce the impact of the disease if a new race capable of infecting a particular variety develops. Diversification of varieties can also play an important role in reducing overall disease pressure, thereby reducing natural selection pressure for one particular race (Raid and Comstock, 2006).

Despite the complex genome structure of sugarcane due to high levels of ploidy, aneuloidy and interspecific hybrid origin, progress in breeding for rust resistance has been rapid. This is largely due to the fact that this trait has high broad and narrow sense heritability (Comstock *et al.*, 1992c; Ramdoyal *et al.*, 2000). Resistance to brown rust is generally considered to be a quantitatively inherited trait (Whittle, 1980; Tai *et al.*, 1981; Chu *et al.*, 1982) and therefore different rating scales based on symptom expression have been developed (Walker, 1987).

Rust resistance variety R570 is one of the most widely cultivated varieties in Réunion and Mauritius and is included in many breeding programmes around the world (Asnaghi *et al.*, 2001). Inheritance studies of resistance involving selfing of R570 showed the presence of a major brown rust resistance gene in this variety (Daugrois *et al.*, 1996). In this study, R570 exhibited resistance against rust isolates from Brazil, Columbia, Florida, Guadeloupe, Réunion Island and Zimbabwe (Asnaghi *et al.*, 2001).

Little or no brown rust had been reported in SA for many years but it recently has become prevalent on a wide range of varieties. It is particularly severe on N29 which was initially considered to have acceptable rust resistance. Pillay *et al.* (2005) identified different genotypes of *P. melanocephala*. Although the pathogenicity of these genotypes was not investigated, this may account for the change in varietal resistance. Due to the presence of different genotypes as well as the recent change in varietal resistance, partial resistance is proposed as a method of rust management in SA.

1.7.1.1. Types of resistance

Plants employ one of two mechanisms of resistance: the first and most exploited is complete / vertical resistance and the second and less studied is partial / horizontal resistance (Agrios, 2005, Do Vale *et al.*, 2001). Vertical resistance, also known as race specific resistance, is based on an incompatible reaction which is usually covered by a gene for gene mechanism (Martin *et al.*, 2003, Perchepped *et al.*, 2006). It is dependent on the presence of both the resistance (R) gene in the plant and the avirulence (avr) gene in the pathogen. Plant R proteins usually recognize the pathogen by recognizing specific effector molecules that are produced during the infection process (Martin *et al.*, 2003). Plants exhibiting vertical resistance usually do not express symptoms of the disease. Partial resistance (PR), also referred to as nonhost resistance, was first characterized by Caldwell in 1968. Unlike VR, it does not offer complete protection against the disease. It is however, usually effective against all races of the pathogen and therefore provides a more durable alternative form of resistance (Liu *et al.*, 2001; Yu *et al.*, 2001; Singh and Rajaram, 2002). It is based on multiple mechanisms, most of which are unknown but are slowly being uncovered (Collins *et al.*, 2003; Lipka *et al.*, 2005; Perchepped *et al.*, 2006; Stein *et al.*, 2006). Incomplete expression, no marked interaction with pathogen isolates and an apparently larger genetic complexity are all characteristics of PR (Jones *et al.*, 1982; Asher and Thomas, 1983; Jones and Davies, 1985; Asher and Thomas, 1987). Usually, a reduction in the rate of development of the disease is noted in partially resistant plants (Kinane and Jones, 2000). Currently, in the SA sugar industry, variety N39 is thought to have partial resistance and N12 complete resistance to brown rust (McFarlane, 2007).

1.7.2. Chemical control

The feasibility of chemical control of brown rust of sugarcane has been investigated since the discovery of the disease. Jiang (1985) investigated the efficacy of several fungicides and found mancozeb 80% WP to be the most effective, increasing the yield of variety F176 by 26%. Frequent applications of the fungicide were however needed and this, coupled with the low net profit obtained after control of the pathogen, limited the use of fungicides in Taiwan (Jiang, 1985).

In the US sugar industry, Raid (1992) investigated the use of mancozeb and propiconazole for the control of rust on the susceptible variety CP72-1210. Although all fungicide treatments resulted in significant reductions in rust severity, treatments with mancozeb provided better control and higher yields than those with propiconazole only.

Five different fungicides were tested for the control of rust in Mauritius. The best control was achieved with fungicides containing fenpropimorph and mancozeb (Saumtally and Autrey, 1999). In Zimbabwe trials included the use of triazole fungicides and these were found to significantly reduce brown rust infections (Zvoutete, 2006).

Trials in SA involved the use of mancozeb and propiconazole (triazole). No reduction in rust severity was noted with an application of a combination of these fungicides every four weeks when compared to the untreated control but a slight increase in yield was seen. Further cost benefit analyses revealed that it was uneconomical to spray fungicides (McFarlane *et al.*, 2006).

The use of chemicals as a form of control has proved to be uneconomical unless used as part of an integrated management program. Early detection of brown rust in susceptible varieties is the key to effective, fungicide control.

1.7.3. Cultural control

Plants usually acquire resistance to the disease with increasing age (Péros and Lombard, 1986). Anderson and Dean (1986) investigated the relationship between brown rust severity and plant nutrients in sugarcane. They found that nitrogen (N), phosphorus (P) and zinc (Zn) were the most important nutrients with regard to rust severity. However, the regression models used in the study did not detect specific nutrient concentration ratios or imbalances and therefore exact nutrient correlations could not be identified. Whether leaf nutrient concentration influences rust intensity or rust intensity influences the nutrient status of the leaf is also unclear, but an association between nutrients and rust severity was noted. In SA, a clearer picture of this

relationship was noted i.e. severe rust was observed when potassium (K) levels in the leaf were high (Cadet *et al.*, 2003). It was hypothesized that K was taken up at the expense of calcium (Ca), magnesium (Mg) and silicon (Si), which have been reported to decrease the severity of foliar diseases in certain crops (Cadet *et al.*, 2003). Increased levels of K in the soil would therefore increase rust severity. In a more recent study, McFarlane *et al.* (2008) found that severe rust infections occurred when high levels K, Ca, Mg and manganese (Mn) were present in the soil. In contrast, severe rust infection was noted with low levels of Iron (Fe). Calcium could be applied before infection of the plant and this would limit the uptake of Ca during infection thereby conferring resistance to later infection (Cadet *et al.*, 2003). Grisham *et al.*, (2006) found that high rust levels were frequently found in fields with optimum or very high levels of plant nutrition in particular sulphur and phosphorous. Rust levels could, therefore be reduced by efficient fertilizer application, avoiding over fertilization.

Silicon has been known to induce resistance to many fungal diseases as well as promote the growth and development of healthy sugarcane. Cadet *et al.* (2003) suggested that application of Si would increase the Si content in leaves and therefore could reduce brown rust infection. In his review on Si, Epstein (1999) presented evidence from different researchers that Si could provide resistance to powdery mildew on cucumber and vines, *Pythium* infections in cucumber and various diseases in greenhouse crops. Silicon has also been shown to decrease the incidence of grey leaf spot (*Pyricularia grisea*) on perennial ryegrass turf (Nanayakkara and Uddin, 2008) and rice blast (*Magnaporthe grisea*), brown spot (*Cochliobolus miyabeanus*) and sheath blight (*Rhizoctonia solani*) in rice (Rodrigues and Datnoff, 2005). Silicon was reported to increase the defense properties of sugarcane against leaf freckle (undetermined pathogen), sugarcane brown rust and sugarcane ringspot (*Leptosphaeria sacchari*) (Matichenkov and Clavert, 2002). In addition Si has been shown to be a beneficial element for sugarcane and has also shown implications for control of the stalk borer *Eldana saccharina* Walker (Matichenkov and Clavert, 2002; Meyer and Keeping, 2005; Kvedaras and Keeping, 2007).

1.7.4. Biological control

Cladosporium tenuissimum is a known hyperparasite of several rust fungi (Nassini *et al.*, 2004). Urediniospore germination of bean rust (*Uromyces appendiculatus*) decreased when it was in contact with the ungerminated conidia of *C. tenuissimum* (Assante *et al.*, 2004). Sharma and Heather (2007) found that conidia of *Cladosporium* spp. were often found in urediniospore collections of *Melampsora arici-populina*, leaf rust of poplar. They demonstrated that the mycelia of *Cladosporium* spp. were able to lyse both germinated and ungerminated urediniospores.

No information is currently available on biological control of brown rust. However, as with the leaf rust of poplar, *Cladosporium* spp. are often found associated with urediniospores of *P. melanocephala* (Pillay *et al.*, 2005). Based on the biological control potential of *Cladosporium* spp. against other rusts, there is an opportunity for research into its activity against brown rust.

1.8. Summary

Sugarcane is a widely grown crop that currently accounts for 78% of the total production of sugar worldwide (Anonymous, 2009c). Brown rust of sugarcane is an important disease in SA due to its prevalence on the susceptible variety N29 as well as a range of other widely-grown intermediate varieties (McFarlane *et al.*, 2006; McFarlane, 2007).

The most reliable method of control is through the use of resistant varieties. Breeding and selection programs aimed at producing resistant varieties are key to this and marker assisted selection is useful in identifying resistant varieties early in the breeding programme. Emphasis is now being placed on breeding varieties with partial resistance to brown rust. This type of resistance is based on many genes (horizontal resistance) and it offers more durable resistance than single gene (vertical) resistance. Many of the newer varieties released in the SA sugar industry have an intermediate resistance rating for brown rust.

Partial resistance does not offer complete control of the pathogen and additional control methods may therefore be required. One of these methods is the use of fungicides. Most fungicides available for the management of brown rust are protectant and not curative and in order for them to be effective, they must be applied before symptoms appear. A model that could predict the risk of a disease outbreak based on environmental conditions at the time would provide an early warning system, informing farmers of the threat of infection. This would improve the efficiency of fungicide applications, reducing costs, increasing profits and reducing the possibility of negative environmental effects. Research on the conditions required for disease development is important before such a model can be developed.

An additional control method that can be employed is cultural control based on nutrition management. A relationship has been established between brown rust severity and nutrients, therefore efficient fertilizer application could aid in reducing the severity of the disease. Silicon, when applied to plants, has been shown to increase resistance to diseases and enhance growth. Application of Si to sugarcane could decrease rust severity by either reducing infection by rust or improving plant vigour.

Effective control of sugarcane brown rust would be more likely using an integrated management strategy. This would include the use of resistant varieties, nutrition management and efficient fungicide application with the aid of a disease prediction model.

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CHAPTER TWO

EFFECT OF TEMPERATURE AND LEAF WETNESS DURATION ON GERMINATION OF *Puccinia melanocephala* UREDINIOSPORES AND DEVELOPMENT OF BROWN RUST ON SUGARCANE

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Abstract

Understanding the conditions required for the development of a particular disease is key to effectively controlling that disease. The aim of this study was to investigate the effect of temperature and wetness duration on the germination of *Puccinia melanocephala* Syd. & P. Syd urediniospores and the development of brown rust on sugarcane (*Saccharum* spp. L.). To confirm the leaf wetness duration (LWD) in the dew chamber, Decagon leaf wetness sensors were placed within the dew chamber and monitored over a 24h period. Urediniospore germination was tested by incubating urediniospores of *P. melanocephala* on multiwell slides at different temperatures for different wetness periods. To investigate the effect of temperature and LWD on disease development, the brown rust-susceptible sugarcane variety N29 was used. Plants were inoculated with *P. melanocephala* urediniospores, maintained at > 98% RH for differing leaf wetness periods at different temperatures. Leaves were assessed for percentage leaf area covered by brown rust pustules using a disease rating program. A significant difference was noted in the sensor output at the different relative humidities, with the sensor output increasing with an increase in RH. However, the leaves were still found to be wet for the entire

period that they were in the dew chamber. Germination and infection occurred over the whole temperature range tested but was significantly higher at 20 and 25°C. An increase in urediniospore germination percentage and percentage leaf area infected was noted with an increase in wetness period. Germination percentage, however, decreased after 14h wetness. This could have been due to the germ tubes breaking off as they were too long. A significant positive effect of LWD on disease severity beyond 9h leaf wetness was only observed at 15°C. From this study, optimum germination percentage was noted at 25°C, 14h leaf wetness and highest percentage area infected was noted at 25°C with 12h LWD. Optimum conditions for rust development identified in this study, together with data obtained from another study, will be used for development of a disease prediction model for brown rust for use in the South African sugar industry.

2.1. Introduction

Many pathogens require a minimum leaf wetness duration (LWD) in order for disease to develop. Brown rust on sugarcane, *Saccharum* spp. L. caused by *Puccinia melanocephala* (Syd. & P. Syd.), requires at least 6-8h of LWD (Purdy *et al.*, 1983; Raid and Comstock, 2000) while soybean rust requires 6-12h (Marchetti *et al.*, 1976). Due to the importance of LWD in disease development, it is often used in disease forecasting models (Magarey *et al.* 2006). Methods to accurately determine LWD are therefore critical.

Development of brown rust on a susceptible sugarcane variety is influenced by many factors including temperature, humidity, host genotype, plant age and soil factors, such as soil pH and nutrient status. However, the most influential of these factors are atmospheric temperature and LWD (Comstock and Raid, 1994).

Infection structures of a rust pathogen usually develop in an orderly manner beginning with formation of a germ tube and its differentiation into an appressorium (Sotomayor *et al.*, 1983). Germination of urediniospores occurs over a fairly wide range of temperatures from 5-34°C (Purdy *et al.*, 1983). Germination decreases rapidly at

temperatures exceeding 30°C (Raid and Comstock, 2000). Appresorium formation also occurs over 5-34°C (Purdy *et al.*, 1983; Sotomayor *et al.*, 1983).

Germination occurs either in water drops or in the water film present on the leaf surface (Purdy *et al.*, 1983; Raid and Comstock, 2000). Several hours of free water on the leaf surface at a favourable temperature are necessary for spore germination, infection and consequent spread of the disease. Long dew periods and rainfall contribute to LWD. However, heavy rainfall is not favourable for rust development as even though it may promote leaf wetness, it tends to wash spores off the leaves. These spores are then unable to germinate and infect the host plant if they land on the soil (Raid and Comstock, 2006).

Brown rust is described as being a warm temperature-high humidity disease. High humidity without the correct temperature will not support development of the disease, illustrating a relationship between temperature and humidity (Purdy *et al.*, 1983). Long periods of warm and humid weather are very favourable for disease development (Sandoval *et al.*, 1983).

Disease forecasting, as part of an integrated management programme, usually involves a warning system based on a probability factor. Farmers would be warned to spray on a specific day when the risk or probability of disease outbreak is high (Putter, 1982). A complete understanding of the conditions required for disease development is therefore essential. Epidemiological studies provide an understanding of pathogen dispersal, infection, colonization, reproduction and disease spread, thereby providing precise information required for integration into a model (Wang and Hartman, 1992).

In South Africa (SA), little research has been conducted on the epidemiology of *P. melanocephala* in controlled environmental conditions, which could be used to provide the necessary data for implementation of a local model.

The aims of this study were 1. To investigate the effect of temperature and leaf wetness duration on germination of *Puccinia melanocephala* urediniospores and 2. The optimum conditions required for the development of brown rust on. The effect of temperature and LWD at a fixed humidity on germination of urediniospores and lesion development were investigated under controlled environmental conditions. Confirmation of LWD in the dew chamber formed part of a preliminary study.

2.2. Materials and Methods

2.2.1. Determination of leaf wetness duration in dew chamber

Temperature in the dew chamber used in these studies is controlled using a reversed cycle refrigeration unit and heating elements. Leaf wetness is achieved using two airbrushes mounted inside the dew chamber on either side. The airbrushes emit water in the form of fine water droplets. The droplets are circulated using two large fans as well as two smaller fans installed on either side of the dew chamber. Humidity is controlled using RH sensors positioned inside the chamber which operate solenoid valves via a control circuit. When the humidity drops to 1.5-2% below the required humidity, the airbrush switches on, increasing the humidity to its required percentage. Similarly, when humidity increases to 1.5-2% above the required humidity, the airbrush switches off, thereby allowing humidity to decrease. Leaf wetness created is therefore a direct result of water being sprayed into the chamber and not created naturally as a result of the leaf temperature going through the dew point.

Leaf wetness was measured in the dew chamber using a Decagon[®] dielectric leaf wetness sensor and the output was monitored using a Campbell Scientific[®] 21X data logger, battery and storage module.

Four temperatures (15, 20, 25 and 30°C) and four RH levels (75% RH, 85% RH and 95% RH and 100% RH) were tested. The dew chamber was run for 24h at each combination of temperature and humidity and mV output was logged on the data logger every 5 min. The leaf was considered wet for those five minutes if a mV output of ≥ 329 ,

85 (Anonymous, 2007) was observed. The total duration of LW over 24h was then determined.

2.2.2. Germination of urediniospores at different temperatures and wetness durations

2.2.2.1. Inoculum source

Urediniospores from golden brown lesions of brown rust with visible urediniospores on the surface were collected from infected sugarcane plants growing within a polytunnel at the University of KwaZulu-Natal (UKZN), Pietermaritzburg, SA.

2.2.2.2. Preparation of urediniospores for germination study

A 3.2×10^5 urediniospores mL^{-1} suspension (20 μl) was placed into each of five wells on a Teflon-coated multi-well slide (Cel-Line Associates). Each well was considered one replicate. Each slide was then placed in a 90 mm Petri dish containing moist Whatmann filter paper (7 cm diameter) and sealed with Parafilm (Pechiney Plastic Packaging). The Petri dishes were then placed in germination chambers at the required temperature for the specified LWD.

2.2.2.3. Experimental design

Four temperatures (15, 20, 25 and 30°C) and nine LWD (1, 3, 6, 9, 12, 14, 16, 18 and 24h) were tested, at a relative humidity of 100%. Each treatment was replicated five times and treatments were arranged in a randomized complete block design.

2.2.2.4. Determination of percent germination

At the end of the required LWD, Petri dishes were removed from the germination chamber and the number of urediniospores that had germinated were counted using a compound microscope (Olympus Provis) at 400X magnification. A urediniospore was

considered to have germinated if the germ tube length was greater than the urediniospore length. At least 100 urediniospores were counted for each replicate.

2.2.3. Epidemiology of brown rust

2.2.3.1. Host material

Single-budded sugarcane setts of variety N29 were grown in seedling trays (155 mm x 210 mm) holding six plants. The seedling trays were placed in plastic containers filled with water. Composted pine bark was used as the growth medium for this study. Plants were watered once a day and fertilized once a week using Hygrotech[®] seedling mix. Plants were grown in a growth room maintained at 28°C and 60% RH, with a 12h photoperiod and a light intensity of 347.17 $\mu\text{mol}/\text{sec}/\text{m}^2$.

2.2.3.2. Inoculum production

Urediniospores of *P. melanocephala* were collected from infected field-grown sugarcane plants, at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, SA. Urediniospores were painted onto the abaxial leaf surface of the plants grown in a tunnel (20-30°C, 50-100% RH with a 12-14h photoperiod) at UKZN. Plants were then placed in a dew chamber for a period of 24h and thereafter transferred to a polytunnel. Plants were maintained in the tunnel and the disease was allowed to develop. Subsequent infection of new plants occurred naturally.

2.2.3.3. Urediniospore collection

Urediniospores were collected from four different types of lesions, i.e. old lesions with evidence of mycelia; dark brown, old lesions with no mycelia; new lesions with urediniospores easily visible, golden brown in colour and very new lesions with urediniospores just visible on the surface and golden brown in colour. Urediniospores were collected using both a wet paintbrush and a vacuum pump. After collection, some urediniospores were heat shocked at 42°C for 2.5 min before plating onto 1.5% water agar and some were plated without heat shocking. Plates were then incubated at 19°C and 25°C. Percentage germination was determined after 24h of incubation by counting

up to 100 urediniospores using a compound microscope (Zeiss, ICS KF2) at 400X magnification. Five plates with three replicates were used for each treatment.

2.2.3.4. Urediniospore concentration

Seven different urediniospore concentrations, i.e. 8×10^4 , 2×10^5 , 3.2×10^5 , 1.85×10^6 , 3.9×10^6 , 8×10^6 and 1.2×10^7 urediniospores m^{-1} were inoculated onto sugarcane plants to identify the optimum urediniospore concentration, giving the best distribution of pustules on the leaf surface of variety N29. This was to ensure that disease parameters could be measured easily and to reduce competition between urediniospores for germination.

2.2.3.5. Inoculation techniques

Urediniospores, at optimum concentration, were inoculated onto the third unfurled leaf of one month old sugarcane seedlings using three techniques i.e. the Andres and Wilcoxson (1984) inoculator, a paintbrush and an airbrush. With the Andres and Wilcoxson inoculator, 0.1 ml of inoculum was sprayed over a 1 cm^2 area onto the middle of the leaf. Urediniospores were painted over the entire leaf surface using a wet paintbrush. The airbrush was used to spray urediniospores over the entire leaf surface, 0.01 ml sprayed once onto middle of the leaf (1 cm^2 area), 0.02 ml sprayed twice onto middle of the leaf (1 cm^2 area) and 0.01 ml sprayed five times over a 9 cm^2 area. Five plants with three replications were used per treatment. Plants were grown in a growth room maintained at 28°C and 60% RH, with a 12h photoperiod and a light intensity of $347.17 \mu\text{mol}/\text{sec}/\text{m}^2$.

2.2.3.6. Inoculation of test plants

Urediniospores of *P. melanocephala* were collected from the inoculum source, suspended in distilled water and adjusted to the optimum concentration using a haemocytometer. The abaxial leaf surface of the third unfurled leaf of N29 sugarcane plants was inoculated 13.5 cm from the base of the leaf. To ensure that the LWD started immediately, plants were sprayed with distilled water before being placed in the dew chamber. The dew chamber was set at the required temperature and humidity 2h before

inoculating the plants to allow the dew chamber to stabilize at the required temperature. Plants were then placed inside the dew chamber for the required LWD and then transferred to a coviron for 14 days. The coviron was maintained at a temperature of 25°C, 80% RH, 12h photoperiod and a light intensity of 66.4 $\mu\text{mol}/\text{sec}/\text{m}^2$.

2.2.3.7. Experimental design

A combination of four temperatures (15, 20, 25 and 30°C) and five LWD (6, 9, 12, 14 and 16h) was tested, at a relative humidity of > 98%. For each combination, six replications with six plants per replicate were used. Experiments were arranged as a randomized complete block design. The trial was repeated.

2.2.3.8. Disease assessment

The inoculated leaf was removed from the plant 14 days post inoculation and disease severity assessed using Assess 2.0: Image analysis software for plant disease quantification. Percentage disease severity of the inoculated area was determined.

2.2.4. Statistical analyses

All data were subjected to a general analysis of variance (ANOVA) using Genstat[®] Executable Release 9 Statistical Analysis Software. All least significant differences were determined at $P < 0.05$. To achieve normality, data were square root transformed before analysis.

2.2.5. 3-D Graphs

3-D graphs were generated using PlotIT[®] 3.2 (Scientific Programming Enterprises).

2.3. Results

2.3.1. Determination of leaf wetness duration

The trial was repeated and results were found to be similar for each temperature and humidity. The results of both trials were therefore pooled and averaged. Only the results from 25°C are graphically represented. A LWD of 24h was noted for all temperatures

and relative humidities tested. However, a significant difference was noted in the sensor output at the different relative humidities, with the sensor output increasing with an increase in RH. The highest sensor output of 401.19 mV was observed for 100% RH and the lowest (328.19 mV) for 75% RH. There was no significant difference between sensor output at 75 and 85% RH. The 95% and 100% treatments were significantly different to both the 75 and 85%, however there was no significant difference between the 95% and 100% (Fig. 2.1).

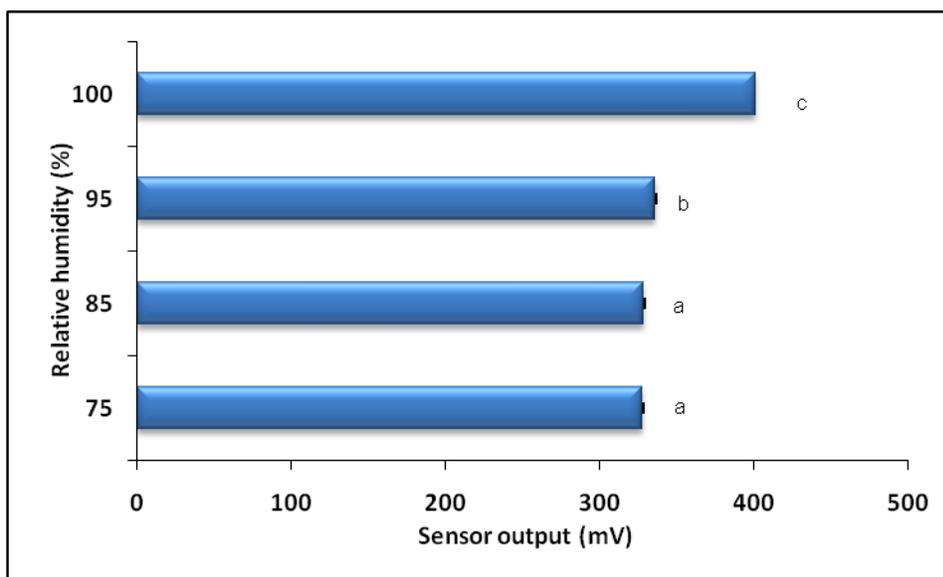


Figure 2.1. Mean sensor output of Decagon leaf wetness sensor over a 24h period measured at different relative humidities in a dew chamber set at 25°C, Bars represent standard error. Treatments with the same letter are not significantly different (P<0.05).

2.3.2. Germination of urediniospores on multiwell slides

Low urediniospore germination percentages were noted at 15 and 30°C, with optimum germination occurring at 20 - 25°C (Fig. 2.2 and Table 2.1). Generally, an increase in percentage germination occurred with an increase in LWD. However, optimum LWD was noted at 14h, for all temperatures, above which percentage germination of urediniospores decreased (Fig. 2.2). The interaction between temperature and leaf

wetness was found to be significant. Optimum germination percentage was noted at 25°C, 14h leaf wetness (Fig. 2.3).

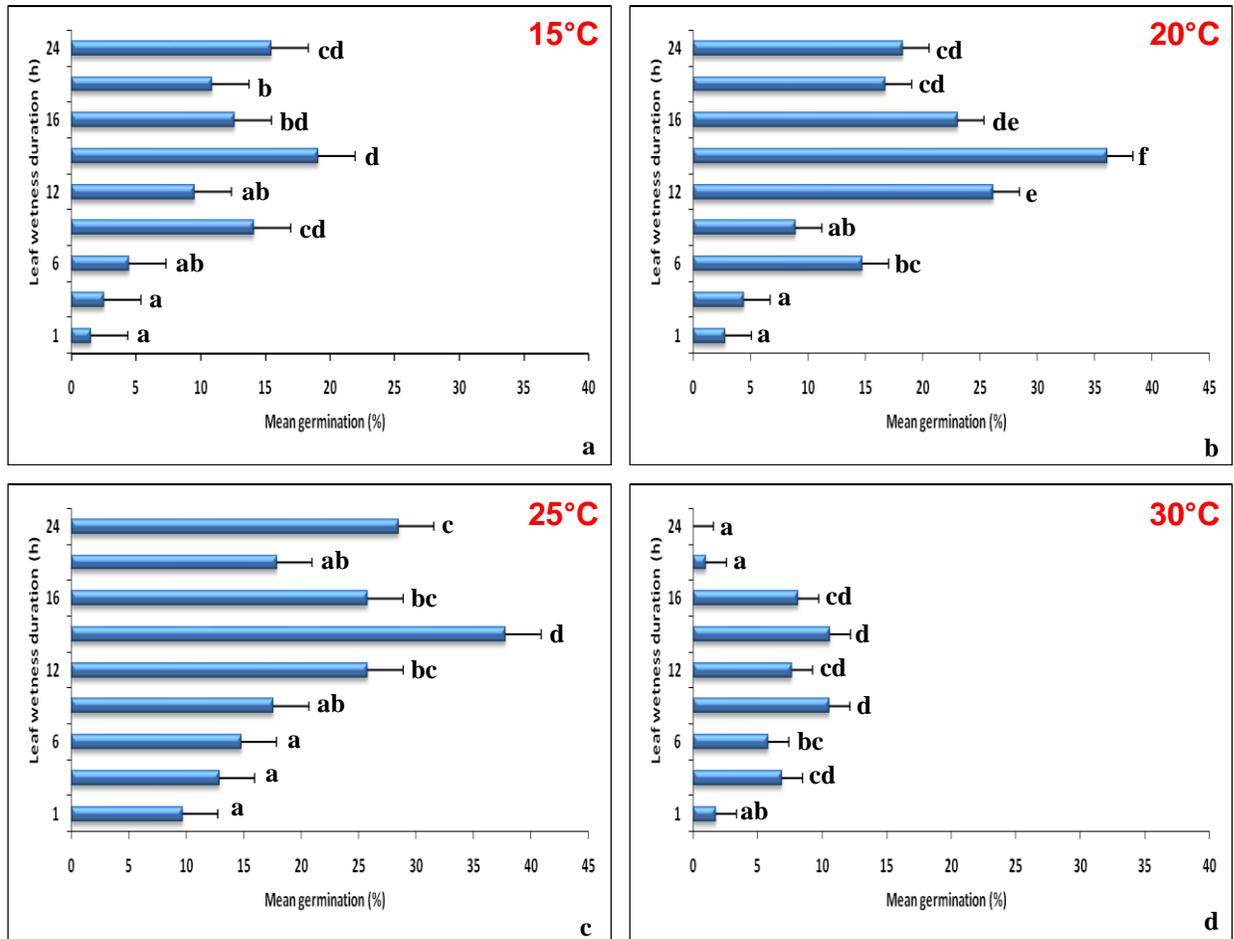


Figure 2.2. Mean germination percentage of *Puccinia melanocephala* urediniospores on multiwell slides incubated at (a) 15°C, (b) 20°C, (c) 25°C and (d) 30°C for varying wetness periods at 100% RH. Bars represent standard error. Treatments with the same letter are not significantly different ($P < 0.05$).

Table 2.1. Statistical probabilities obtained after analysis of data obtained from N29 sugarcane plants inoculated with *Puccinia melanocephala* and exposed to different temperatures and leaf wetness durations at > 98% relative humidity (RH) using a general analysis of variance (ANOVA) in randomized blocks. Data for 20°C and 30°C were square root transformed before analysis

	15°C	20°C	25°C	30°C
Grand Mean (%DS)	10.03	16.82	21.2	5.83
F probability	< 0.01	< 0.01	< 0.01	< 0.01
I.s.d.	8.21	6.66	8.82	4.57
% cv	21	12.4	8.3	16.3

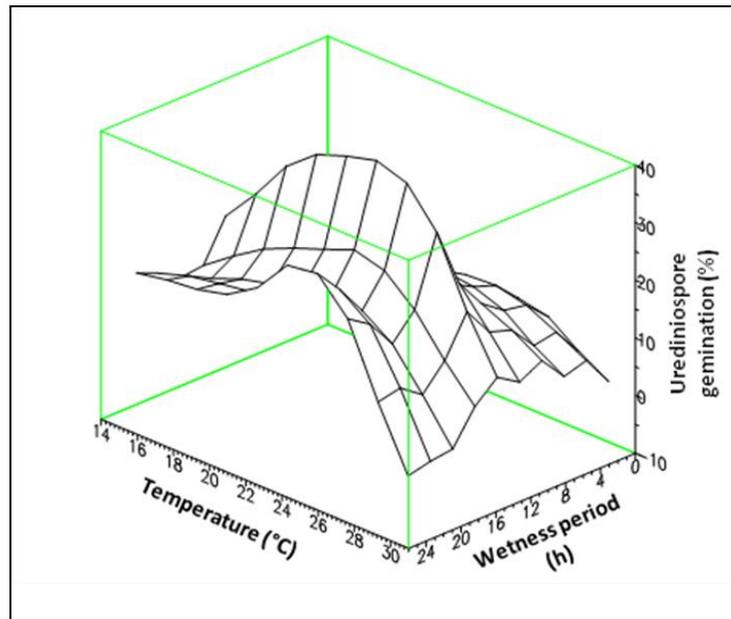


Figure 2.3. Effect of temperature and wetness period on germination of *Puccinia melanocephala* urediniospores on multiwelled slides at 100% RH.

2.3.3. Urediniospore collection, urediniospore concentration and inoculation technique

Urediniospores collected from the freshest lesions using a vacuum pump germinated best (Table 2.2). Heat shocking had no significant effect on urediniospore germination. The best urediniospore concentration was found to be 3.2×10^5 urediniospores mL^{-1} as this resulted in evenly distributed lesions over the inoculated area. The most convenient and accurate inoculation technique was the airbrush method where urediniospores were sprayed five times over a 9 cm^2 area. This technique was therefore used for all further inoculations of plants.

Table 2.2. Observation of germination efficiency of urediniospores collected from lesions at different stages and grown on water agar, incubated at 20°C for 14h

Old lesions + mycelia	Old lesions - no mycelia	New lesions- urediniospores easily visible	Very new lesions - urediniospores barely visible
Very few urediniospores germinated (<10%), short germ tubes observed	Very few urediniospores (<10%) but germ tubes from urediniospores were observed to be longer	Higher germination percentage (>50%). Long germ tubes. Very good germination of urediniospores	Very few urediniospores germinated (<10%), but germ tubes from urediniospores were long

2.3.4. Disease development at different temperatures and leaf wetness durations

Infection occurred at all temperatures tested but a higher disease severity was observed at 20°C and 25°C compared to 15°C and 30°C (Fig. 2.4 and 2.5, Table 2.3). Mean infection at 15°C and 30°C was found to be < 1% and was also found to be significantly less than infection at 20°C and 25°C (Fig. 2.5). A significant difference was noted between all LWD at 15°C and there was no significant difference between LWD at 20°C (Fig. 2.5a and b). For 25°C and 30°C, no significant difference was noted between 6h and 9h LWD (Fig. 2.5c and d). At 25°C, there was no significant difference in disease severity at 12h, 14h and 16h LWD. However, the disease severity was found to be significantly higher when compared to 6h and 9h (Fig. 2.5c). At 30°C, 12h and 14h were

significantly different to 6h but 16h was not significantly different to 6h or 9h LWD (Fig. 2.5d). Infection was found to be highest at 20°C (Fig. 2.5). Data for 20°C and 30°C were square root transformed before analysis but data presented in Figure 2.5 are untransformed means.

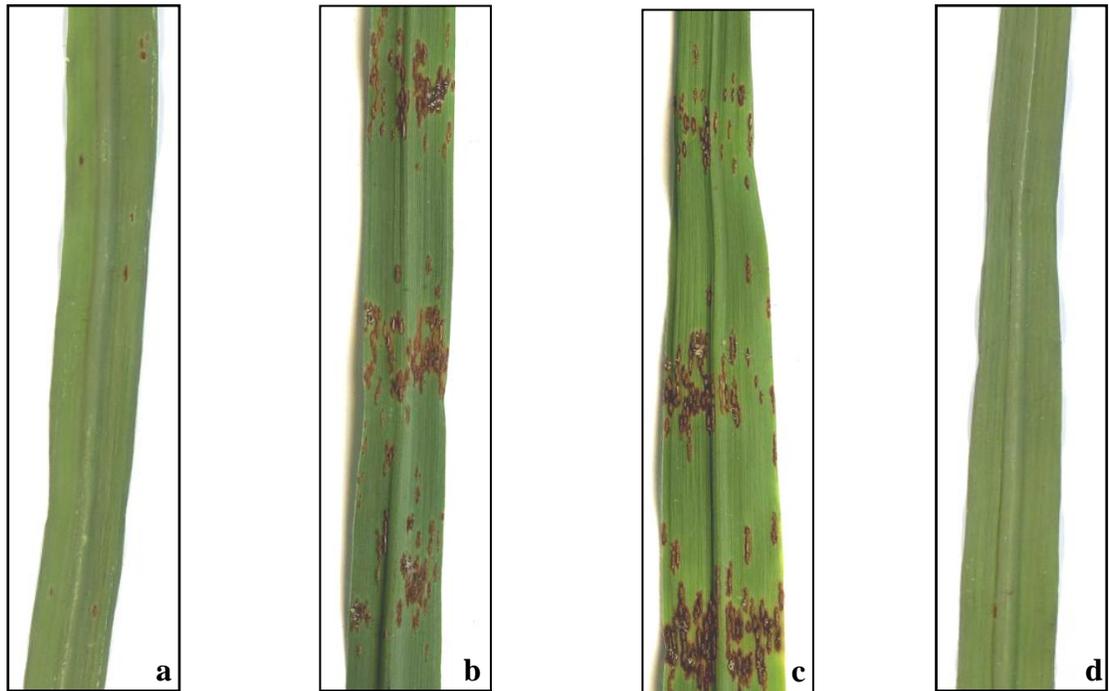


Figure 2.4. Sugarcane leaves showing infection after 16h at (a) 15°C, (b) 20°C, (c) 25°C and (d) 30°C at > 98% RH.

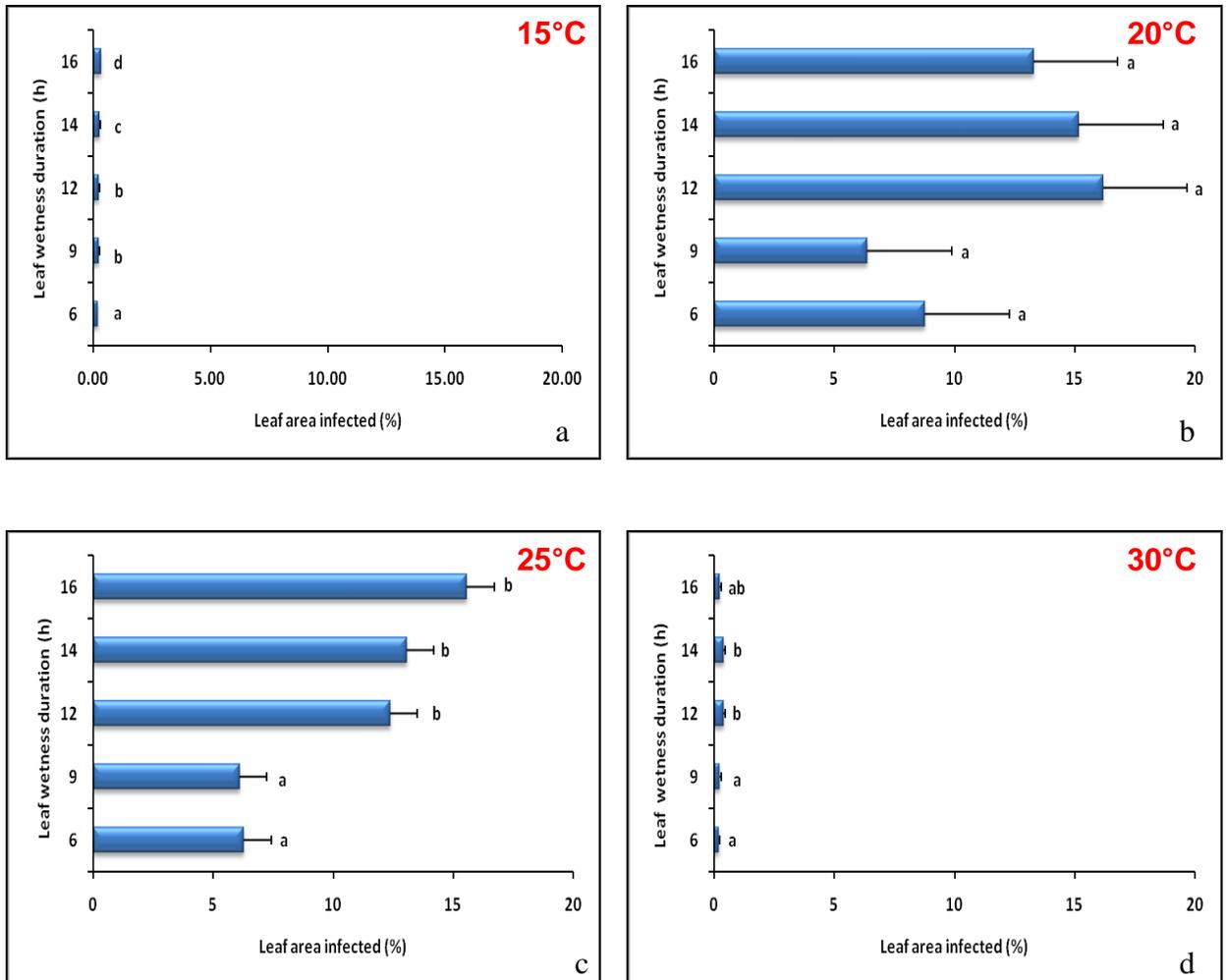


Figure 2.5. Mean percentage leaf area infected of N29 sugarcane plants inoculated with *Puccinia melanocephala* and incubated at (a) 15°C, (b) 20°C, (c) 25°C, (d) 30°C and > 98% relative humidity for 6, 9, 12, 14 and 16h. Plants were thereafter maintained at 25°C, 80% RH for two weeks. To normalize the data from 20°C and 30°C, they were square root transformed before analysis. Bars represent standard error. Treatments with the same letter are not significantly different, $P < 0.05$.

Table 2.3. Statistical probabilities obtained after analysis of data obtained from N29 sugarcane plants inoculated with *Puccinia melanocephala* and exposed to different temperatures and leaf wetness durations at > 98% relative humidity (RH) using a general analysis of variance (ANOVA) in randomized blocks. Data for 20°C and 30°C were square root transformed before analysis

	15°C	20°C	25°C	30°C
Grand Mean (%DS)	0.27	12	10.69	0.30
F probability	0.003	0.152	< 0.001	0.037
I.s.d.	0.06	1.33	3.33	0.15
% cv	19.1	33.2	25.8	23

The interaction between LWD and temperature was found to be statistically significant. An increase in percentage leaf area infected was noted with an increase in LWD. The highest percentage area infected was noted at 25°C with 12h LWD (Fig. 2.6).

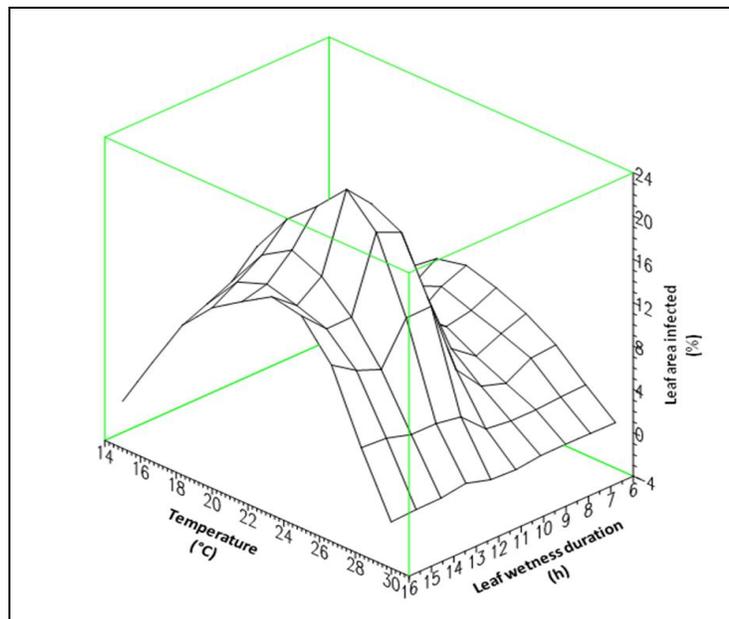


Figure 2.6. Effect of temperature and leaf wetness on percentage leaf area infected by *Puccinia melanocephala* at > 98% RH.

2.4. Discussion

During the LWD studies it was confirmed that leaves were wet for the entire period that they were in the dew chamber. This is of great importance as LWD is a critical variable in epidemiological studies and must be measured accurately. Therefore the leaf wetness period described in the study is an accurate representation of actual leaf wetness and not the time that the plants were in the dew chamber for. The significant difference noted in the sensor output (Fig. 2.1) indicates that the sensor is able to detect the increased / decreased moisture levels due to the increased / decreased humidity levels. Data like this could be useful when using an automatic weather in the field as it would enable one to estimate humidity levels based on sensor output. This further illustrates the reliability of the dew chamber to produce an accurate controlled environment in which epidemiological studies can be performed.

A general increase in urediniospore germination (Fig. 2.2) and disease severity (Fig. 2.5) was noted with an increase in LWD. The presence of water is essential for the germination of rust spores (Purdy *et al.*, 1983). Sotomayor *et al.* (1983) found that *P. melanocephala* urediniospores germinated over a temperature range of 5-30°C if liquid water was present for at least 6h. In addition, it was found that urediniospores would germinate in as little as 1h LWD. These results were confirmed in this germination study as urediniospores germinated at all tested temperatures (15-30°C) and LWD (1-24h) except 30°C with 24h LWD. The drop in spore germination at >14 h leaf wetness could be attributed to the germ tubes becoming too long and breaking off before they could be counted. Hence, spores that were counted as ungerminated could have been spores that had germ tubes but they broke off prior to being counted.

Previous studies by Purdy *et al.* show that infection of sugarcane by *P. melanocephala* occurs over a temperature range of 5-34°C, with the optimal range being between 15-25°C (Purdy *et al.*, 1983). This was also confirmed in this study as it was shown that N29 sugarcane leaves were infected by *P. melanocephala* over the entire study temperature range of 15-30°C, with optimal infection occurring at 20°C (Fig. 2.5).

Germination and infection in this study both occurred optimally at 20-25°C and 9-12h LWD (Fig. 2.3 and 2.6), indicating a link between urediniospore germination and disease development. Even though spore germination occurred at 15°C and 30°C (Fig. 2.2), very little infection was noted at 15°C and 30°C (Fig. 2.5). This could be attributed to two factors: the first being that germination percentages were much lower at 15°C and 30°C (Table 2.1) and the second being that the germ tubes developed more slowly at these temperatures and were therefore too short to reach the stomata during the specified leaf wetness and therefore penetrate the leaf and cause infection. This point is further illustrated by the point that an increase in leaf wetness duration showed an increase in disease severity as well. At 15°C, this increase was significant (Fig. 2.2a). This indicates that the more time the spore was exposed to wetness, the more time the germ tube had to develop and the longer it became. These longer germ tubes could then find the stomata, penetrate the leaf and cause infection.

However, the effect of temperature and LWD on disease development is due, not only to the effect of these parameters on urediniospore germination but also appressorial formation. It was found in the study by Sotomayor *et al.*, (1983) that appressoria formed over the same temperature range as urediniospore germination. Appressorial formation is essential for infection and subsequent disease development as it forms the basis on which the penetration peg is formed (Purdy *et al.*, 1983). Disease development in this study could therefore be attributed to both urediniospore germination and appressorial formation (based on literature), which is greatly affected by temperature and wetness period.

From the study, it was determined that the optimum temperature for development of brown rust on sugarcane is 20-25°C. Only a small difference in disease severity was noted between these temperatures. In addition, LWD of ≥ 12 h did not have a significant positive effect on disease severity, except at 30°C. This study is consistent with other studies but further research in this area is required. Relative humidity has been known to influence the development of diseases and therefore humidity ranges below 98% RH should be tested. Preliminary studies show that infection does not occur below 95% RH,

but this must be proven using full trials. Leaf wetness durations of < 6h could also be tested.

The results generated from this study together with data from a separate study being conducted at the South African Sugarcane Research Institute will be used in the development and implementation of a disease prediction model which would allow for more efficient control of brown rust. Studies conducted locally, using a local rust isolate is important when the model must be implemented locally.

2.5. References

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CHAPTER THREE

UPTAKE AND DEPOSITION OF SILICON IN N29 SUGARCANE PLANTS

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Abstract

Silicon (Si) has been shown to reduce the incidence and severity of pests and diseases in a number of crops. Improved understanding of the uptake of Si in the different plant parts as well as the deposition of Si within the leaves of the plant would aid in establishing the potential of Si as a control measure against brown rust of sugarcane, a foliar disease caused by the fungus *Puccinia melanocephala* Syd. & P. Syd.. Silicon was applied to plants of the rust susceptible sugarcane (*Saccharum* spp.) variety N29 at eight different concentrations. In the first trial, old leaves, young leaves and stalks were digested using dry ashing, wet nitric acid and microwave digestion methods. Roots were digested using the microwave method only. Digested samples were analyzed using inductively coupled plasma optical emission spectrometry. In the second trial, transverse sections of the leaf were viewed using an environmental scanning electron microscope. Silicon deposition in the upper epidermis, lower epidermis and mesophyll were measured using energy dispersive X-ray mapping. Silicon deposition was quantified using AnalySiS Version PRO 3.2. The microwave method was found to be the optimum digestion method showing the Si accumulation to be roots > old leaves > stalks > young leaves. Significant differences were found between the three tissue types with the most Si being deposited in the lower epidermis and the least in the mesophyll. Results also

showed that Si uptake in the different plant parts and deposition in the leaves did not significantly increase when Si was applied at a concentration of $\geq 1600 \text{ mg } \ell^{-1}$. Based on evidence of uptake of Si by sugarcane plants and its preferential deposition in the lower epidermis of the leaf, application of Si to sugarcane may provide an additional method of control for *P. melanocephala*, a pathogen that usually infects through the undersurface of the leaf.

3.1. Introduction

Although all plants growing in soil will take up silicon (Si), Si accumulation in tissues of different plants varies. Silicon uptake and deposition is an important characteristic of the family Poaceae, to which sugarcane (*Saccharum* spp.) belongs (Motomura *et al.*, 2006). Silicon is absorbed from the soil via the roots in the form of silicic acid [$\text{Si}(\text{OH})_4$], an uncharged monomer molecule (Ma and Yamaji, 2006; Motomura *et al.*, 2006). This uptake occurs when the solution pH is below 9 (Ma and Yamaji, 2006). Silicon is then translocated to the shoot via the xylem. When the concentration of silicic acid exceeds 2mM, silicic acid polymerizes to form silica gel ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) which is then deposited in the cell.

This process of silification in the above-ground parts of grasses and sedges differs depending on cell type, tissue, organ and species in which deposition occurs as well as the amount of Si present in the culture medium (Sakai and Sanford, 1984). Large amounts of Si are deposited in the cell walls of grasses, particularly in the walls of specialized epidermal cells of the shoot. In sugarcane, Si deposits are highest in the inner tangential walls of the root epidermis and in the silica cells of the leaf and stem epidermis (Kaufman *et al.*, 1981). There are seven major types of epidermal cells found in leaf blades viz. silica cells, cork cells, long cells, trichomes, guard cells, subsidiary cells and buliform cells (Sakai and Sanford, 1984). However, research has been aimed at silica cells only. Silica cells are unique, differing from other epidermal cells in that loss of cytoplasm and cell death occurs just before Si accumulation. Silicon is located in the

cell lumen of silica cells, yet another unique feature of these epidermal cells (Sakai and Sanford, 1984).

Silicon has been shown to be beneficial to plants by alleviating plant stress, ensuring better growth and improving plant resistance to pests and diseases. These beneficial effects of Si, whether direct or indirect, have been demonstrated on a wide variety of crops including barley, cucumber, oats, rice, sugarcane and wheat (Datnoff *et al.*, 2001, 2007; Ma and Takahashi, 2002)

The aim of this study was to determine the difference in accumulation of Si in the roots, stalks, old and young leaves and deposition of silicon in the upper epidermis, lower epidermis and mesophyll tissue of N29 sugarcane plants.

3.2. Materials and methods

3.2.1. Test plants

One month old sugarcane seedlings of the variety N29 were transferred to composted pine bark (Gromor) in six-celled seedling trays (155 mm × 210 mm). After transplanting, seedling trays were placed in plastic containers (80 × 150 × 210 mm) (Food Packaging Distributors) and filled with water. They were fertilized once a week using Hortichem 3:1:3 (N:P:K) and Si. Trays were placed in a glasshouse (23-27°C, 80% RH) for the duration of the trial. Plants were grown for a further six weeks before they were analyzed.

3.2.2. Application of silicon

Silicon was applied as soluble potassium silicate (K_2SiO_3) at seven different concentrations. An additional treatment of Calmasil[®] (Omnia Fertilizer (Pty.) Ltd.), incorporated into the growing medium at planting at a dosage of 52 g 5 kg⁻¹ potting mix was used. Potassium silicate (600 ml), at the specific concentration required, was added to the plastic container once a week. Liquid already present in the container was removed prior to the addition of the solution.

3.2.3. Experimental design

The trial consisted of eight treatments i.e. 100, 200, 400, 800, 1016 (Appendix 1), 1200, 1600, 2000 mg t^{-1} Si and a control to which no Si was added. The trial was replicated four times and six plants per replication were used.

3.2.4. Harvesting of plants

Roots, stalks and leaves were collected six weeks after transplanting (i.e. they were 2.5 months old). For the Si uptake trial, all leaves above and including the dewlap leaf were considered to be young while leaves below the dewlap leaf were considered to be old. Tissue samples were dried overnight at 80°C. Plant sections were first ground in a coffee grinder followed by an analytical plant mill and passed through a 2 mm mesh. For the deposition trial, the third leaf below the dewlap leaf was removed from the 0, 400, 800, 1200, 1600, 2000 mg t^{-1} Si treatments. Transverse sections of the leaf were cut and freeze dried overnight to preserve structure.

3.2.5. Digestion of plant samples to determine silicon uptake

3.2.5.1. Dry ashing digestion

Ground plant tissue (500 mg) was added to ceramic crucibles and ashed overnight at 650°C. Ashed samples were then transferred to nickel crucibles together with 5 ml sodium hydroxide (NaOH, 15% w/v). The crucibles were placed on a sand heater at approximately 50°C until all the NaOH had evaporated. The digested mixture was allowed to cool and then rinsed out into 15 ml plastic bottles using 10 ml ultra pure water. Five millilitres of hydrochloric acid (HCl) was added to clear samples before analysis.

3.2.5.2. Nitric acid digestion

Ground plant tissue (100 mg) was placed in a glass Erlenmeyer flask together with 5 ml nitric acid (HNO_3 , 70% v/v). The flask was placed on a sandbath and heated to approximately 125°C. Nitric acid was continuously added until the plant material was completely digested. The nitric acid was allowed to evaporate and upon cooling, 10 ml NaOH (15% w/v) was added. The flask was then placed back onto the sandbath and the

NaOH allowed to evaporate. The digestate was then rinsed with 10 ml double-distilled water and transferred to plastic bottles. Five blanks containing no plant material were digested with each set of samples.

3.2.5.3. Microwave digestion

Samples were digested using the CEM Microwave accelerated reaction system (Mars) 5 microwave (Appendix 2). Ground plant tissue (100 mg), together with 3 ml HNO₃ (70% v/v) and 2 ml hydrogen peroxide (H₂O₂) was added to the microwave digestion tube. Tubes were capped, microwaved and the acid digestion completed. Upon completion, vessels were removed and 20 ml NaOH (10% w/v) was added to the tubes. The tubes were recapped and returned to the microwave for completion of the alkali digestion. The digestate was cleared using 3 ml HNO₃ (v/v).

3.2.6. Analysis of silicon uptake and deposition in the leaves

Silicon uptake was measured by diluting samples 10 fold and analyzing for Si content using a Varian 720 ICP-OES at 252.411nm. Freeze dried leaves were viewed using a Philips XL30 Environmental Scanning Electron Microscope (HOLLAND) and Si deposition within the leaf was mapped using Energy Dispersive X-RAY Microanalysis (EDX mapping) using EDAX PHOENIX version 3.2. digital microanalyzer, at an accelerating voltage of 15 kV, working distance of 10mm and chamber pressure between 0.9–1.0 torr, the temperature was approximately 25°C and RH 5%. The maps were created at 43 minutes per scan. To quantify Si deposition, maps were analysed for percent area of Si deposits in the upper and lower epidermis as well as the mesophyll using AnalySiS Version PRO 3.2. Regions of interest were marked off in each tissue type and the percent area was determined using set threshold settings (Fig. 3.1). For each leaf, five regions of interest were measured in the upper and lower epidermis and three regions of interest within the mesophyll. The average percentage area covered by Si deposits was determined for each area.

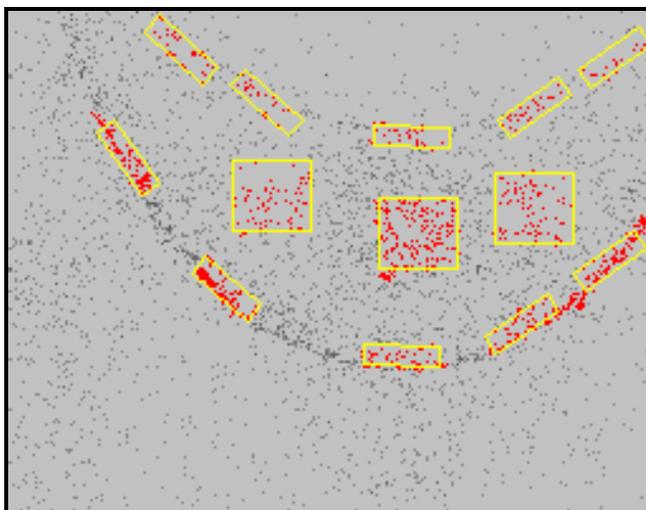


Figure. 3.1. Regions of interest within the upper and lower epidermis and mesophyll used for measurement of percent area covered by silicon deposits. Silicon deposits are indicated as grey dots and regions of interest are indicated by yellow blocks. Silicon deposits counted appear as red dots.

3.2.7. Statistical analyses

Data were normalised by transforming to square root percentage area before conducting a general analysis of variance (ANOVA), regression and correlation analyses using Genstat[®] Executable Release 9 Statistical Analysis Software. The least significant difference was determined at $P < 0.05$.

3.3. Results

The dry ashing method and the microwave method showed similar trends of an increase in Si uptake with an increase in concentration of Si added to the plant. Both methods also showed the Si accumulation in the different plant parts was as follows: old leaves > stalks > young leaves. Roots, digested using the microwave method, showed higher Si accumulation than other plant parts. The wet digestion method did not show any obvious trends.

3.3.1. Dry ashing digestion

In general, an increase in Si concentration in the different plant parts was noted with an increase in the concentration of Si added (Fig. 3.2), with most of the Si being accumulated in the old leaves. Accumulation of Si in the plant parts of the Calmasil treatment ($1016 \text{ mg } \ell^{-1}$) was lower than expected. There was also no significant difference in concentration of Si in the old leaves in the $1200 \text{ mg } \ell^{-1}$ and $1600 \text{ mg } \ell^{-1}$ treatments with a significant decrease in Si accumulation in the $2000 \text{ mg } \ell^{-1}$ treatment. There was no significant difference between the $1600 \text{ mg } \ell^{-1}$ and $2000 \text{ mg } \ell^{-1}$ in the young leaves and between $1200 \text{ mg } \ell^{-1}$ and $1600 \text{ mg } \ell^{-1}$ in the stalks. Although Si was present in the controls, a significant difference in Si accumulation between the controls and the Si treated plants was noted. The interaction between treatments (concentration of Si added * concentration of Si in plant parts * concentration of Si in different plant parts) was also found to be significant.

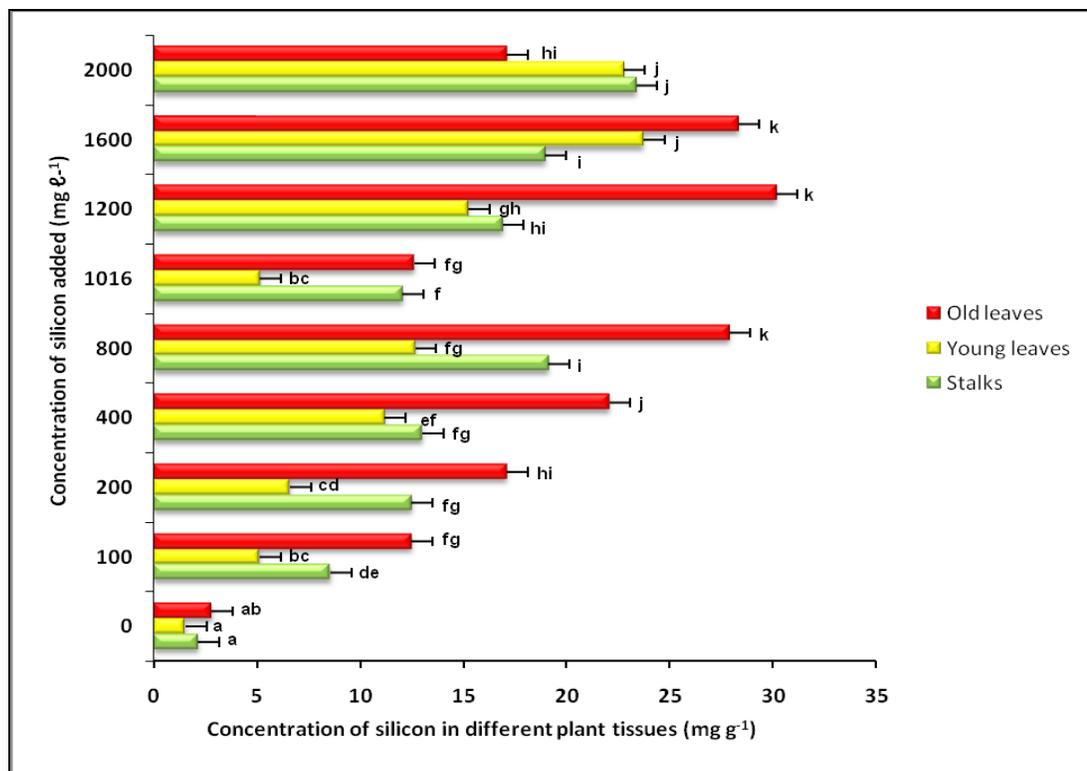


Figure. 3.2. Mean accumulation of silicon in stalks, old leaves and young leaves of N29 sugarcane plants subjected to varying concentrations of silicon, as determined by dry ashing digestion and ICP analysis. ‘Old leaves’ were all leaves below the top visible dewlap (TVD) and ‘young leaves’ were all leaves above and including the TVD. Bars represent standard error. Means with the same letter are not significantly different ($P < 0.05$).

3.3.2. Nitric acid digestion

No significant difference was noted between the controls and the treatments or between the treatments alone. In addition, no obvious trends in Si accumulation in the plant parts in response to Si added could be established (Fig. 3.3).

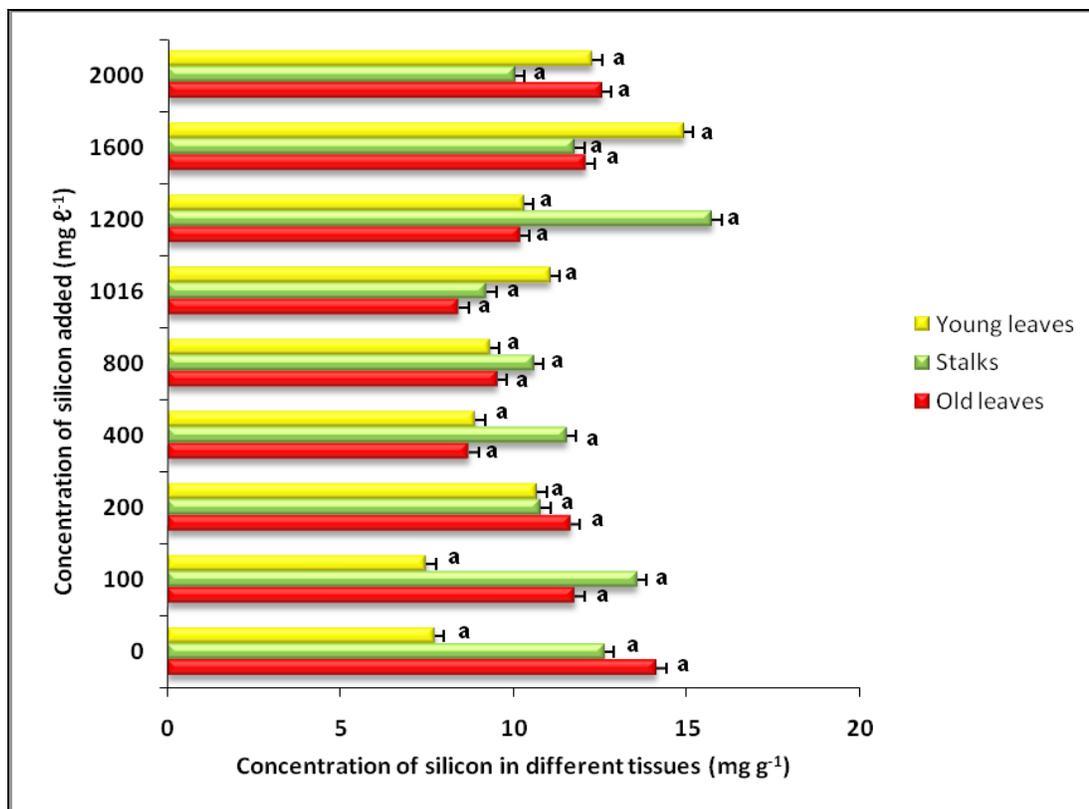


Figure. 3.3. Mean accumulation of silicon in stalks, old leaves and young leaves of N29 sugarcane plants subjected to varying concentrations of silicon, as determined by wet, nitric acid digestion and ICP analysis. ‘Old leaves’ were all leaves below the top visible dewlap (TVD) and ‘young leaves’ were all leaves above and including the TVD. Bars represent standard error. Means with the same letter are not significantly different ($P < 0.05$).

3.3.3. Microwave digestion

A significant increase in Si concentration in the different plant parts with an increase in concentration of Si added was noted (Fig. 3.4). Significant differences were noted between the control and all treatments $> 100 \text{ mg l}^{-1}$ for the stalks, 200 mg l^{-1} for the leaves and 400 mg l^{-1} for the roots. No significant difference in Si accumulation was observed between the 1600 and 2000 mg l^{-1} treatments for all the plant parts. The interaction between treatments (concentration of Si added * concentration of Si in plant parts * concentration of Si in different plant parts) was significant.

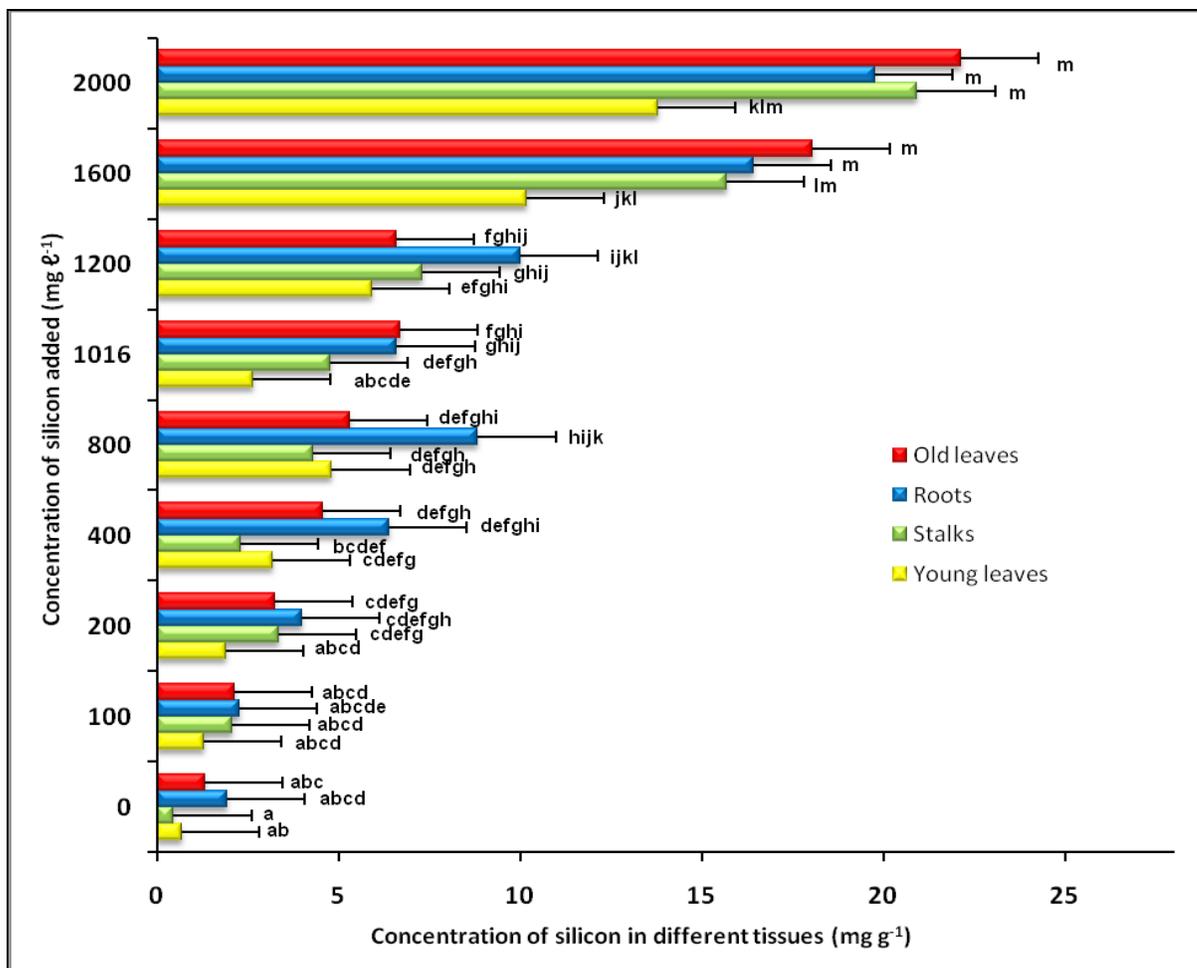


Figure 3.4. Mean accumulation of silicon in roots, stalks, old leaves and young leaves of N29 sugarcane plants subjected to varying concentrations of silicon, as determined by microwave digestion and ICP analysis. ‘Old leaves’ were all leaves below the top visible dewlap (TVD) and ‘young leaves’ were all leaves above and including the TVD. Bars represent standard error. Means with the same letter are not significantly different ($P < 0.05$).

3.3.4. Silicon maps

The difference in Si accumulation in the different tissue types was tested in both the midrib and the leaf blade. Similar trends were obtained for both leaf areas however these differences were more pronounced in the midrib. For this reason, only data for this region are presented. An increase in Si deposition was noted with an increase in Si

concentration. Silicon deposition was observed to be the highest in the lower epidermis and lowest in the mesophyll (Fig. 3.5a and b).

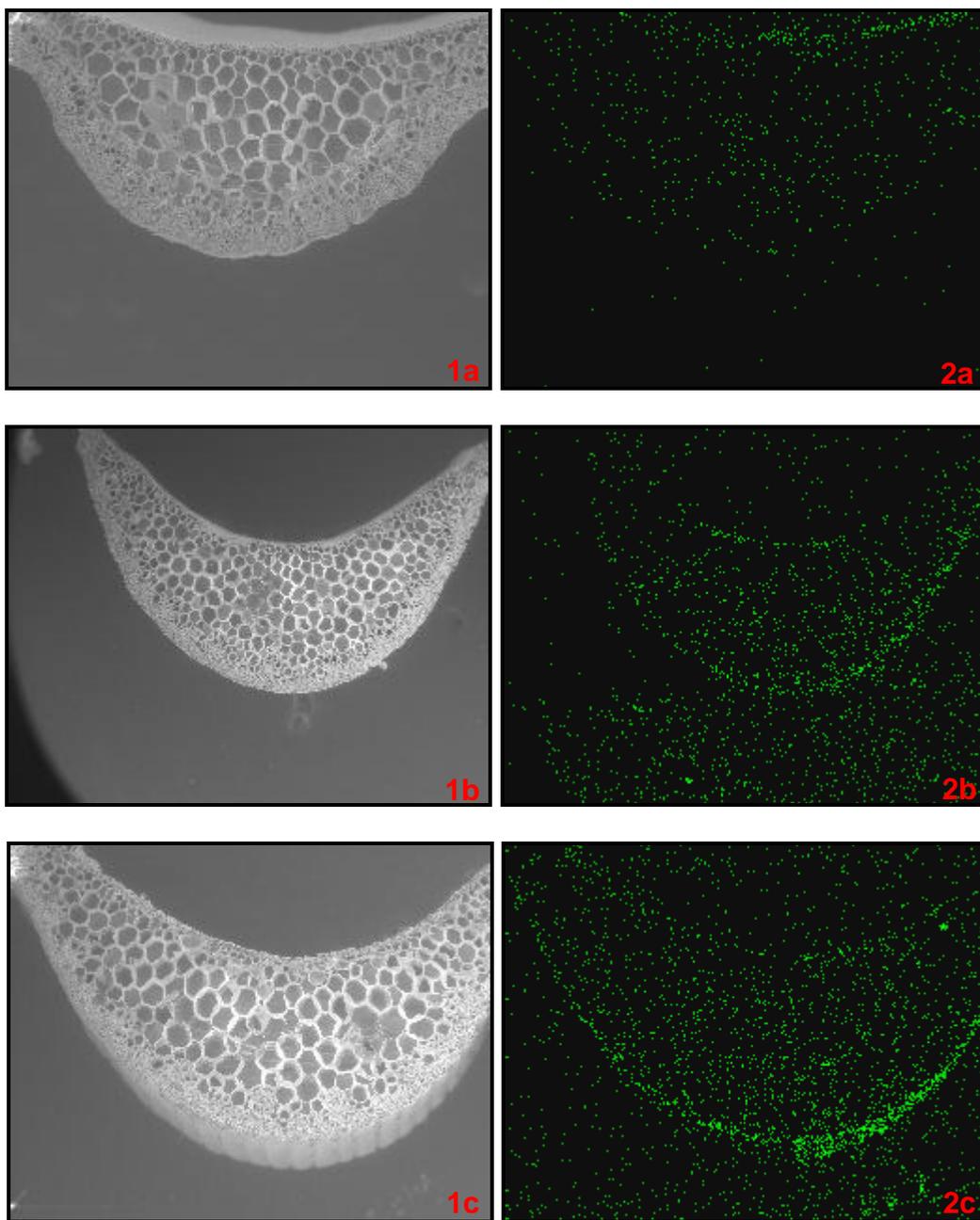


Figure 3.5a Midribs of N29 sugarcane leaves treated with (1a) 0, (1b) 400 and (1c) 800, silicon, applied as potassium silicate and their corresponding X - ray maps (2a-c).

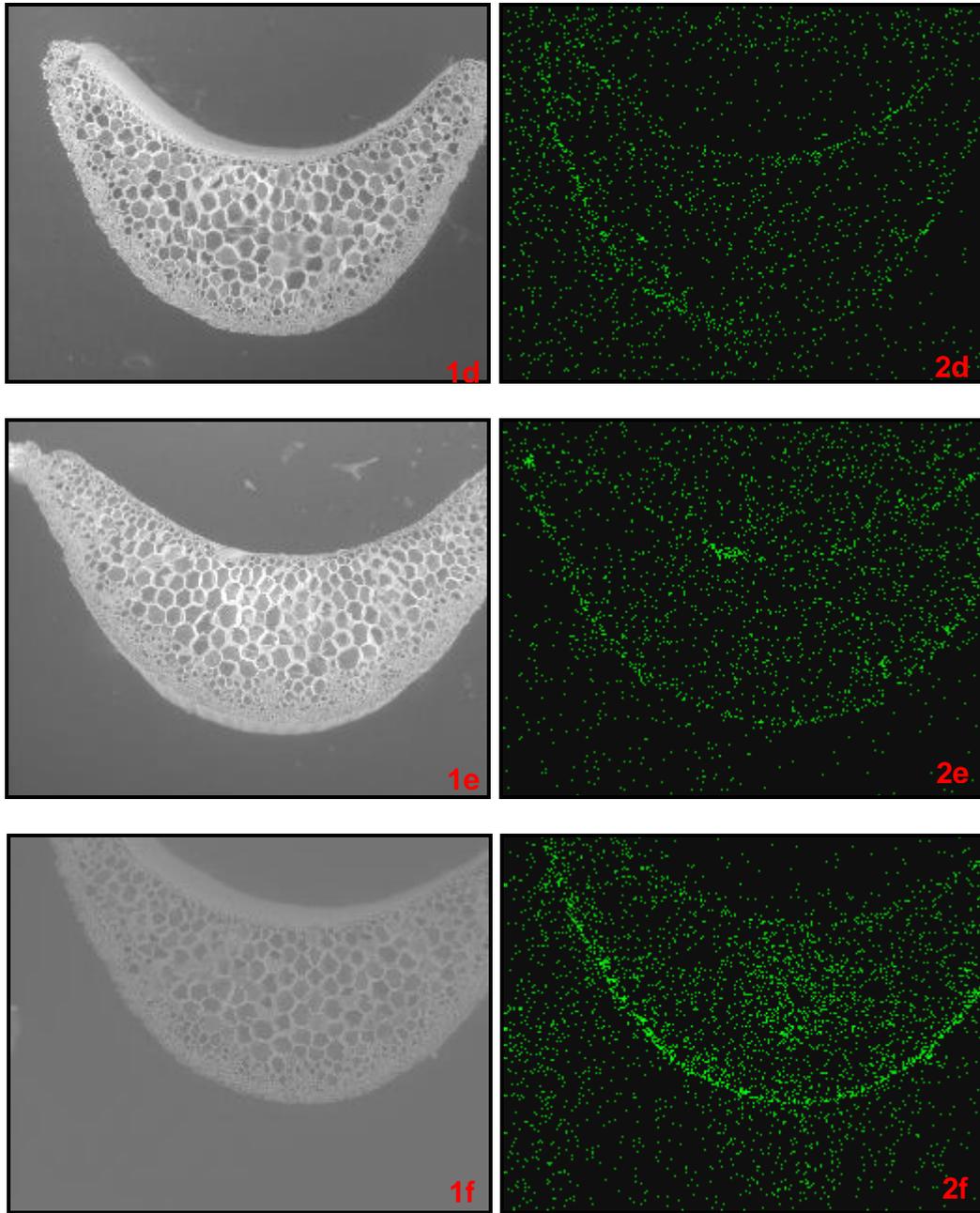


Figure 3.5b Midribs of N29 sugarcane leaves treated with (1d) 1200, (1e) 1600 and (1f) 2000 mg ℓ^{-1} silicon, applied as potassium silicate and their corresponding X - ray maps (2d-f).

3.3.5. Quantification of silicon deposition

Significant differences were noted between treatments as well as for the interaction between the treatments. A significant increase in Si deposition was noted with an increase in Si concentration (Fig. 3.6). The lowest percentage area covered by Si was recorded in the mesophyll of the control (0 mg ℓ^{-1} Si), while the highest was noted in the lower epidermis of the 2000 mg ℓ^{-1} Si treated plants. There was no significant difference in the mean percent area covered between 1600 and 2000 mg ℓ^{-1} Si. This trend was observed in all three tissue types (Fig. 3.6).

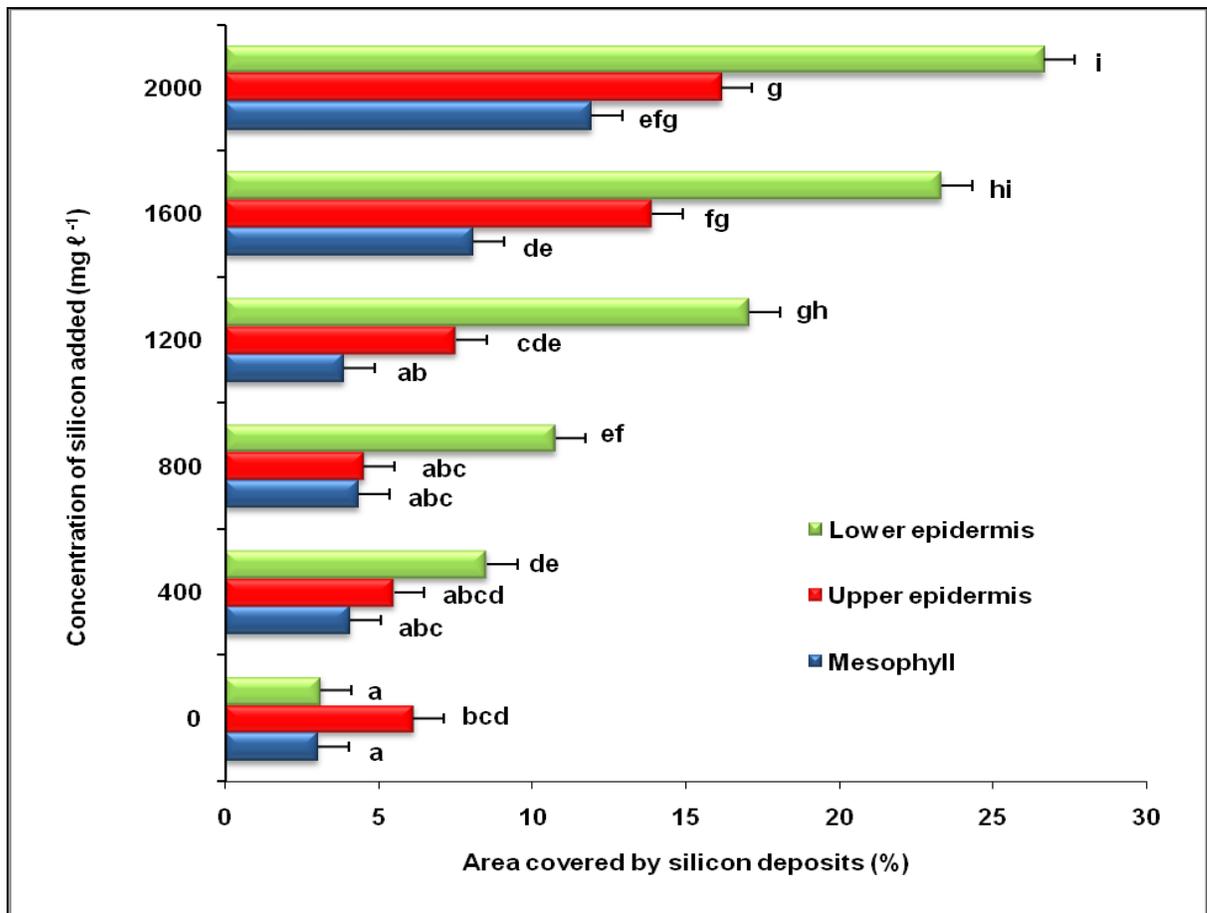


Figure. 3.6. Mean percentage area covered by silicon deposits in the upper epidermis, lower epidermis and mesophyll of leaves from N29 sugarcane plants in response to varying concentrations of Si applied as potassium silicate. Bars represent standard error. Means with the same letter are not significantly different ($P < 0.05$).

3.3.6. Regression and correlation analyses

Regression analyses between uptake in the different plant parts (Figs. 3.7-3.12) as well as deposition in the different tissue types (Figs. 3.13-3.15) were all significant ($P < 0.05$).

3.3.6.1. Silicon uptake

The highest regression co-efficient ($R^2=0.785$) with the most accurate prediction line was observed for the regression between the concentration of Si in stalks and young leaves (Fig. 3.8a). The lowest regression co-efficient ($R^2=0.365$) with the least accurate prediction line was observed for the regression between the concentration of Si in roots and stalks (Fig. 3.9). A stronger relationship between actual values and predicted values (regression line) was obtained at the lower concentrations of Si. All regression lines except for the relationship between stalks and young leaves (Fig. 3.8d) began to plateau at the higher Si concentrations (Figs. 3.7-3.9).

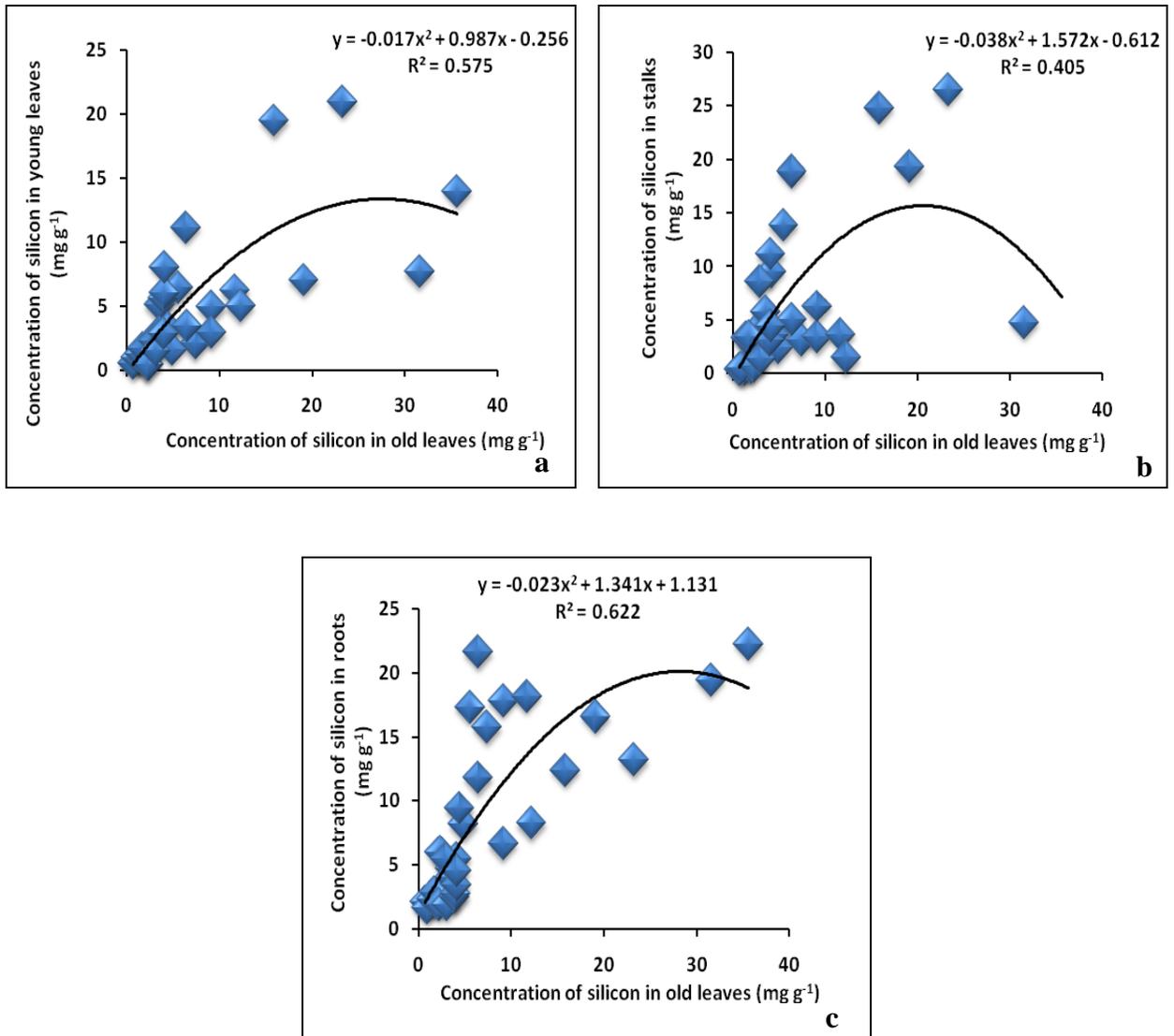


Figure. 3.7. Regression analysis of the concentration of silicon (Si) in (a) young leaves, (b) stalks and (c) roots and concentration of Si in old leaves in response to varying concentrations of Si applied as potassium silicate and calcium silicate.

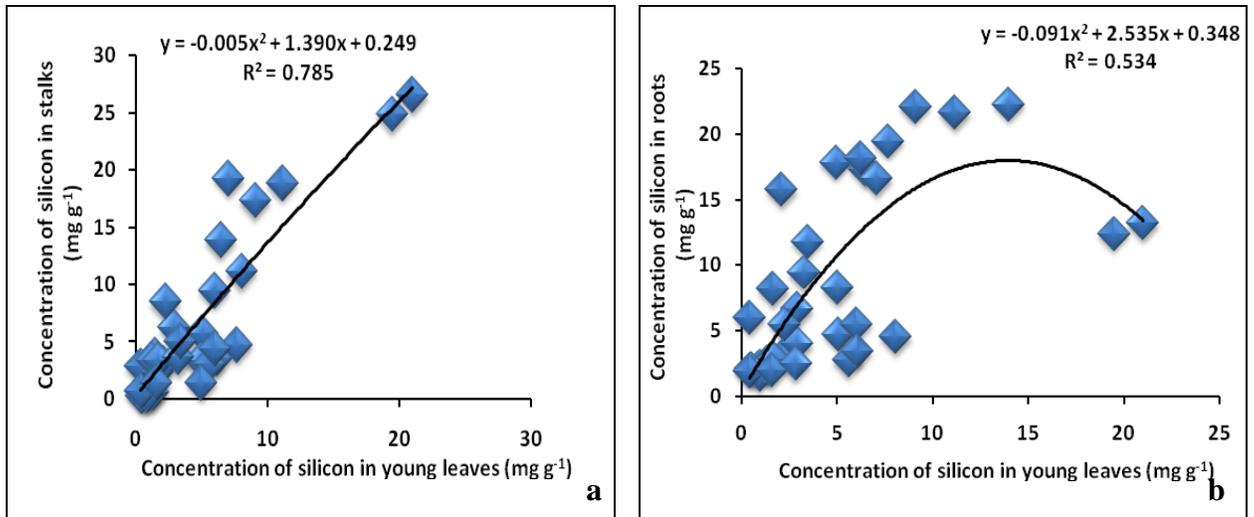


Figure. 3.8. Regression analysis of the concentration of silicon (Si) in (a) stalks and (b) roots and concentration of Si in young leaves in response to varying concentrations of Si applied as potassium silicate and calcium silicate.

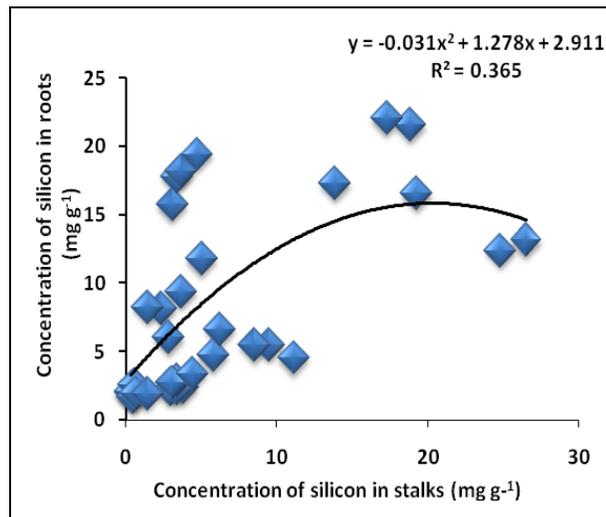


Figure. 3.9. Regression analysis of the concentration of silicon (Si) in roots and concentration of Si in stalks of N29 sugarcane plants in response to varying concentrations of Si applied as potassium silicate and calcium silicate.

All correlations showed a significant positive relationship between variables indicating that an increase in Si accumulation in one plant part results in a corresponding increase in another. As with regression analysis, the highest correlation ($r=0.8869$) was found for the relationship between the concentration of Si in stalks and concentration of Si in young leaves and the lowest correlation ($r=0.5162$) for the relationship between the concentration of Si in stalks and the roots (Table 3.1).

Table 3.1. Correlation matrix showing correlations between the concentration of silicon (Si) added ($\text{mg } \ell^{-1}$) and the concentration of Si accumulated in the different plant parts ($\text{mg } \text{g}^{-1}$) of N29 sugarcane plants in response to varying concentrations of Si applied as potassium silicate and calcium silicate

Correlation co-efficient				
	Silicon added	Old leaves	Roots	Stalks
Old leaves	0.6836			
Roots	0.7561	0.6817		
Stalks	0.8016	0.5341	0.5162	
Young leaves	0.7765	0.6634	0.5223	0.8869

3.3.6.2. Silicon deposition

Regression co-efficients for Si deposition were generally low but significant. As with Si accumulation, the relationship between actual and predicted values (regression line) was stronger at the lower concentrations. The highest regression co-efficient ($R^2=0.635$) was obtained for the regression between the percentage area covered by Si deposits in the mesophyll and lower epidermis (Fig. 3.10b). The lowest ($R^2=0.470$) was obtained for the regression between percentage area covered by Si deposits in the mesophyll and upper epidermis (Fig. 3.11).

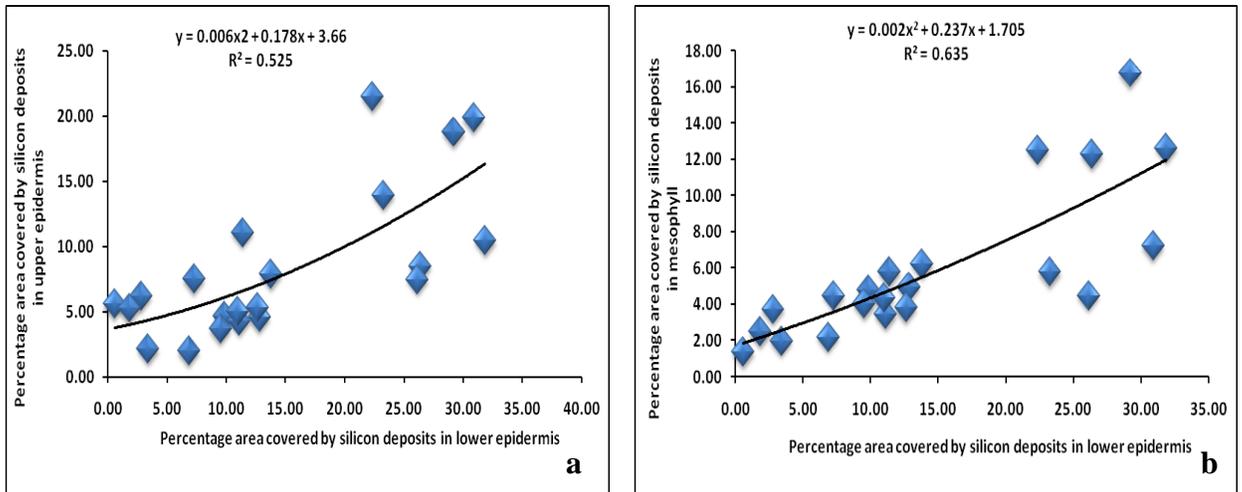


Figure. 3.10. Regression analysis of percentage area covered by silicon (Si) deposits in (a) upper epidermis and (b) mesophyll and lower epidermis of leaves from N29 sugarcane plants in response to varying concentrations of Si applied as potassium silicate.

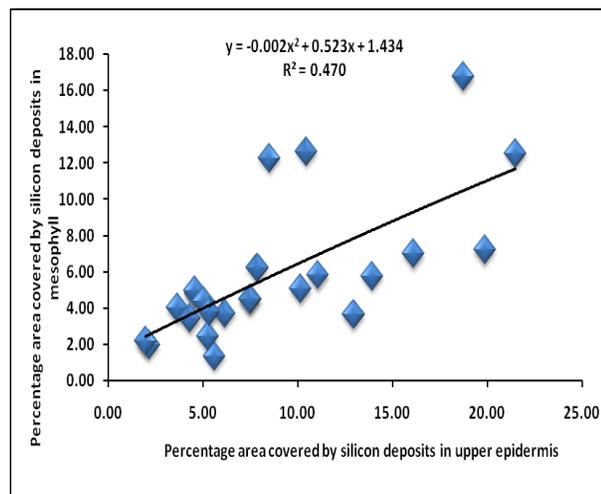


Figure. 3.11. Regression analysis of percentage area covered by silicon (Si) deposits in the mesophyll and upper epidermis of leaves from N29 sugarcane plants in response to varying concentrations of Si applied as potassium silicate.

Significant positive correlations were obtained for all Si deposition relationships. Despite the low regression co-efficients, high correlation co-efficients were obtained. The strongest relationship ($r=0.8634$) was obtained between the concentration of Si added and the percentage area covered by Si deposits in the lower epidermis. The weakest relationship ($r=0.7084$) was obtained for the relationship between the concentration of Si added and the percentage area covered by Si deposits in the upper epidermis (Table 3.2).

Table 3.2. Correlation matrix showing correlations between the concentration of silicon (Si) added ($\text{mg } \ell^{-1}$) and the percentage area covered by Si deposits in the different tissue types of leaves from N29 sugarcane plants in response to varying concentrations of Si applied as potassium silicate and calcium silicate

Correlation co-efficient			
	Silicon added	Lower epidermis	Mesophyll
Lower epidermis	0.8634		
Mesophyll	0.7437	0.7952	
Upper epidermis	0.7084	0.718	0.7387

3.4. Discussion

An increase in Si accumulation in different plant parts was noted in response to Si application (Fig. 3.4). Similarly, an increase in Si deposition in the different cell types occurred with an increase in concentration of Si applied (Fig. 3.6). Savant *et al.* (1999) stated that sugarcane is known to be a Si accumulator, which responds strongly to Si supply and that Si is accumulated in the form of silica gel which is localized in specific cell types. This relationship was illustrated by the strong positive correlations obtained between the concentration of Si added and the uptake by the different plant parts as well as the deposition in the different leaf tissue types. These relationships were significant (Tables 3.1 and 3.2).

Silicon accumulation was found to be roots>old leaves>stalks>young leaves. This was expected as Si was added to the roots and therefore the highest concentration could be expected in this area. Old leaves and stalks had more time to accumulate the added Si compared to the younger leaves, accounting for their high levels of Si. Ma *et al.* (1989) stated that younger leaves accumulate less Si than older leaves. Later Chen (1990) and Epstein (1991) stated that in rice; most of the Si is accumulated in the leaves, followed by the hulls, roots and culms (Datnoff *et al.*, 2007).

The area of highest Si deposition was the lower epidermis, followed by the upper epidermis with the least being found in the mesophyll (Fig. 3.6). These results are consistent with other studies described in the literature. These studies have shown that Si is deposited in high concentrations in the dumb-bell shaped silica cells of the leaf and stem epidermis (Artschwager, 1930; Wong *et al.*, 1971a; Wong *et al.*, 1971b). Wong *et al.* (1971a) found that any Si available to the plant would first be deposited in the silica cells, then the trichomes and finally the stomatal walls. Studies on other crops such as rice, bamboo, cucumber and *Impatiens* have also indicated high levels of Si deposition in the epidermis (Sameuls *et al.*, 1991; Lux *et al.*, 2003; Frantz *et al.*, 2005; Ma and Yamaji, 2006; Motomura *et al.*, 2006). Studies by Elawad and Green (1979) showed that Si accumulates more in the epidermal cells than in any other tissue type in the leaf.

The different plant parts analyzed for Si uptake i.e. roots, stalks, old leaves and young leaves showed significant regression co-efficients when plotted against each other (Fig. 3.7. – 3.9). The highest regression co-efficient was found between the stalks and young leaves (Fig. 3.7a) indicating that this would be the best predictive tool to use. In young plants, determination of Si in the young leaves could therefore be used to predict levels of Si in the stalk and vice - versa. This would be useful for stalk pests/pathogens of sugarcane such as *E. saccharina* (the sugarcane stalk borer) as one could test the young leaves for Si and use the results to make predictions about Si concentrations in the stalk. For rust, however, which is a foliar disease, the regression model between old leaves and young leaves would be more useful. Although low, the regression co-efficient was found to be significant and Si concentrations measured in the old leaves could be

used to predict Si concentrations in the young leaves, saving time and improving efficiency. Similarly, deposition of Si in the different tissue types i.e. upper epidermis, lower epidermis and mesophyll showed significant regressions (Figs. 3.10 – 3.11). The highest regression was between the mesophyll and the lower epidermis (Fig. 3.10b). This is useful for research purposes as it would save time and resources, only having to test one part of the plant for Si using the results to estimate Si concentrations in the other plant parts.

The dry ashing method showed a decrease in Si accumulation in the old leaves at 2000 mg ℓ^{-1} Si added. In this sample, and other samples to which a higher concentration of Si was supplied, a black, indigestible precipitate formed. This precipitate was tested using EDX analysis which showed that Si was present. However the form in which it was present could not be identified. It is therefore proposed that the reduction in Si accumulation at the higher concentration of Si added was due to an underestimation of Si in these samples. There are however, no reports in the literature of such precipitation of Si but it was evident that this precipitation was associated with higher concentrations of Si in plant tissues. Although the wet digestion method digested the tissue with higher Si concentrations without producing similar precipitate, no obvious trends were obtained (Fig. 3.2) using this method. This was probably due to the fact that glass flasks were used. Nitric acid digests Si in the glass and as a result, overestimates the amount of Si present in the tissue. This was expected and to quantify this excess Si, at least five blanks with no plant material were included for every 15 samples. The blanks showed a higher Si content than the samples. It is hypothesised that Si from glass is digested at a faster rate than Si from plant tissue. With no plant material present, the nitric acid digested Si in the glass first while in the samples, the plant material was digested first and then the glass, resulting in a lower Si content recorded in these samples. However, no literature has been found to support this hypothesis. Due to the above findings, the traditional dry ashing digestion method and the nitric acid wet digestion method were not the best methods for determining Si content in the different tissues in this study. The microwave digestion method proved to be effective, even in the presence of high Si-

containing tissues as the containers used did not contain Si and were resistant to acid digestion, eliminating overestimation of Si levels.

In these experiments using variety N29, uptake and deposition appeared to plateau between 1600 and 2000 mg ℓ^{-1} Si (Figs. 3.2 and 3.4). This information is vital in terms of the economics of Si fertilizer application. If a limit is established, better choices in terms of recommended concentrations can be made and the expense of over application of Si with limited benefits could be avoided.

This study showed that Si accumulates in the leaves of sugarcane plants in response to Si supply and is deposited predominantly in the lower epidermis of the leaf. This has important implications for rust control, as *P. melanocephala* urediniospores infect through the abaxial leaf surface. Field trials investigating the uptake of Si and its effect on the severity of brown rust are required before Si can be included in an integrated pest management programme in sugarcane.

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CHAPTER FOUR

EFFECT OF SILICON ON THE SEVERITY OF BROWN RUST OF SUGARCANE

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Abstract

Silicon (Si) has been identified as a key element in the control of a wide range of diseases and pests. This study focused on the effect of Si on the severity of brown rust of sugarcane caused by *Puccinia melanocephala* Syd. & P. Syd. The trial, repeated twice, consisted of eight treatments which included two different forms of Si and a control to which no Si was added. Each treatment was replicated four times with six plants per replicate in a randomized complete block design. Plants were naturally infected by spores from brown rust-infected plants within the polytunnel. Plants were rated weekly for disease severity. A significant reduction in disease severity compared with the control was noted for all plants treated with Si at a concentration of $\geq 400 \text{ mg Si } \ell^{-1}$ for both the final disease severity index and AUDPC. Disease severity was significantly reduced from a rating of 8.5 in the control to 6.4 in plants treated with Si at $2000 \text{ mg Si } \ell^{-1}$, while AUDPC showed a decrease from 161.3 in the control to 121.4. No significant difference in disease severity was noted between 1600 and $2000 \text{ mg Si } \ell^{-1}$. Silicon at a concentration of $1016 \text{ mg Si } \ell^{-1}$, applied once off as Calmasil[®] provided the same control as K_2SiO_3 applied weekly at a Si concentration of $1200 \text{ mg Si } \ell^{-1}$. A significant negative relationship with high regression co-efficients was obtained for regression analyses between the concentration of Si added and disease severity index

and AUDPC, indicating a strong relationship between application of Si and disease severity. A similar relationship was noted for disease severity index and Si accumulation and deposition. Results from this study clearly indicate a reduction in brown rust severity with an increase in Si content in the plant. Further investigation into this could result in the development of a new control measure for the control of brown rust within the South African sugar industry.

4.1. Introduction

Brown rust of sugarcane, caused by *Puccinia melanocephala* H. & P. Sydow, was accidentally introduced into South Africa (SA) from India in 1941, and was first reported on variety Co301 (Bailey, 1995; Saumtally and Autrey, 1999). It reappeared in SA in the mid-1970s on the variety N55/805 which exhibited severe symptoms (Bailey, 1995). It was then regarded as an economically minor disease as mean yield losses on this variety were estimated to be only 5% (Bailey, 1979). However, the disease became prevalent again in 2000, shortly after the release of variety N29. Environmental conditions were ideal for rust development (Cadet *et al.*, 2003). In addition, it later became apparent that several genotypes of *P. melanocephala* were present in the industry (Pillay *et al.*, 2005), which is assumed to be the main reason for the increased susceptibility observed in N29. Although N29 has many other good traits, growers have been dissuaded from planting the variety (Cadet *et al.*, 2003). Yield losses are estimated to be as high as 26% when N29 is severely affected (Rutherford *et al.*, 2003, McFarlane *et al.*, 2006). Brown rust continues to be prevalent on N29 (Anonymous, 2006) and is also common, but less severe, on varieties with intermediate resistance.

Currently there is a shift towards using integrated management strategies to control plant diseases. Varietal resistance and fungicides can be effective against brown rust in sugarcane. In addition, an association between nutrients and rust severity has been identified (Anderson and Dean, 1986; Cadet *et al.*, 2003, McFarlane *et al.*, 2008) and could play an important role in the management of brown rust. High levels of nitrogen (N), phosphorus (P), copper (Cu), manganese (Mn), zinc (Zn), sulphur (S), potassium

(K), calcium (Ca) and magnesium (Mg) were found to be associated with high rust severity (Anderson and Dean, 1986; Grisham *et al.*, 2006; McFarlane *et al.* 2008) while high levels of Mn and low levels of iron (Fe) in the soil were found to be associated with low rust severity (McFarlane *et al.* 2008). Silicon (Si) has been reported to induce resistance to many fungal diseases in rice, cucumber, vines, turfgrass and various greenhouse crops as well as promote development of healthy crops including sugarcane (Epstein, 1999). Cadet *et al.* (2003) suggested that applications of Si would increase the Si content in the leaves and could therefore reduce infection. However, in a later study, intensive sampling showed that high levels of Si were present in leaves showing severe rust infections (McFarlane *et al.*, 2008). This was possibly in response to rust infection. A study by Raid *et al.* (1992) showed that Si had no effect on brown rust development.

The aim of this study was to assess the effect of Si on the severity of brown rust in the susceptible sugarcane variety N29.

4.2. Materials and Methods

4.2.1. Test plants

Single budded sugarcane setts of variety N29 were grown in 155mm × 210mm seedling trays holding six plants. The seedling trays were placed in plastic containers (8 × 15 × 21 cm) (Food Packaging Distributors) filled with water. Composted pine bark (Gromor) was used as the growth medium for this study. Plants were grown in a polytunnel (25°C, 50-100% relative humidity with a photoperiod of 12-14 h). Plants were fertilized weekly using Hortichem (Ocean Agriculture) 3:1:3 (N:P:K). Plants were exposed to natural infection with *P. melanocephala*, from infected spreader plants growing within the tunnel, at the same time that Si was applied.

4.2.2. Application of Silicon

Silicon was applied to the plants in two forms i.e. as potassium silicate (K₂SiO₃) or as Calmasil[®] (Omnia Fertilizer (Pty.) Ltd.), a commercially available form of calcium silicate. Potassium silicate (600 ml), at the specific concentration required, was added to the

plastic containers once a week, for seven weeks. Any liquid already present in the container was removed prior to addition of the Si. At planting, Calmasil[®] was incorporated into the pine bark at one concentration of 52 g 5 kg⁻¹ (1016 mg ℓ⁻¹ Si).

4.2.3. Experimental design

The trial consisted of nine treatments i.e. 100, 200, 400, 800, 1200, 1600 and 2000 mg Si ℓ⁻¹, Calmasil[®] at a concentration of 52 g 5 kg⁻¹ of pine bark and a control to which no Si was added. The trial was replicated four times with six plants per replication. The trials were repeated twice with treatments arranged in a Randomized Complete Block Design.

4.2.4. Brown rust ratings

Plants were rated for brown rust severity once a week for eight weeks using a rating scale ranging from 1-9 (Table 4.1) which was based on a combination of a modified soybean rust rating scale and one used by Tai *et al.* (1981). Area under disease progress curve (AUDPC) was calculated using a trapezoidal integration program (Berger, 1981) for the different concentrations of Si applied.

4.2.5. Statistical analyses

Data were subjected to analysis of variance (ANOVA) using Genstat[®] Executable Release 9 Statistical Analysis Software 11th edition. The data were tested for normality and equality of variances. The least significant difference was determined at P<0.05 where appropriate. Correlations were calculated using Genstat while polynomial regression was calculated using Microsoft Excel. Uptake and deposition data for the regression and correlation analyses was obtained from trials in Chapter 3.

Table 4.1. Rating scale used to assess brown rust severity on N29 sugarcane plants caused by *Puccinia melanocephala*

Rating	Description
0	No visible chlorosis / pustules
1	Chlorosis / necrosis present, no pustules
2	Few / abundant necrotic flecks, pustules visible, spores not visible with the naked eye
3	Necrotic flecks develop into small, spore bearing lesions, spores visible with the naked eye
4	Pustules small and sparse, spores visible with the naked eye
5	Pustules increase in number as well as size on older leaves, infection spreading to less mature leaves
6	Lesions on tips of lower leaves starting to coalesce, chlorosis and necrosis on less mature leaves
7	Tips of older leaves dead and dry, on less mature leaves pustules sporulating profusely
8	Some old leaves dead and dry, all old leaves densely covered with pustules. Tips of less mature leaves densely covered with pustules, lesions starting to coalesce
9	All old leaves dead. Many less mature leaves showing death at tips of leaves

4.3. Results

The results from the repeated trials confirmed those of the first trial. Data used for further analyses was therefore an average of the results obtained for the three trials.

A significant decrease in disease severity and AUDPC was noted with an increase in Si concentration (Fig. 4.1). The highest final disease severity index and AUDPC was obtained for the control (0 mg Si ℓ^{-1}) and the lowest for 2000 mg Si ℓ^{-1} . A significant reduction in disease severity compared with the control was noted for all plants treated

with Si at a concentration of $\geq 400\text{mg Si } \ell^{-1}$ for both the final disease severity index and AUDPC. Plants to which Si was applied at $2000 \text{ mg} \ell^{-1}$ showed a lower disease incidence than plants containing Si applied at $1600 \text{ mg Si } \ell^{-1}$. This difference was however, not significant. Silicon at a concentration of $1016 \text{ mg Si } \ell^{-1}$, applied as Calmasil[®] provided the same control as K_2SiO_3 applied at a Si concentration of $1200 \text{ mg Si } \ell^{-1}$ (Fig. 4.1).

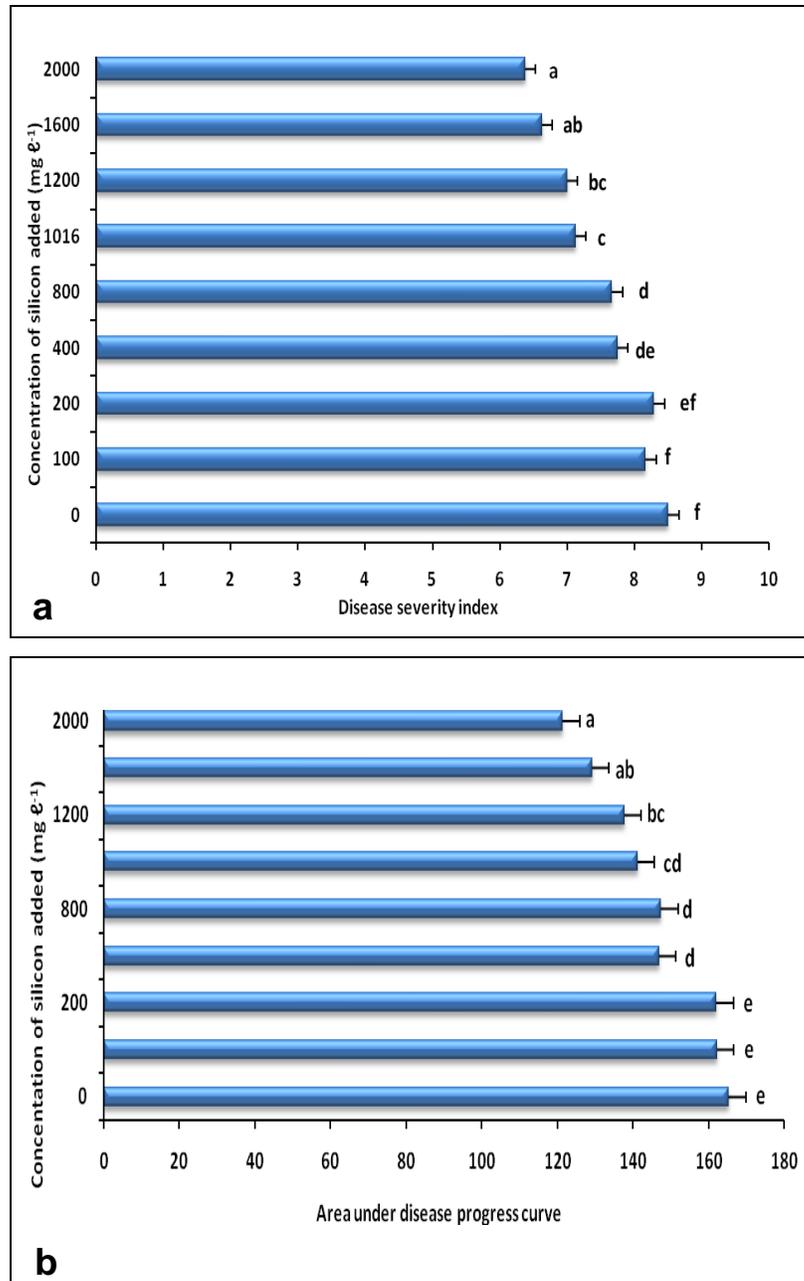


Figure 4.1. Disease severity index (a) and area under disease progress curve (b) of N29 sugarcane plants naturally infected with *Puccinia melanocephala* treated with different concentrations of silicon, applied as potassium silicate and calcium silicate. Treatments with the same letter are not significantly different at $P < 0.05$. Error bars represent the standard error.

Regression analyses for disease severity and Si accumulation and deposition showed a significant negative relationship with high regression co-efficients, indicating a strong relationship between application of Si and disease severity. A decrease in disease severity was noted with an increase in Si accumulation and deposition. A higher regression co-efficient was obtained for Si deposition than Si accumulation.

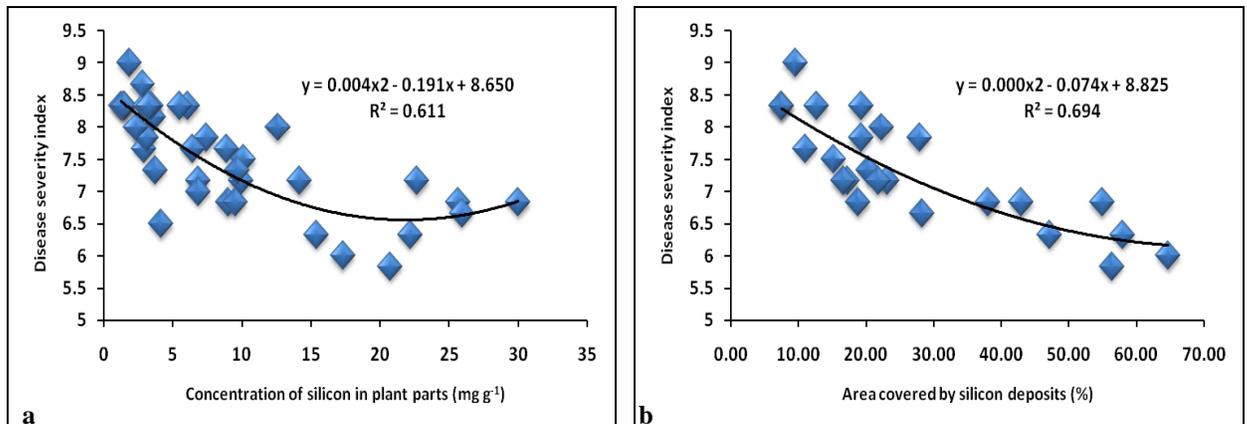


Figure 4.2. Regression analyses of disease severity index and (a) concentration of Si in the different plant parts and (b) percent area covered by Si deposits in leaf tissue of N29 sugarcane plants naturally infected with *Puccinia melanocephala* subjected to varying concentrations of Si applied as potassium silicate and calcium silicate.

A significant negative relationship was obtained between the concentration of Si added and both the disease severity index and AUDPC. High regression co-efficients and therefore accurate prediction tools were obtained for both variables. A higher co-efficient was obtained for the relationship with disease severity index than AUDPC (Fig. 4.3).

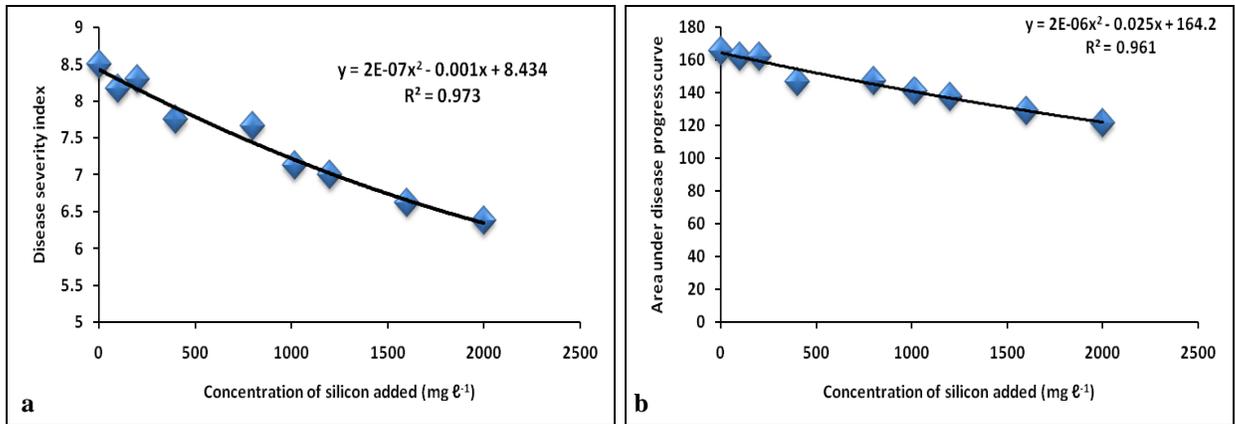


Figure 4.3. Regression analyses of the concentration of silicon added and (a) disease severity index; (b) area under disease progress curve of N29 sugarcane plants naturally infected with *Puccinia melanocephala* treated with different concentrations of silicon, applied as potassium silicate and calcium silicate.

Significant correlation co-efficients were obtained for all analyses. The highest correlation co-efficient ($r=0.9061$) was obtained between the concentration of Si added and its accumulation in the different plant parts while the lowest ($r=0.7093$) was for accumulation in the plant and deposition in the leaf tissue. The correlation between the concentration of Si added, accumulation and deposition was found to be positive while the correlation between disease severity and these variables was negative (Table 4.2).

Table 4.2. Correlation matrix showing correlations between the concentration of silicon (Si) added ($\text{mg } \ell^{-1}$), the concentration of Si accumulated in the different plant parts (mg g^{-1}), the percent area covered by Si deposits in the leaf tissue and disease severity index.

Correlation co-efficient			
	Concentration of silicon added	Silicon accumulation	% Area covered by silicon deposits
Accumulation	0.9061		
% Area covered by silicon deposits	0.8228	0.7093	
Disease severity index	-0.8923	-0.7274	-0.819

4.4. Discussion

This study focused on the ability of Si to reduce brown rust severity on N29 sugarcane plants. Results show a significant decrease in disease severity as well as AUDPC with an increase in Si concentration (Fig. 4.1). Older leaves on control plants were densely covered with pustules and lesions and in some cases the leaves died. Pustules and lesions also extended to less mature leaves. An average disease rating of 8.5 was assigned to the untreated control plants. Plants treated with $2000 \text{ mg } \ell^{-1}$ showed coalescing lesions on older leaves but few or no lesions on less mature leaves, indicating that Si not only reduced disease incidence but also slowed down disease progress. A once-off application of Calmasil at a concentration of $1016 \text{ mg Si } \ell^{-1}$ at planting provided the same control as a weekly application of $1200 \text{ mg Si } \ell^{-1}$. The once-off application would be more practical in the field as it could be incorporated into the soil at planting. Better rust control might be achieved by increasing the rate of Calmasil applied.

There was no significant difference in rust severity between the $1600 \text{ Si mg } \ell^{-1}$ and $2000 \text{ Si mg } \ell^{-1}$ treatments. These results are consistent with previous findings (Chapter 3)

where it was noted that there was no significant difference in uptake or deposition of Si when between 1600 mg ℓ^{-1} and 2000 mg ℓ^{-1} was applied. Increased potassium (K) rates can increase or decrease disease severity depending on the crop, disease and environment which surrounds it (Prabhu *et al.*, 2007). Due to the application of K_2SiO_4 as the treatment in the study, high rates of K together with Si were applied. The effect of the K on disease severity within this study could not be established due to the absence of a K control. However, Cadet *et al.* (2003) found that rust severity significantly increased with increasing levels of K within the leaves. High levels of K were also found to be associated with high rust severity in other studies (Anderson and Dean, 1986; Grisham *et al.*, 2006; McFarlane *et al.* 2008). In addition, a calcium silicate treatment (1016 mg Si ℓ^{-1}) was included in the study which controlled the disease as effectively as the K_2SiO_4 treatments (Fig. 4.1). This suggests that K was not responsible for the observed decrease in brown rust severity but rather that Si had a negative effect on disease development.

A significant positive relationship (variables directly proportional to one another) was shown for concentration of Si added and uptake and deposition of Si within the plant while a significant negative relationship (variables inversely proportional) was observed for the disease severity index and the concentration of Si added and uptake of Si within the plant and deposition of Si within the leaf (Table 4.2). This indicates that the uptake and deposition of Si within the plant was as a result of the Si added to the plant while reduction of disease severity could be attributed to the uptake and deposition of Si within the plant tissues. Using the regression equation generated for the concentration of Si added and the disease severity index (Fig. 4.3), one could estimate what concentration of Si would be required to bring disease levels down to levels that will not cause economic damage (if one knew what that disease rating was). This would be quite accurate due to the high regression coefficient obtained (Fig. 4.3).

Anderson and Dean (1986) showed a relationship between rust severity and nutrients. Silicon could be used to reduce rust incidence due to its reported ability to induce disease resistance in other crops. In this study the application of high levels of Si led to

a reduction in disease severity and disease progress. Brown rust is known to be more severe in younger plants, with plants 2-6 months old being most susceptible (Péros and Lombard, 1986). Therefore, the application of K_2SiO_3 during the early stages of plant growth or even as a once-off treatment of Calmasil may reduce brown rust severity to acceptable levels. If used as part of an integrated management programme, Si could be effective in the control of brown rust of sugarcane.

Numerous studies have been conducted on Si and pest resistance in sugarcane and Si and disease resistance in other crops (Epstein, 1994; Holland and Munkvold, 2001; Liang, 1999; Liang *et al.*, 2003, Meyer and Keeping, 2005; Savant *et al.*, 1997; 1999). Only one study has been reported to date on the effect of Si on brown rust in sugarcane. This research by Raid *et al.* (1992) indicated that soil amendment with calcium silicate slag at a rate of 6.7 t ha^{-1} had no effect on brown rust severity. In this study, best control was achieved with an application rate equivalent to $21 \text{ t ha}^{-1} K_2SiO_4$ ($2000 \text{ mg } \ell^{-1}$), just over three times that used by Raid *et al.* (1992). The lack of control in that study could therefore be attributed to the low rate of Si applied. However, it must be noted that significantly lower disease than the control was obtained with an application rate of 4.2 t ha^{-1} ($400 \text{ mg } \ell^{-1}$), which is closer to the current application rate of Calmasil (3 t ha^{-1}) (personal communication¹) used in the SA sugar industry. An application at this rate would be more practical as part of an integrated management strategy where Si could be used to reduce levels of rust. The uptake of Si into the plant can be adversely affected by the soil conditions and this would need to be considered when applying the product.

Results from this study clearly indicate a reduction in brown rust severity with an increase in Si content in the plant. However, this relationship needs to be confirmed in large-scale field trials before Si can be used as part of an integrated management strategy against brown rust. Differences in the response to Si and its effect on disease severity by different sugarcane varieties would need to be investigated. In addition, the mechanism by which Si affords this protection against the disease would be of interest.

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CHAPTER FIVE

IDENTIFICATION OF GENE SEQUENCES IN VARIETIES WITH VARYING RESISTANCE TO RUST EXPRESSED IN RESPONSE TO BROWN RUST INFECTION

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Abstract

Brown rust, caused by *Puccinia melanocephala*, is currently a major problem in the South African sugarcane industry due to its prevalence on the susceptible commercial variety N29 as well as a range of other widely grown intermediate varieties. The most economical and effective method of control is with the use of resistant varieties. This study focused on identifying genes sequences expressed in response to brown rust infection. Three sugarcane varieties with varying degrees of resistance were used: N29 (susceptible), N39 (intermediate) and N12 (resistant). Plants were challenged with rust and total RNA was extracted from challenged plants as well as from uninoculated controls. To isolate differentially expressed genes, two cDNA libraries were created, one for variety N39 and another for variety N12. After cDNA subtraction, differentially expressed sequences were PCR amplified, cloned and transformed colonies transferred to nylon membranes. To screen the libraries and test efficiency of the library, colonies were hybridized to radioactively labeled total cDNA probes of both the driver and the tester. Distinct differences, consistent with what was expected, in infection were noted between the different varieties. The range of products amplified differed between the two

libraries and two different amplification profiles were observed. Two efficient subtracted cDNA libraries were created and differentially expressed genes were identified within the libraries. Transcripts identified from the N12 library were predominantly stress / disease associated while transcripts from the N39 library were related to general plant function. This study is the first step towards the use of marker assisted selection for the early identification of brown rust resistance within breeding lines.

5.1. Introduction

Brown rust of sugarcane (*Saccharum* spp., L.), caused by *Puccinia melanocephala* Syd. and P. Syd., was first reported in South Africa (SA) in 1941 on the Co301 variety. It is currently a major problem in the SA sugar industry due to its prevalence on the variety N29, a variety that covers an estimated 6000 hectares of the SA sugar industry (personal communication²). It also infects several other widely grown varieties with intermediate resistance (Bailey, 1995; Saumtally and Autrey, 1999, McFarlane *et al.*, 2006; McFarlane, 2007). Currently, in the SA sugar industry, the most economical method of control is through the use of resistant varieties. However, even though many resistant varieties have been identified, resistance has not been durable and has often been known to break down due to increased disease pressure and therefore mutation of the fungus (Raid and Comstock, 2006). Emphasis has been on identifying varieties with partial resistance to the rust, which offers a more durable solution to the problem.

Marker assistant selection (MAS) is a tool used by plant breeders to assist with selecting varieties that display a particular trait of interest, for example disease resistance. It involves the use of a molecular marker, identified to be associated with the trait, to indirectly select for that particular trait (Anonymous, 2009). Markers used should be easy to identify and linked to desirable genetic traits that are not easily identified (D'Arcy *et al.*, 2001). It has many applications including its use in developing varieties resistant to various pathogens such as bacterial blight in rice, barley stem rust and bean common mosaic virus (Melchinger, 1990; Kelly, 1995; Penner *et al.*, 1995; Miklas *et al.*, 1996, Sanchez *et al.*, 2000). Studies by McIntyre *et al.* (2005a) suggest that markers identified

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on the sugarcane genome could be broadly effective in selecting brown rust and parenchyma root rot resistant genotypes within breeding programmes. However, for brown rust resistance, only two of the seven markers tested remained significantly associated with resistance (McIntyre *et al.* 2005a). This is because the markers are only linked to the genes of interest and are not the actual genes conferring resistance. This is one of the major limitations of marker assisted selection. However, if the actual genes associated with resistance could be identified for its use as the marker, this problem could be overcome. Marker assisted selection could be used by plant breeders within the SA sugar industry to facilitate the selection of rust resistant varieties. Identification of genes conferring this resistance (either partial or complete) is therefore imperative in order to facilitate the use of MAS for the development of rust resistant varieties.

Identification of cDNA sequences differentially expressed between resistant and susceptible genotypes can be a useful tool to identify genes associated with resistance to particular pathogens. Suppression subtractive hybridization (SSH), is a highly effective method used for generating cDNA libraries and identifying differentially expressed gene sequences (Diatchenko *et al.*, 1996). It has been used extensively in identifying resistance genes associated with various different pathogens including wheat leaf rust (*Puccinia triticina*) on barley (Neu *et al.*, 2003) and tobacco blue mould (*Nicotiana megalosiphon*) on tobacco (Borrás *et al.*, 2007). It has also been used to identify defense-related genes in rice (Xiong *et al.*, 2001). Genes found to be associated with rust resistance, as identified by SSH, could be used to develop molecular markers for MAS of rust resistant varieties.

The aim of this study was to identify genes expressed by the sugarcane varieties N12 and N39 in response to infection by *P. melanocephala*. This was done using the SSH technique. The two varieties chosen were primarily for their different resistance ratings with respect to brown rust. N12 is known to be completely resistant, while N39 is known to have intermediate resistance. Variety N12 shows no signs of infection. Variety N39 shows limited spore development and observations have indicated that infection develops more slowly in this variety. Variety N29 shows rapid infection and spore

development. In addition, an infection of N29 with rust persists in the plant while N39 grows out of the infection. The use of these varieties would allow for the identification of differences in expressed genes between the different resistance mechanisms. This study is the first step towards the development of markers associated with brown rust resistance.

5.2. Materials and Methods

5.2.1. Host material

Single bud sugarcane setts of varieties N29, N39 and N12 were grown in 30cm pots containing potting soil. Variety N29 is a brown rust susceptible sugarcane variety, N12 a resistant variety whilst N39 has an intermediate resistance rating for brown rust. The pots were placed in a greenhouse at an average temperature of 28°C and fertigated twice daily for 5 min. One month after planting, plants were inoculated with *P. melanocephala* 1ml of water urediniospores at a concentration of 7.75×10^6 , using the leaf whorl technique (Fig. 5.1, Sood *et al.*, 2007). Leaves were collected for RNA extraction when N29 showed fully developed lesions, which was after approximately two weeks.



Figure 5.1. Leaf whorl technique used to inoculate test plants with *Puccinia melanocephala* urediniospores.

5.2.2. Total RNA extractions

Total RNA was extracted from inoculated leaves using a modified method of Bugos *et al*, 1995. Fresh leaf tissue (2 g) was ground to a fine powder using liquid nitrogen in a mortar and pestle. The ground tissue was transferred to a 50 ml Corning[®] tube together with 10 ml TENS homogenization buffer (0.1M Tris-HCl pH 7.5, 1mM EDTA, 0.1M NaCl, 0.1% SDS), 70 µl mercaptoethanol and 10 ml of phenol:chloroform:isoamylalcohol (25:24:1). The mixture was homogenized with a Vortex mixer at high speed for two minutes. To this, 700 µl of 3M sodium acetate (pH 5.2.) was added, mixed well and stored on ice for 15 minutes. Tubes were centrifuged at 12 000 g for 15 minutes at 4°C. The aqueous phase was transferred to a new 50 ml Corning[®] tube and an equal volume of isopropanol added. This was incubated at -70°C for 30 minutes. The RNA was recovered by centrifugation at 10000g for five minutes at 4°C. The pellet was washed with 70% ethanol, centrifuged at 10 000 g for five minutes and air-dried. The dried pellet was resuspended in 750 µl DEPC treated water and transferred to a microcentrifuge tube. The microcentrifuge tubes were then centrifuged at 10 000 g for 5 minutes at 4°C, to remove any insoluble material.

5.2.3. RNA quantification and gel electrophoresis

RNA was quantified using the Nanodrop[®] ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.). The quality of the RNA was examined by denaturing agarose gel electrophoresis. Ten micrograms of RNA (total volume of 6 µl) was mixed with 12.5 µl of formamide, 2.5 µl of 10 × 3-(N-morpholino) propanesulfonic acid (MOPS) buffer and 4 µl of formaldehyde and incubated at 55°C for 15 minutes. The mixture was chilled on ice and 2.5 µl loading dye added. RNA was fractionated on a 1.2% (w/v) agarose gel, containing 17% formaldehyde. The gel was run in a 1 × MOPS buffer, stained with 100 mg ml⁻¹ ethidium bromide (EtBr) for 30 minutes and viewed using an Alpha Imager[™] 2200 (Alpha Innotech Corporation) at 302nm.

5.2.4. Isolation of poly (A)⁺ RNA

Isolation of poly (A)⁺ RNA from total RNA was performed using the Dynabeads[®] poly (A)⁺ RNA Purification Kit (Dynal) as per the manufacturer's recommended protocol. The

poly (A)⁺ RNA was eluted in 10 µl of elution buffer and quantified with the Nanodrop[®] ND-1000 Spectrophotometer.

5.2.5. Construction of subtracted cDNA libraries

Two subtracted cDNA libraries were made, one for the resistant variety, N12 and one for the intermediate variety, N39.

5.2.5.1. cDNA subtractive hybridizations

cDNA subtractive hybridizations were conducted using the Clontech PCR-Select cDNA subtraction kit (Clontech Laboratories Inc.). The recommended protocol was followed unless otherwise indicated. Two different subtractions and a control subtraction were conducted. For this study, the following terminology describes the cDNA populations used for the subtractive hybridizations. The ‘tester’ refers to the cDNA pool that contains the differentially expressed transcripts of interest. The ‘driver’ cDNA is used to remove all common transcripts between the tester and driver pools. The first subtraction used N12 challenged with brown rust as the tester. The driver was an equal mixture of cDNA from N12 (unchallenged) and N29 (challenged). For the second subtraction the tester was N39 (challenged) and the driver an equal cDNA mixture of N39 (unchallenged) and N29 (challenged). The control subtraction used the control tester and driver from the Clontech kit.

5.2.5.2. Cloning of subtracted cDNA products

Subtracted cDNA products from both subtractive hybridizations were cloned into the pGEM EcoRI restriction site using the Promega pGEM T-easy cloning kit (Promega Corporation). The cDNA (3 µl) was ligated to the vector overnight and thereafter transformed into *Escherichia coli* strain JM109 supercompetent cells. Transformation conditions were as follows: 50 µl of competent cells were mixed with 2 µl of ligation mix. Mixed cells were transferred to an ice-cold micropulser cuvette and left on ice for 10 minutes. Electroporation was performed with a BIO-RAD Micropulser[™] (Bio-Rad Laboratories), using the bacterial, Ec2 and time settings. Directly after electroporation, 450 µl of Luria Bertani (LB) broth was added to the cuvette. The contents were mixed,

transferred to a microfuge tube and placed in a shaking incubator for one hour at 37°C. To isolate transformed colonies 50 µl of the cloned products were plated directly onto LB agar plates containing 100 mg l⁻¹ ampicillin, 20 mg ml⁻¹ X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and 0.5mM isopropyl-beta-D-thiogalactopyranoside (IPTG). The remaining cells were centrifuged at 12 000 g for two minutes, pellet resuspended in 50 µl of LB broth and plated on LB/ampicillin/X-gal/IPTG plates. Plates were incubated overnight at 37°C and then inspected for blue/white colonies. Transformed (white) colonies were transferred to 100 mm² square plates containing LB agar supplemented with 100 mg l⁻¹ ampicillin.

5.2.6. Preparation of membranes

Transformed colonies were transferred to Hybond N⁺ (AEC, Amersham) membranes by colony lifts. Membranes were placed on the surface of pre-cooled 100 mm² plates containing colonies and several orientation marks placed using a needle. After one minute, the membrane was removed and colonies denatured for two minutes by placing on a filter paper stack saturated with denaturation buffer (1.5M NaCl + 0.5M NaOH). They were then neutralized for five minutes in the same way with neutralization buffer (1.5M NaCl + 1.5M NaOH). To remove cellular debris, membranes were rinsed with 2 x SSC pH 7.8 (0.03M Na₃C₆H₅O₇ + 0.3M NaCl). Membranes were air-dried DNA side up, on filter paper, and cross linked using a Hoefer Scientific Instruments UV Crosslinker (Hoefer Inc.).

5.2.7. cDNA probe synthesis and hybridization

Radioactively-labeled cDNA probes of the tester and driver from each subtraction were generated using ³³P-dCTP and the Qiagen LabelStar Array kit (Qiagen), according to the recommended protocol. Membranes containing cDNA clones were prehybridized at 65°C for eight hours in a solution of 0.5M sodium phosphate buffer (pH 7.2), 7% (w/v) sodium dodecyl sulphate (SDS), 0.9mM EDTA and 10 µg ml⁻¹ denatured salmon sperm DNA (final concentrations). The labeled tester cDNA was added and incubated overnight at 65°C. To remove excess, unhybridized probe, membranes were washed with 1 x SSC, 0.1% (w/v) SDS followed by 0.5 x SSC, 0.1 % (w/v) SDS. Each wash was

performed twice, for 20 minutes at 65°C. Membranes were stripped using the hot SDS procedure. A boiling solution of 0.1% (w/v) SDS was poured onto the membrane and allowed to cool. The membranes were then rinsed briefly in 2 × SSC. Membranes were thereafter probed with labeled driver cDNA using the same procedure.

Membranes were exposed to super resolution cyclone phosphor screens for one week. Results were captured on the Packard Cyclone™ Storage Phosphor Screen (Packard Instruments) and analyzed using OPTIQUANT™ software (Cyclone, Packard). Clones that were identified as positively hybridized were clones that appeared darker where colonies were present.

5.2.9. DNA sequence analysis

Both strong and weakly hybridizing colonies were selected for DNA sequence analysis. Overnight cultures of individual clones were prepared by inoculating 5 ml of LB broth and incubating overnight at 37°C. Plasmid DNA was extracted using the Qiagen Miniprep plasmid extraction kit (Qiagen). DNA sequencing of plasmid DNA was performed by dye terminator cycle sequencing using the BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems) as per the manufacturer's protocol. cDNA inserts were sequenced in both directions using the M13 forward and reverse universal primers. Cycle sequencing was performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) and sequence analysis performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

5.2.10 Sequence data analysis

Sequences obtained were edited using Chromas Version 2.31 to remove vector and ambiguous sequences. cDNA sequences were compared to the NCBI GenBank database using the BLASTX (nr protein databases) and BLASTN (non mouse, non human nucleotide database) algorithms (Altschul, 1997). Putative identities were assigned manually based on the match with the highest sequence similarity (the first hit). An e value $\leq 10^{-5}$ was indicative of a statistically significant sequence homology.

5.3. Results

5.3.1. Infection of leaves by brown rust

A distinct difference in infection response of the three varieties was observed visually two weeks after plants were inoculated with *P. melanocephala* urediniospores (Fig. 5.2). The level of infection observed in each variety was consistent with the level of resistance to brown rust exhibited by the varieties. The brown rust susceptible variety N29 exhibited fully developed lesions with spores visible on the surface, as indicated by the arrow position in Figure 5.2. N39 (intermediate resistance/susceptibility) exhibited chlorosis only with some brown lesions just starting to develop. N12, the brown rust resistant variety, showed no signs of infection. It was thus expected that differences in gene expression in response to brown rust infection would be detected in leaves harvested at this stage.

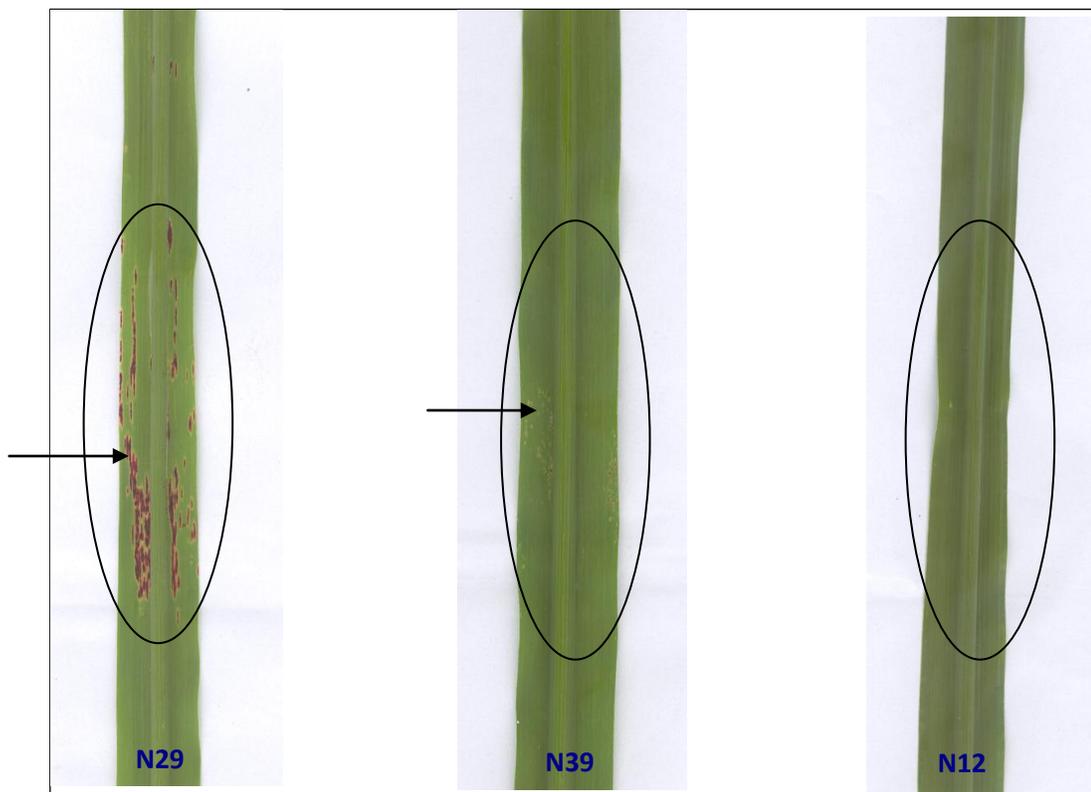


Figure 5.2. Leaves of sugarcane varieties N29, N39 and N12 two weeks post inoculation with *Puccinia melanocephala*.

5.3.2. Construction of subtracted cDNA libraries

Suppression subtractive hybridisation (SSH) was used to isolate differentially expressed cDNA sequences in the resistant (N12) and intermediate (N39) varieties. After performing the subtractive hybridizations, different volumes of the subtracted products were used as the template for PCR amplification. Three different volumes of template were tested: 1, 3 and 5 μl . The amount of PCR product amplified did not appear to differ markedly with different template volumes (Fig 5.3). Furthermore, the size range of PCR products amplified was the same, regardless of the volume of template used (Fig. 5.3). For N39, the amplified products ranged between 150 and 3000 kb whilst for N12 they were between 150 and 2000 kb. In addition, for N12, two clusters of subtracted cDNAs of approximately 325 kb (Fig. 5.3a) and 650 kb (Fig. 5.3b), respectively, appeared to be preferentially amplified. These could represent highly expressed sequences.

To construct the cDNA libraries, 3 μl of PCR product (produced using 1 μl of subtracted cDNA template) was ligated to the pGEM vector. When the subtracted cDNA products were cloned into the vector, the majority of the colonies appeared white (transformed) with only a few blue (non-transformed) colonies observed. This suggests that the transformation had been effective. Transformed colonies (9 from each library) were randomly selected from each library and insert sizes determined by PCR amplification using the universal M13 forward and reverse primers. Insert sizes ranged from 350-800 kb for the N12 subtracted library and 350-1000 kb for the N39 subtracted library. These insert sizes corresponded favourably with the size range of amplified products shown in Fig 5.3, suggesting that cDNA inserts representative of the PCR amplified products had been successfully cloned.

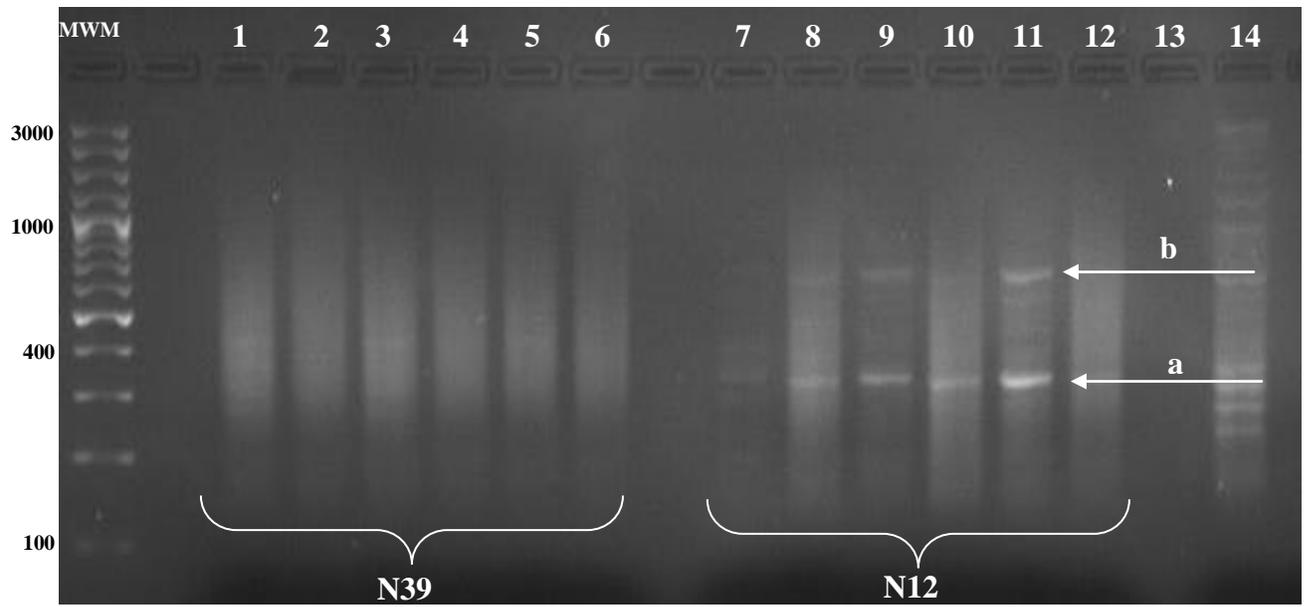


Fig. 5.3. Agarose gel electrophoresis of subtracted cDNA fragments after polymerase chain reaction amplification.

Lane:

- | | |
|----------------------------------|------------------------------------------------------|
| 1 - N39 Subtracted (1 μ l) | 8 - N12 Unsubtracted (1 μ l) |
| 2 - N39 Unsubtracted (1 μ l) | 9 - N12 Subtracted (3 μ l) |
| 3 - N39 Subtracted (3 μ l) | 10 - N12 Unsubtracted (3 μ l) |
| 4 - N39 Unsubtracted (3 μ l) | 11 - N12 Subtracted (5 μ l) |
| 5 - N39 Subtracted (5 μ l) | 12 - N12 Unsubtracted (5 μ l) |
| 6 - N39 Unsubtracted (5 μ l) | 13 - Negative control (no DNA template) |
| 7 - N12 Subtracted (1 μ l) | 14 - Positive control (control subtraction from kit) |

5.3.3. Screening for differentially expressed genes

Subtracted libraries were screened for differentially expressed cDNAs by randomly selecting transformed colonies and transferring them to nylon membranes. Onto these membranes, 1200 colonies were transferred from the N12 subtracted library and 800 colonies from the N39 library. The N12 library was then screened by hybridizing the membranes to radioactively labeled cDNA probes generated using poly (A)⁺ RNA from N12 leaves inoculated with *P. melanocephala* (tester). To test the efficiency of the subtractive hybridization process, the same membranes were stripped and re-probed with radioactively labeled cDNA generated using a combination of poly (A)⁺ RNA from

N29 leaves (inoculated with *P. melanocephala*) and N12 leaves (not inoculated with *P. melanocephala*). If the subtraction process was successful it would be expected that a difference in the types of differentially expressed genes would exist between the two libraries. The N39 subtracted library was screened by hybridizing the membranes to radioactively labeled cDNA probes generated using poly (A)⁺ RNA from N39 leaves inoculated with *P. melanocephala* (tester). Stripped membranes were then hybridized to radioactively labeled cDNA generated using a combination of poly (A)⁺ RNA from N29 leaves (inoculated with *P. melanocephala*) and N39 leaves (not inoculated with *P. melanocephala*). Results using the tester cDNA indicated that a high proportion of clones on the membrane hybridized to the cDNA probes from both N12 and N39 libraries (Fig.5.4). In contrast, the driver cDNA did not hybridize to any of the clones present on the membrane for both driver cDNA probes. These results suggest that the subtraction process had been efficient for both libraries.

When probed with the tester cDNA, different signal intensities between hybridizing clones were observed with some clones hybridizing strongly to the tester (Fig. 5.4., 1) and some showing weaker hybridization signals (Fig. 5.4., 2). Differences in signal intensity were more marked between clones from the N12 library (Fig. 5.4., 1 and 2) than those from the N39 library (Fig. 5.4., 3 and 4). Regardless of their signal intensity, 100 clones that hybridized to the cDNA probes were randomly selected from each library for DNA sequence analysis.

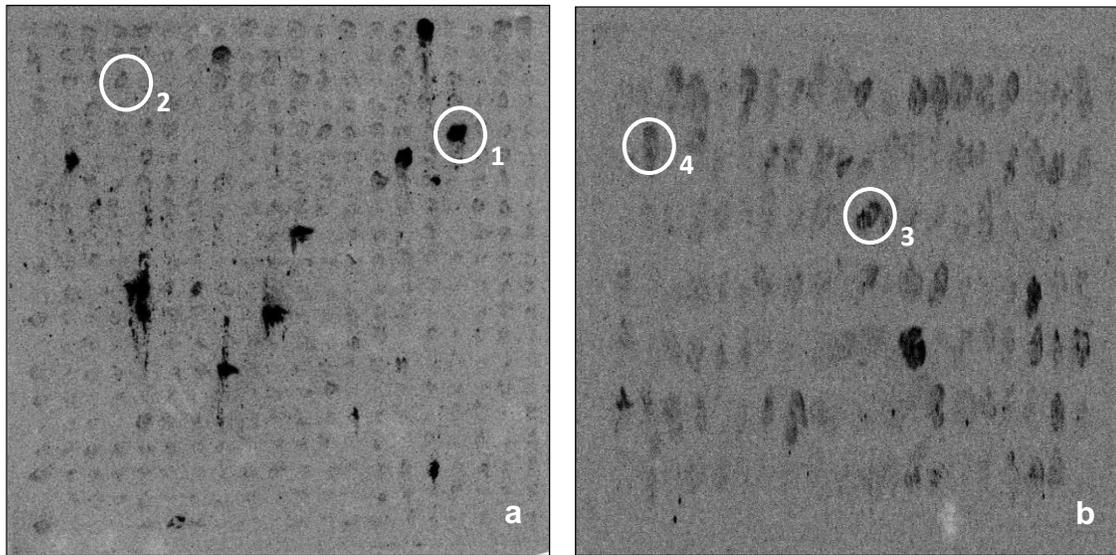


Fig. 5.4. Membranes containing N12 (a) and N39 (b) subtractive libraries probed with radioactively labeled tester DNA, showing a clear distinction between strongly hybridizing (1) and weakly hybridizing (2) clones for the N12 library and a less clear distinction for the N39 library (3 and 4).

5.3.4. Putative identification of differentially expressed genes

High quality DNA sequences were subjected to a BLASTN (nucleotide) and BLASTX (peptide) search of the public gene databases (GenBank) to identify homologous sequences. Matches to both nucleotide and peptide sequences were obtained and sequences grouped into functional categories based on homology to nucleotide sequences or homology to peptide sequences from Genbank (Tables 5.1 and 5.2, respectively). Four groups of homologous nucleotide sequences and seven groups of homologous peptide sequences were compiled for the N12 subtracted library (Tables 5.1 and 5.2). In contrast, homologous sequences identified from the N39 subtracted library could not be grouped based on putative function in the same manner as the N12 library (Tables 5.3 and 5.4). This is because the putative functions of the homologous sequences identified were too broad to allow for further classification. Those sequences were therefore grouped only according to peptide or nucleotide sequence homology.

Of the 23 nucleotide sequences identified from the N12 subtracted library, 22 sequences were homologous to uncharacterized cDNA clones (Table 5.1). The majority of these sequences were either from *Saccharum officinarum* cDNA clones (13) or cDNA clones from libraries generated in response to a stress (4). The different stresses were a pathogen, drought, anaerobic conditions and brassinolide (Table 5.1). One cDNA sequence was observed to be homologous to a plant defense response nucleotide sequence from *Oryza australis* (Table 5.1). Five cDNA sequences were observed to be homologous to plant defense or disease resistance or stress associated peptides (Table 5.2). These included a secretory peroxidase from *Oryza sativa*, an abscisic stress ripening protein from *Zea mays* and a putative flavonone 3-hydroxylase also from *Oryza sativa* (Table 5.2). Of the 14 cDNA clones from the N39 subtracted library found to be homologous to nucleotide sequences, 10 were homologous to nucleotide sequences from uncharacterized *Saccharum officinarum* cDNA clones and one cDNA clone from oxidatively stressed roots and leaves from *Sorghum bicolor* plants (Table 5.3). In contrast to the clones from the N12 library, no sequences were homologous to peptide sequences associated with plant defense or disease resistance. Peptide sequences identified from the N39 library were associated with general plant metabolic processes such as a photosystem I reaction and ferredoxin-NADP reductase, leaf isozyme from *Zea mays* (Fig. 5.4). One homologous peptide sequence was in common between the two libraries: phosphoenolpyruvate carboxylase from *Eulalia aurea*. A difference, in the type of transcripts putatively identified, was noted for the two libraries. Transcripts identified from the N12 library were mostly associated with stress / disease resistance while transcripts from the N39 library were related to general plant function.

Table 5.1. Putative differentially expressed gene sequences in N12 sugarcane plants in response to rust, as identified using BLASTN analysis

Clone	Sequence homology/match	GenBank Accession	Sequence identity (%)	E value
Plant defence response genes				
63F	<i>Oryza australis</i> - Alcohol dehydrogenase class 3	ACM17531.1	84.7	$2 \times e^{-15}$
cDNA clones - stress related				
32F	<i>Sorghum bicolor</i> cDNA clone - Pathogen induced	BE600647.1	96	$4 \times e^{-102}$
82F	<i>Sorghum bicolor</i> cDNA clone - Drought-stressed after flowering	CF757872.1	93	$1 \times e^{-41}$
31R	<i>Sorghum bicolor</i> cDNA clone - brassinolide-treated seedlings	CX613873.1	99	0
8F	<i>Sorghum bicolor</i> cDNA clone - Anaerobic roots	CX611355.1	95	$4 \times e^{-55}$
Saccharum officinarum cDNA clones				
65F	<i>Saccharum officinarum</i> cDNA clone S- CSGRT2061H05	CA145146.1	98	$8 \times e^{-104}$
69F	<i>Saccharum officinarum</i> cDNA clone - SCPRRT3026A02	CA259763.1	97	$2 \times e^{-76}$
34F	<i>Saccharum officinarum</i> cDNA clone - SCSFRT2070A10	CA144741.1	95	$2 \times e^{-82}$
33F	<i>Saccharum officinarum</i> cDNA clone - SCBFST3133G06	CA181560.1	99	$5 \times e^{-102}$
20F	<i>Saccharum officinarum</i> cDNA clone - SCRUAD1133E02	CA217710.1	99	$1 \times e^{-112}$
23F	<i>Saccharum officinarum</i> cDNA clone - SCJFRZ3C08A03	CA160270.1	98	$4 \times e^{-50}$
30F	<i>Saccharum officinarum</i> cDNA clone - SCJFSB1014F09	CA169644.1	81	$6 \times e^{-93}$
70R	<i>Saccharum officinarum</i> cDNA clone - SCJFRZ3C05C08	CA159787.1	99	$5 \times e^{-158}$
75F	<i>Saccharum officinarum</i> cDNA clone - SCSFSB1071A12	CA172109.1	89	$3 \times e^{-51}$
3	<i>Saccharum officinarum</i> cDNA clone - SCJLRT3077A06	CA265874.1	98	$1 \times e^{-137}$
7	<i>Saccharum officinarum</i> cDNA clone SCRFLB1056G01	CA114701.1	100	$9 \times e^{-94}$
10F	<i>Saccharum officinarum</i> cDNA clone - SCUTST3089B04	CA187181.1	99	$5 \times e^{-91}$
14F	<i>Saccharum officinarum</i> cDNA clone - SCJLFL4098E03	CA253171.1	93	$3 \times e^{-60}$
Other cDNA clones				
71R	<i>Saccharum hybrid cultivar</i> cDNA clone - MCSA050H02	CF573786.1 CD227886.	97	$1 \times e^{-65}$
8	<i>Sorghum bicolor</i> cDNA clone - CCC1_56_C12_A007	1	93	$2 \times e^{-56}$
19	<i>Zea mays</i> cDNA clone - ZM_BFc0156P04	EE176979.2 GH726969.	72	0.002
87F	<i>Triticum aestivum</i> cDNA clone - G356107P17	1	99	0
35F	<i>Oryza sativa Indica Group</i> cDNA	FG965457.1	99	$1 \times e^{-145}$

* Putative identification indicates the best match to a sequence in the NCBI non mouse non human nucleotide database

* Values $> e^{-5}$ are considered not statistically significant

Table 5.2. Putative differentially expressed protein sequences in N12 sugarcane plants in response to rust, as identified using BLASTx analysis

Clone	Protein homology/match	GenBank Accession	Score (bits)	E value
Plant defense				
50R	<i>Oryza sativa</i> - secretory peroxidase	EAY96656.1	46.2	1 × e ⁻⁴
83F	<i>Zea mays</i> - Catalase isozyme 3	P18123.2	114	4 × e ⁻²⁴
Stress associated				
19	<i>Zea mays</i> – Abscisic stress ripening protein	ACF82608.1	44.3	0.009
Disease resistance				
34F	<i>Oryza sativa</i> - Putative flavanone 3-hydroxylase	AAN74829.1	489	1 × e ⁻⁰⁴
92R	<i>Eulalia aurea</i> - Phosphoenolpyruvate carboxylase	CAM84060.1	139	6 × e ⁻³²
Signalling proteins				
20F	<i>Zea mays</i> - Serine/threonine-protein kinase SAPK6	ACG33803.1	82.4	1 × e ⁻¹⁴
35F	<i>Lilium</i> - putative senescence-associated protein	ABO20851.1	184	2 × e ⁻⁴⁵
69F	<i>Oryza sativa</i> - Vesicle coat complex	EAY73532.1	148	3 × e ⁻³⁴
71R	<i>Oryza sativa</i> - Serine/threonine kinase	EAZ44163.1	531	7 × e ⁻⁶
10F	<i>Zea mays</i> - Soluble inorganic pyrophosphatase	ACG41455.1	1.34	2 × e ⁻³⁰
82F	<i>Zea mays</i> - Guanylate kinase	ACG39888.1	41.6	4 × e ⁻⁷
Mediation proteins				
31R	<i>Zea mays</i> - Cytochrome P450 monooxygenase	T02955	171	2 × e ⁻⁴¹
Membrane proteins				
32F	<i>Oryza sativa</i> - Cytochrome b6/f complex subunit V	YP_654227.1	97.1	3 × e ⁻¹⁸
33F	<i>Zea mays</i> - Transposon protein mutator sub-class	ACG42214.1	72.4	1 × e ⁻¹¹
3	<i>Zea mays</i> - MTN3	ACG28570.1	52	6 × e ⁻¹⁰
7	<i>Zea mays</i> - 20S proteasome alpha subunit	NP_001105138.1	128	2 × e ⁻²⁸
Other				
14F	<i>Zea mays</i> - Transcription initiation factor IIB	ACG36522.1	155	1 × e ⁻³⁶
87F	<i>Magnetospirillum gryphiswaldense</i> - conserved hypothetical protein	CAJ30045.1	63.9	1 × e ⁻⁸

* Putative identification indicates the best match to a sequence in the NCBI non redundant protein database

* Values > e⁻⁵ are considered not statistically significant

Table 5.3. Putative differentially expressed gene sequences in N39 sugarcane plants in response to rust, as identified using BLASTx analysis

Clone	Sequence homology/match	GenBank Accession	Sequence identity (%)	E value
101	<i>Zea mays</i> - hypothetical protein mRNA clone			
F	1535323	EU955520.1	87	$3 \times e^{-135}$
107	<i>Zea mays</i> - ferredoxin--NADP reductase, leaf isozyme			
F		ACG33858.1	36.2	0.94
134	<i>Zea mays</i> - chloroplast ferredoxin 1 precursor			
F		ACA34367.1	79	$1 \times e^{-13}$
141	<i>Sorghum bicolor</i> cDNA clone – Oxidatively-stressed leaves and roots			
F		CN134755.1	83	$1 \times e^{-108}$
110	<i>Saccharum officinarum</i> cDNA clone			
F	SCEQSD2075A03 5'	CA288521.1	99	$3 \times e^{-52}$
113	<i>Saccharum officinarum</i> cDNA clone			
F	SCAGFL8005H07 5'	CA289508.1	96	$3 \times e^{-114}$
114	<i>Saccharum officinarum</i> cDNA clone			
F	SCCCST1004E12 5'	CA173801.1	100	$2 \times e^{-147}$
115	<i>Saccharum officinarum</i> cDNA clone			
F	SCJFAD1C09D09 3'	CA218502.1	100	$1 \times e^{-41}$
125	<i>Saccharum officinarum</i> cDNA clone			
F	SCVPFL4C11A04 5'	CA280771.1	98	$3 \times e^{-51}$
133	<i>Saccharum officinarum</i> cDNA clone			
F	SCQSLB2054C02 5'	CA270380.1	95	$1 \times e^{-58}$
136	<i>Saccharum officinarum</i> cDNA clone			
F	SCCCRT1C05A11 5'	CA190694.1	98	0
138	<i>Saccharum officinarum</i> cDNA clone			
R	SCAGFL8042B04 5'	CA293241.1	99	$5 \times e^{-97}$
139	<i>Saccharum officinarum</i> cDNA clone			
F	SCEZSD2079F08 5'	CA288901.1	99	$8 \times e^{-180}$
140	<i>Saccharum officinarum</i> cDNA clone			
F	SCRLFL4106G04 5'	CA253865.1	97	$2 \times e^{-89}$

* Putative identification indicates the best match to a sequence in the NCBI non mouse non human nucleotide database

* Values $> e^{-5}$ are considered not statistically significant

Table 5.4. Putative differentially expressed protein sequences in N39 sugarcane plants in response to rust, as identified using BLASTx analysis

Clone	Sequence homology/match	GenBank Accession	Score (bits)	E value
93F	<i>Eulalia aurea</i> - phosphoenolpyruvate carboxylase	CAM84060.1	139	$6 \times e^{-32}$
103F	Putative C4 phosphoenolpyruvate carboxylase	CAC08829.1	265	$1 \times e^{-69}$
138R	<i>Zea mays</i> - VTC2	ACG38291.1	62.8	$9 \times e^{-9}$
139F	<i>Zea mays</i> - ABCF-type protein	NP_001105535.1	142	$3 \times e^{-32}$
109F	<i>Zea mays</i> - rhythmically expressed protein	ACG25671.1	250	$3 \times e^{-65}$
112F	<i>Zea mays</i> - nucleic acid binding protein	ACG44387.1	234	$2 \times e^{-60}$
117F	<i>Zea mays</i> – GADPH (AA 1-337)	NP_001105413.1	265	$2 \times e^{-69}$
101F	<i>Zea mays</i> - hypothetical protein	ACG27638.1	89.7	$7 \times e^{-17}$
113F	<i>Zea mays</i> - ribosome-like protein	ACG40299.1	72.4	$1 \times e^{-11}$
140F	<i>Oryza sativa</i> - Esterases and lipases	EEC73699.1	70.1	$6 \times e^{-11}$
133F	<i>Oryza sativa</i> - hypothetical protein	EEC71142.1	77	$1 \times e^{-12}$
136F	<i>Oryza sativa</i> - Glutamine amidotransferases class-II (GATase)	NP_001065744.1	261	$1 \times e^{-68}$
98F	Photosystem I reaction	O65107.1	137	$3 \times e^{-40}$
141F	FKBP - type peptidyl-prolyl cis-trans isomerase	ACG27545.1	67	$5 \times e^{-10}$
114F	<i>Zea mays</i> - ferredoxin--NADP reductase, leaf isozyme	ACG33858.1	36.2	0.94

* Putative identification indicates the best match to a sequence in the NCBI non redundant protein database

* Values $> e^{-5}$ are considered not statistically significant

5.4. Discussion

Control of brown rust in the SA sugar industry is currently achieved through the use of chemical means and resistant varieties. Chemical control is expensive and often not economically feasible. The use of resistant varieties is often the preferred method of control. Methods to improve ways of selecting resistant varieties are therefore important. Against this backdrop, this study aimed to identify genes that were differentially expressed between brown rust resistant (complete and intermediate) and susceptible varieties in response to brown rust infection. This was achieved through the construction of two sugarcane cDNA libraries enriched in sequences specifically expressed in response to rust infection. The resistant varieties used to produce the libraries were N12

(complete resistance) and N39 (intermediate resistance) and the susceptible variety was N29,

A random selection of clones (100 from each library) were screened and from this process, 41 differentially expressed transcripts were identified from the N12 library and 29 identified from the N39 library (Tables 5.1-5.4). For the N12 library 24% of the transcripts identified were stress related or associated with disease resistance (Tables 5.1 and 5.2). Fernandez *et al.*, (2004) found that 24% of the transcripts identified from the cDNA library created during that study were related to plant defense. Similarly to this study, the study by Fernandez *et al.*, (2004) looked at differentially expressed genes in coffee (*Coffea arabica* L.) plants in response to infection by the coffee leaf rust fungus, *Hemileia vastatrix*. That study however looked at the genes involved in the hypersensitive reaction exhibited by coffee plants and samples for creation of the library were taken at different times post inoculation. In the study by Santaella *et al.* (2004), differentially expressed genes expressed in cassava plants (*Manihot esculenta*) in response to bacterial blight (*Xanthomonas axonopodis* pv. *Manihotis*) 4% of the total transcripts identified were disease / defense related. This study also involved the use of a resistant and susceptible variety. In contrast, for the N39 library, only 3.4% of the transcripts were stress - related (Table 5.3 and 5.4). Transcripts identified from the N39 library were related to general plant function. Other transcripts identified from the N12 library were homologous to uncharacterized cDNA clones but 4 of them were found to be stress associated and from libraries similar to the libraries created during this study.

There are two types of resistance mechanisms employed by plants: the first and most exploited is complete / vertical resistance and the second and less studied is partial / horizontal resistance (Agrios, 2005, Ribeiro Do Vale *et al.*, 2001). Vertical resistance, also known as race specific resistance, is based on an incompatible reaction which is usually covered by a gene for gene mechanism (Martin *et al.*, 2003, Perchepped *et al.*, 2006). It is determined by the present of both the resistance (R) gene in the plant and the avirulence (avr) gene in the pathogen. The R gene in the plant recognizes the avr gene in the plant and elicits a local defense response which is often associated with

rapid programmed cell death, termed hypersensitive response (Perchepped *et al.*, 2006). One of the earliest responses in plants is the generation of active oxygen species which not only have toxic effects on the pathogen but also play a role in signaling (Durant *et al.*, 2000). Plant R proteins usually recognize the pathogen by recognizing specific effector molecules that are produced during the infection process (Martin *et al.*, 2003). Partial resistance (PR), also referred to as nonhost resistance, was first characterized by Caldwell in 1968. It does not offer complete immunity but is usually effective against all races of the pathogen and therefore provides a more durable alternative form of resistance (Liu *et al.*, 2001; Yu *et al.*, 2001; Singh and Rajaram, 2002). It relies on multiple mechanisms, most of which are unknown but are slowly being uncovered (Collins *et al.*, 2003; Lipka *et al.*, 2005; Perchepped *et al.*, 2006; Stein *et al.*, 2006). This type of resistance is similar to the innate immunity present in mammalian cells and includes multiple signal transduction events such as oxidative burst, ion fluxes, activation of MAP kinase cascades coupled with the transcriptional induction of pathogen-responsive genes and localized callose deposition at the cell wall (Asai *et al.*, 2002; Zipfel *et al.*, 2004; Lix *et al.*, 2005). Because PR is usually present at various levels in all parts, breeders do not have to find primitive genotypes from centres of diversity or related wild species. It also can be found in genotypes that have had their race specific resistance broken down (Mikulová *et al.*, 2008). Incomplete expression, no marked interaction with pathogen isolates and an apparently larger genetic complexity are all characteristics of PR (Jones *et al.*, 1982; Asher and Thomas, 1983; Jones and Davies, 1985; Asher and Thomas, 1987). It is usually expressed as a reduction in the rate of development of disease in the host (Kinane and Jones, 2000).

A resistant plant therefore exhibits complete resistance and no symptoms develop on the plant as the pathogen is completely inhibited. This is mediated by one / few resistance genes specific to the pathogen to which it is resistant to (Agrios, 2005). The transcripts identified from the N12 library could be grouped into a wide range of functional groups. These ranged from disease / defense related genes / proteins, to signaling / membrane / mediation proteins and uncharacterized cDNA clones either from *Saccharum officinarum* or from other plants but identified from stress related cDNA

libraries. These types of transcripts were expected as N12 exhibits complete resistance which is illustrated by the lack of symptoms exhibited when infected with *Puccinia melanocephala* urediniospores (Fig. 5.2). N39 exhibits partial resistance which is illustrated by the presence of less pronounced symptoms when infected with *Puccinia melanocephala* urediniospores (Fig. 5.2). The types of transcripts identified for N39 was also consistent with the type of resistance exhibited by this variety. These transcripts could not be grouped into functional categories and those transcripts that were identified were related to general plant function.

Other transcripts identified during this study were also identified in other studies of similar nature. These include Cytochrome P450 (Table 5.2) which was also identified by Hahn and Mendgen (1997), Fernandez *et al.* (2004) and Santaella *et al.* (2004). In addition, Fernandez *et al.*, (2004), showed that the sequence that matched the cytochrome P450 was up regulated during infection of coffee plants with the leaf rust fungus. The up regulation of cytochrome P450 after infection with a pathogen is also supported in other literature (Birch *et al.*, 1999; Cheong *et al.*, 2002; Takemoto *et al.*, 1999; Xiong *et al.*, 2001). They are also known to be involved in the biosynthesis of plant defense compounds (Chapple, 1998; Kahn and Durst, 2000). Kinases were also identified by Fernandez *et al.*, and Santaella *et al.* (2004). Santaella *et al.* (2004) identified a probable serine/threonine- specific protein kinase and a similar serine/threonine protein kinase was also identified during this study (Table 5.2). These authors stated that it might be involved in signal transduction pathways initiated after recognition of elicitors. In addition, the Pto gene which confers resistance to *Pseudomonas syringae* in tomato showed sequence similarity with serine threonine protein kinase (Martin *et al.*, 1993). Other transcripts of interest identified during this study that may be associated with resistance mechanisms were plant catalases (Table 5.2) and phosphoenolpyruvate carboxylase (Table 5.2 and Table 5.4). Plant catalases play a critical role as one of the antioxidant defense genes expressed in response to physiological and environmental stress (Higo *et al.*, 1994). The phosphoenolpyruvate carboxylase found in both libraries is thought to play a role in plant defense but its exact role has not yet been established (Krome *et al.*, 2007). Its levels in cucumber leaves

increased in the presence of fungal extracts of *Cercospora beticola* and *Fusarium graminearum* and the plant activator benzothiadiazole (Krome *et al.*, 2007). This indicates that genes / proteins that have previously been shown to be associated with disease resistance have been identified during this study. This indicates potential for the future use of molecular markers for the early identification of rust resistance.

Even though the results of this study provided some useful preliminary data on the types of genes expressed during the *Puccinia melanocephala* – sugarcane interaction, further research within this field is still required. A difference in the types of genes expressed was noted between the completely and partially resistant varieties and this should be further investigated. Furthermore, the results should be further examined using more detailed gene expression analyses to establish the change in levels of the various transcripts during infection with *P. melanocephala*. These could include tests such as northern blot and microarray analyses and / or q-PCR analysis. Thereafter once expression levels of the different sequences have been identified, a link between these sequences and rust resistance would need to be established. This is essential for one to evaluate their ability for use as molecular markers for early rust identification. Casu *et al.*, (2005) and McIntyre *et al.* (2005a and b) illustrate the potential for use of MAS in sugarcane. This study provides the necessary preliminary data on which further research into MAS for rust resistance could be developed.

5.6. References

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CHAPTER SIX

GENERAL DISCUSSION

Currently, great emphasis is being placed on the use of integrated management strategies to control pests and diseases. Initially, integrated pest management (IPM) or integrated disease management (IDM) was based solely on timing of fungicide applications, where fungicides were applied only when the pest or disease reached levels that cause economic damage. However, presently IPM/IDM is described as a disease management system that, in the context of the environment and population dynamics of the pathogen, utilizes all possible compatible control options to reduce the pathogen/pest population to below economic damage causing levels (Anonymous, 2009). The results of this study provide a sound basis for development of an integrated management strategy for the control of brown rust within the SA sugar industry. However, further research is still required.

The epidemiology studies showed that at least 6h leaf wetness and moderate temperatures (20-25°C) were required to obtain high brown rust infection levels in sugarcane seedlings (Chapter Two). This indicates that fairly specific environmental conditions are required for plants to show high disease levels. These results show potential for development of a disease prediction model which could be used as part of an IDM programme. Even though this study did not test humidities below 98%, and humidity has been identified as a factor of development of disease (Purdy *et al.*, 1983) it still provides valuable information in terms of the factors that influence brown rust development. Preliminary studies do indicate that infection percentages are very low at these humidities but these must be confirmed by full trials. Another key feature is that the findings of this study confirmed those of research carried out in other countries. In addition, current trials are being conducted at the SA Sugarcane Research Institute, Mt Edgecombe where rust levels in the field are being monitored in relation to environmental conditions. The results generated from this study, together with future research under controlled conditions and the field trials being conducted, could be used

in the development of the model, particularly with regards to the parameters of the model. This work conducted locally, using a local rust isolate is imperative for the implementation of the model locally.

Once developed and tested, the model would predict when disease levels are likely to increase, thereby causing economic damage. The growers would then be warned of these high infection levels and that fungicide application was required. This ties in with classical IDM in which fungicide application is timed. This type of disease management has a two-fold benefit: the first being the most beneficial to the grower, in that it would reduce the frequency of fungicide applications (between different seasons), thereby reducing input costs and increasing profit margins and secondly this would have a beneficial impact on the environment with the reduction in chemicals being used. This is of equal importance with the current environmental situation.

Cultural control such as nutritional control methods are usually an important component of any IDM strategy. Even though they often do not provide adequate control to reduce yield loss or if they do, it is not economically feasible, within IDM they can be utilized to reduce disease pressure within the system. However, when investigating the possibility of using a nutrient as a control measure against a disease, one must first test the plant's ability to accumulate the nutrient in large enough quantities for it to be effective. One must also have some background of the ability of that nutrient to decrease disease severity. Silicon (Si) has been shown to induce resistance to fungal diseases in many crops (Epstein, 1999) and sugarcane is known to be a Si accumulator (Motomura *et al.*, 2006). The results of this study confirm that sugarcane is able to accumulate Si and that this Si is accumulated in the leaves (Chapter Three). This shows potential for the use of Si to be used as a control measure against *Puccinia melanocephala*, a foliar pathogen.

This resulted in the testing of Si as a control measure against *Puccinia melanocephala*, the causal agent of brown rust. Application of Si, in the form of potassium silicate and calcium silicate, to sugarcane plants significantly reduced brown rust severity. Even though plants treated with the once-off application of calcium silicate did not show the

best control, it did show significantly less disease than control plants, to which no Si was added (Chapter Four). This fits in perfectly with the concept of IDM, as an application of calcium silicate at planting could reduce disease incidence in the early stages of the crop (first six months) when the crop is most vulnerable to infection by rust. Even if favourable conditions were present for rust development and growers would need to spray, there would be a lower disease incidence and therefore less fungicide would need to be used. This would once again help to increase growers' profits and reduce negative impacts on the environment.

These results, however, are preliminary as they are based only on controlled glasshouse studies. It has been established that Si must be taken up by the plant in order for it to control the disease. In the field, problems have been experienced with Si uptake by sugarcane, in response to Si application. This will therefore have to be further investigated. Initially, pot trials with larger pots as well as growing the cane for a longer period could be conducted. Thereafter, it could be taken to the field and this relationship between Si and brown rust severity could be further investigated. One could also take time to investigate the mode of action by which Si reduces disease severity. This is a relatively new field of research and has not been properly established as yet. Initially, it was thought that Si acted as a physical barrier only, but more recent evidence has shown that Si actively reduces disease incidence by up - regulating defense related genes (Belanger, 2008).

Within the SA sugar industry, the most economical method of control for brown rust is the use of resistant varieties. However, resistance to diseases is tested much later in the breeding process, during the screening phase of the varieties. Development of a protocol to easily identify resistance earlier in the breeding process would refine the process and make it more efficient. One such way is with the use of marker assisted selection. Marker assisted selection (MAS) is a tool used by breeders to efficiently select varieties with resistance to a particular trait. However, molecular markers used during MAS are usually only linked to the gene of interest. As a result, some markers are lost as they are not always found to be associated with resistance. This is illustrated in

studies by McIntyre *et al.* (2005), where only two of the seven markers tested were found to be consistently associated with resistance. If one can identify the genes involved in resistance, then the gene of interest can be used as the marker.

The study conducted in Chapter 5 is the first step towards the identification of molecular markers for the early detection of brown rust resistance. During this study, two subtracted cDNA libraries were generated between a resistant and a susceptible sugarcane variety and a variety with intermediate resistance and a susceptible variety. cDNA clones that were differentially expressed in response to brown rust infection were identified. The putative gene identities of the differentially expressed cDNA clones from each subtracted library were different and several genes of interest that could possibly be used to develop markers were identified. Further studies are required before markers can be developed. Initially, the cDNA clones identified must be tested for their association with brown rust. This could be done using northern blot or microarray analysis. Once the association with brown rust resistance is established, the identified sequences must then be tested for their suitability as markers. The identified markers must then be tested for their accuracy in detecting brown rust resistance within breeding lines. Within the context of IDM, resistant or partially resistant varieties could be used to initially reduce brown rust resistance, thereby eliminating or reducing fungicide applications. Investigating the factors contributing to partial resistance is valuable in terms of rust resistance as durability of resistance has been a problem with brown rust. partial resistance is less susceptible to breaking down and therefore further investigation of this avenue would be of great use.

The results generated in this entire study provide a sound foundation on which an integrated management strategy for the management of brown rust within the SA sugar industry can be built. It investigated aspects of epidemiology, cultural control and resistance with respect to brown rust, all essential components of a good IDM strategy.

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APPENDIX 1

Calculation for Calmasil:

52 g Calmasil in 5000 ml pine bark

x g Calmasil in 1800 ml

$$\begin{aligned}\text{Therefore } x &= (52 \text{ g} \times 1800 \text{ ml}) \div 5000 \text{ ml} \\ &= 18.72 \text{ g}\end{aligned}$$

If 9.8 % Si present in Calmasil

Then 1.83 g Si present in 1800 ml pine bark

In 100 ml pine bark:

1.01667 g Si per 100 ml pine bark

Therefore concentration of Si = 1016.67 mg ℓ^{-1}

APPENDIX 2

Conditions used for digestion of plant samples:

- 500W-1 000W Stepwise for 5 min
- Maintain at 1 000W for 10 min
- Cool at fan setting 2 for 15 minutes

N.B. The same operating conditions were used for both the acid and alkali digestion.

DEPOSITION OF SILICON IN LEAVES OF SUGARCANE (*SACCHARUM* SPP. HYBRIDS) AND ITS EFFECT ON THE SEVERITY OF BROWN RUST CAUSED BY *Puccinia melanocephala*

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Sugarcane is considered to be a silicon (Si) accumulator, and extensive research has been conducted on the role of Si nutrition in this crop. This study focused on the uptake and deposition of Si in the leaves of sugarcane plants and the associated effect on the severity of brown rust. Two trials were conducted, each consisting of varying treatments of potassium silicate (K₂SiO₃), applied weekly. For the disease severity trial, an additional treatment of Calmasil[®] was applied. Si uptake and deposition increased significantly with an increase in Si added. Using X-ray mapping, it was found that significantly more Si was deposited in the lower epidermis than in the upper epidermis and mesophyll. Disease severity was significantly reduced in plants treated with Si at 2000 mg/L. These results suggest that Si nutrition may play an important role in the management of brown rust.

Keywords: sugarcane, brown rust, silicon, Si, deposition, plant resistance

Introduction

Brown rust of sugarcane, caused by *Puccinia melanocephala* H&P Sydow, was accidentally introduced into South Africa from India in 1941 and was first reported on variety Co301 (Bailey, 1995; Sauntally and Autrey, 1999). Even when it reappeared in the mid-1970s, brown rust was regarded as an economically unimportant disease. However, there was a resurgence of the disease in 2000, shortly after the release of variety N29.

Although brown rust in sugarcane is best managed through the use of varietal resistance, fungicides and nutrition management can be effective against the disease. An association between nutrients and rust severity has been identified (Anderson and Dean, 1986; Cadet *et al.*, 2003, McFarlane *et al.*, 2008). Silicon (Si) has been shown to induce resistance to fungal diseases in other crops (Datnoff *et al.*, 2007) as well as to promote the development of healthy sugarcane (Meyer and Keeping, 2001). Cadet *et al.* (2003) suggested that applications of Si could increase its content in the leaves and thus reduce the rate of rust infection. In addition, sugarcane is known to be a silicon accumulator, retaining up to 3% Si in the leaves (Savant *et al.* 1999; personal

communication³). Silicon deposits are highest in the inner tangential walls of the root epidermis and in the silica cells of the leaf and stem epidermis (Kaufman *et al.*, 1981).

The aims of this study were to investigate the uptake of Si by N29 sugarcane plants, identify the leaf tissues in which Si accumulates and assess the effect of Si application on brown rust severity.

Materials and Methods

Two trials were conducted, one to investigate uptake and deposition of Si and the other to investigate the effect of Si on brown rust severity. Each trial consisted of eight treatments, i.e. applications of K₂SiO₄ containing 100, 200, 400, 800, 1 200, 1 600 and 2 000 mg/L Si and a control with no added Si. The trial was replicated four times with six plants per replication. For the disease severity trial, an additional treatment of Calmasil[®] at a rate of 10.49 g/kg was incorporated into pine bark at planting. Trays were arranged in a randomised complete block design and all data was subjected to analysis of variance (ANOVA) using Genstat[®] Executable Release 9 statistical analysis software (Lawes Agricultural Trust, 2003). Least significant difference was determined at P<0.05.

For the uptake and deposition trial, the third leaf below the dewlap leaf was removed from the 0, 400, 800, 1 200, 1 600 and 2 000 mg/L Si treatments six weeks after planting. Transverse sections of the leaf were prepared and analysed for Si deposition in the different leaf tissues using energy dispersive X-ray microanalysis. To quantify Si deposition, the X-ray maps were analysed for percentage area covered by silicon deposits using image analysis. The total Si content in the remaining leaves, roots and stems was determined using inductively coupled plasma optical emission spectrometry (ICP-OES) (Haysom and Ostatek-Boczynski, 2006).

For the disease severity trial, plants were naturally infected with *P. melanocephala* from infected spreader plants growing within the tunnel. Plants were rated for brown rust weekly for eight weeks using a modified rating scale based on a soybean rust rating scale developed by Cedara Agricultural College and also the scale devised by Tai *et al.* (1981).

Results and Discussion

The deposition of Si and the response to Si application in the leaf was significantly different in the different tissue types, with the highest deposition observed in the abaxial epidermis and the lowest in the upper adaxial epidermis (Figure 1). The concentration of Si in the different plant parts increased with an increase in applied Si, and appeared to plateau between 1 600 and 2 000 mg/L (data not presented).

These results are consistent with previous studies, which showed that Si is deposited in high concentrations in the dumb-bell shaped silica cells of the leaf and stem epidermis (Artschwager, 1930; Wong You Cheong *et al.*, 1971a, b). However, the relative number of silica cells in the upper and lower leaf epidermis may differ between sugarcane varieties (Kaufman *et al.*, 1979). Wong You Cheong *et al.* (1971a) found that any Si available to the plant would first be deposited

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in the silica cells, then the trichomes and finally the stomatal walls. Studies on other crops have also indicated high levels of Si deposition in the epidermis (Samuels *et al.*, 1991; Lux *et al.*, 2003; Frantz *et al.*, 2005; Ma and Yamaji, 2006; Motomura *et al.*, 2006). Higher deposition in the lower epidermis could be attributed to a greater number of silica cells in this region (Kaufman *et al.*, 1979; Ferreira *et al.*, 2007).

That Si accumulation plateaued between 1 600 and 2 000 mg/L was evident in both the uptake and deposition results. This information is important when determining the rate of application, and could impact on the economics of Si fertiliser use.

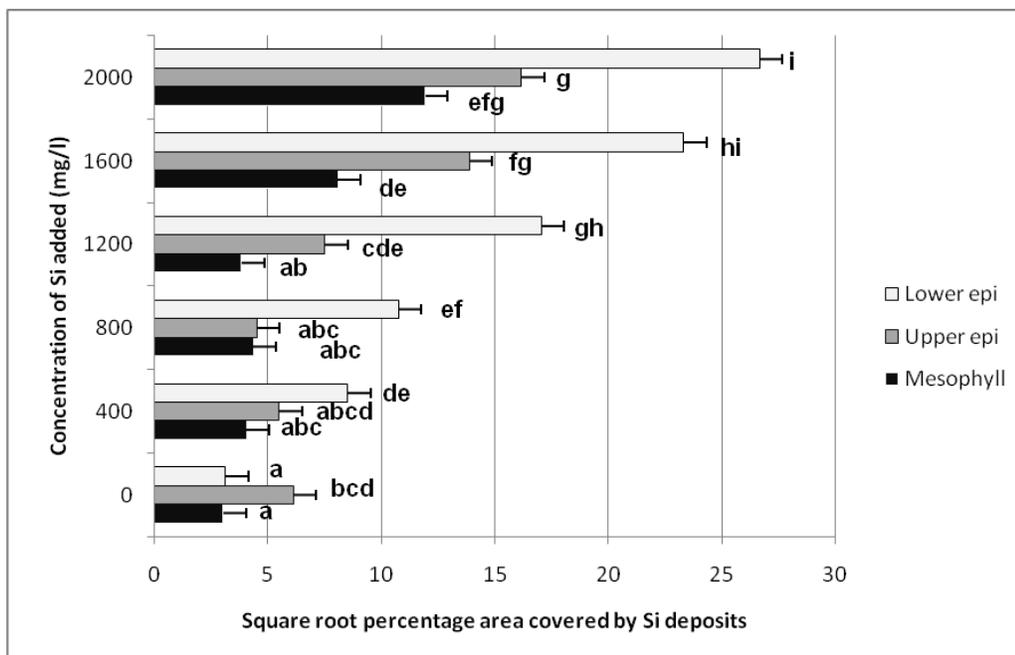


Figure 1. Percentage area (square root transformed) covered by Si deposits in the upper epidermis, lower epidermis and mesophyll of leaves from variety N29 sugarcane plants in response to varying concentrations of Si applied as potassium silicate (ANOVA). Treatments with the same letter are not significantly different.

An increase in Si concentration resulted in a significant decrease in brown rust severity (Figure 2).

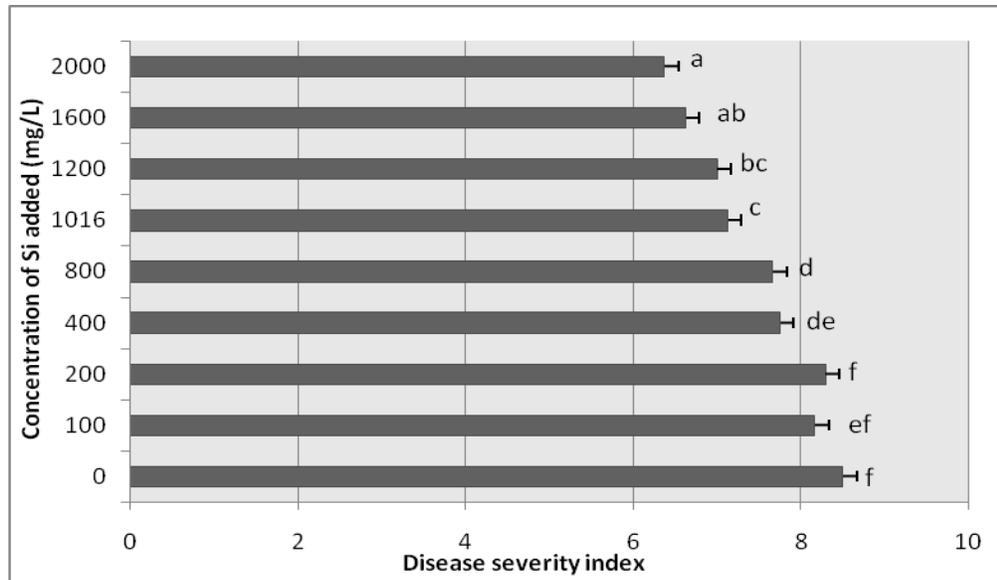


Figure 2. Disease severity index of variety N29 sugarcane plants treated with different concentrations of Si, applied as potassium silicate and calcium silicate (ANOVA). Treatments with the same letter are not significantly different. Treatments with the same letter are not significantly different.

Older leaves on control plants were densely covered with pustules and lesions, and in some cases were necrotic. The pustules and lesions extended to less mature leaves, and an average disease rating of 8.5 was assigned to the control. Rust severity in plants treated with 2 000 mg Si/L was rated as 6.4, with lesions present on older leaves but few or no lesions on younger leaves, indicating that Si not only reduced disease incidence but also slowed down disease progress. A once-off application of Calmasil[®] (1 016 mg/L) was as effective as a weekly application of K₂SiO₄ at a concentration of 1 200 mg/L.

Anderson and Dean (1986) showed a relationship between rust severity and nutrient status of the soil. Cadet *et al.* (2003) suggested that Si could have an effect on brown rust incidence in sugarcane, given its ability to induce disease resistance in other crops. In this study, the application of high levels of Si led to a reduction in disease severity and disease progress. Brown rust tends to be more severe in two to six month old cane (Péros and Lombard, 1986). Therefore, the application of K₂SiO₃ during the early stages of plant growth or even as a once-off treatment of calcium silicate may reduce brown rust levels. The once-off application would be more practical and cost-effective as the Si could be incorporated into the soil at planting. Better rust control might be achieved by increasing the rate of Calmasil[®] applied. There was no significant difference in control between 1 600 Si mg l⁻¹ and 2 000 mg Si/L. These results are consistent with the results from the uptake and deposition trial. If used as part of an integrated management programme, our results indicate that Si could be effective in the control of brown rust of sugarcane, particularly since the pathogen infects the plant through the lower epidermis where the greatest Si deposition occurred.

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Movement of silicon through *Saccharum officinarum* (sugarcane) and its effect on *Puccinia melanocephala* (brown rust)

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Sugarcane (*Saccharum officinarum* L.) is known to absorb more silicon (Si) than any other mineral nutrient. In addition, Si has also been identified as a key element in the control of various diseases and pests. This study focused on the uptake and deposition of Si in sugarcane as well as its effect on the severity on brown rust of sugarcane, caused by *Puccinia melanocephala* H. & P. Sydow. Both trials consisted of 9 treatments i.e. 100, 200, 400, 800, 1200, 1600, 2000 mg l⁻¹ potassium silicate (K₂SiO₃), applied once a week for 8 weeks and Calmasil[®], a commercially available form of calcium silicate, applied at the recommended dosage of 52 g 5 l⁻¹ incorporated in the potting soil at planting. The concentration of Si in the Calmasil[®] was calculated to be 1017 mg l⁻¹. Each trial was replicated 4 times with 6 plants per replicate in a randomized complete block design. The trials were repeated twice. For the disease severity trial, plants were placed in a tunnel and naturally infected by *P. melanocephala* growing on other sugarcane plants. From 3 weeks after planting, plants were rated weekly for 5 weeks for disease severity using a rating scale. Significant differences were noted between treatments. Disease severity was reduced from 8.5 in the control to 6.4 in plants treated with Si at 2000 mg l⁻¹. For the Si uptake trial, total Si accumulation in leaves and stems was measured using ICP-AES. Si uptake increased with increased Si concentration but between 1600 and 2000 mg l⁻¹ there was no increase in Si uptake. The area of highest deposition of Si within the tissues will be assessed by cutting transverse sections of stems and leaves and evaluated using energy dispersion X-ray (EDX) analysis with environmental scanning electron microscopy. Current trials are focusing on the possible ability of Si to catalyze the plant's defense response to brown rust.

Identification of expressed gene sequences associated with brown rust resistance

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Brown rust, caused by *Puccinia melanocephala*, is currently a major problem in the South African sugarcane industry due to its prevalence on the susceptible commercial variety N29 as well as a range of other widely grown intermediate varieties. The most economical and effective method of control is with the use of resistant varieties. This study focused on identifying gene sequences associated with brown rust resistance. Three sugarcane varieties with varying degrees of resistance were used: N29 (susceptible), N39 (intermediate) and N12 (resistant). Plants were challenged with rust at a concentration of 7.75×10^6 urediospores per ml using the leaf whorl inoculation technique. Differentially expressed gene sequences were identified using suppression subtractive hybridization. Total RNA was extracted from challenged plants as well as from uninoculated controls. Two sets of subtractions were conducted, one for variety N39 and another for variety N12. For both subtractions, the tester was the challenged resistant (N12) and intermediate (N39) plants. The driver was a combination of the cDNA of the challenged susceptible plants (N29) and the unchallenged resistant and intermediate plants. After cDNA subtraction, differentially expressed sequences were PCR amplified and cloned into a plasmid vector. Individual transformed colonies were transferred to nylon membranes by a colony lift procedure. To identify the differentially expressed genes, colonies were hybridized to radioactively labeled total cDNA probes from N12 and N39. Colonies were also screened with the respective driver cDNAs to eliminate any cross-hybridising cDNA clones. A large number of positive hybridizing clones have been identified after N12 and N39 screening. Identified differentially expressed genes will be presented and discussed within the context of brown rust resistance.