INVESTIGATING INDUCED RESISTANCE IN SUGARCANE

by

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DISSERTATION SUMMARY

Five potential resistance-inducing chemicals were applied to two sugarcane varieties (N12 and N27) in a pot trial with the aim of inducing resistance to nematodes in naturally-infested soil. BION® (acibenzolar-S-methyl), methyl jasmonate, cis-jasmone and 2,6-dichloroisonicotinic acid (INA) were applied as a foliar spray and suSCon® max*i* (imidacloprid) applied to the soil. All chemicals were tested at two rates and plants were sprayed one week prior to being harvested at 7, 9 and 11 weeks of age. *Meloidogyne* and *Pratylenchus* infestation of sett and shoot roots was determined at each harvest. The activity of four pathogenesis-related proteins was examined at 7, 9 and 11 weeks using separate assays, these enzymes where chitinase, β-1,3-glucanase, peroxidase and polyphenol oxidase. Methyl jasmonate treatment produced significant increases in β-1,3-glucanase, chitinase and peroxidase activity. All other elicitor treatments showed little difference in enzyme activity from the Control. The effect of each treatment on plant growth was examined by recording the dried root and shoot biomass of each plant. No significant differences were seen ($p<0.05$; Holm-Sidak test). However, root and shoot dried biomass was highest in the N12 variety treated by suSCon® max*i.*

The infection of sugarcane with *Ustilago scitaminea* (sugarcane smut) is commonly identified visually by the presence of a smut whip. Identification of sugarcane smut infection can be determined prior to whip development by staining tissue sections with lactophenol cotton blue and examining plant tissues microscopically. This allows for a rapid determination of smut infection which can aid breeding programs. Smut infection is achieved *in vitro* by soaking sugarcane setts in smut spores collected from infected whips. Four methods of inoculation were examined. The method that most consistently caused infection involved allowing setts to germinate for 24 hours, before puncturing a bud with a toothpick, followed by submerging the sett in $1x10^8$ smut spores per mℓ. An elicitor of systemic acquired resistance called BION®, and an insecticide with resistance-inducing properties called Gaucho® (imidacloprid) were used as a sett soak treatments to induce resistance to sugarcane smut. The effect of each treatment at three concentrations on plant germination and growth was examined in the NCo376 variety. Smut spore germination on agar was examined in the presence of both treatments at three concentrations. Sugarcane setts were treated with a concentration

that did not significantly reduce the germination of smut spores or sugarcane setts. Plants were infected with smut post treatment and allowed to grow for approximately one month until plants were between 8 and 10 cm in height. Smut infection was assessed by cutting longitudinal sections through the base of the shoot and staining each section with cotton blue lactophenol. Treatment with BION® and Gaucho® did not reduce smut infection.

DECLARATION

I, Gareth John Edmonds, 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.

G.J. Edmonds

Prof. M.D. Laing (Supervisor)

Dr P.M. Caldwell (Co-supervisor)

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FOREWORD

The research presented in this thesis was undertaken at the University of KwaZulu-Natal, Discipline of Plant Pathology under the academic supervision of Dr P.M Caldwell and Prof. M.D. Laing, and was conducted at the Crop Biology Resource Centre of the South African Sugarcane Research Institute (SASRI), under the co-supervision of Dr R.S. Rutherford and Dr S. Berry.

Controlled induction of disease resistance has been achieved in a number of plant species and has resulted in reduced pathogen infection and damage. Chemical inducers of disease resistance have been used on sugarcane plants in only two other studies, with very limited goals. In this study the use of several chemical treatments were investigated with the goal of inducing disease resistance in sugarcane for the control of sugarcane smut and two genera of nematodes.

The study consists of four chapters and an overview. Chapter One consists of a review of the current literature available on induced resistance, nematodes in sugarcane and sugarcane smut, which are the core scientific fields covered in this dissertation.

In Chapter Two, a study is presented on the effects of the application of five putative resistance inducing chemicals on the levels of four pathogenesis related (PR) proteins: chitinase, β-1,3-glucanase, polyphenol oxidase and peroxidase activity. The chemicals were applied to two sugarcane varieties. The specific aims were to determine whether any of the chemicals, at one of several tested concentrations, increased the concentrations of any of the PR proteins, and whether this changed with different sugarcane varieties.

In Chapter Three a study is presented of the effect of applications of resistance inducing chemicals on both the biomass of two sugarcane varieties, and on the numbers of *Pratylenchus* and *Meloidogyne* nematodes infesting sugarcane roots. The specific goal was to determine whether any of the chemicals, at any dose, and on either of the sugarcane varieties, became more resistant to nematodes, or affected plant growth.

In Chapter Four the initial goal was to develop a reliable means of inoculating sugarcane with smut that bypassed physical resistance features of buds, and to be able to quantify the level of infection of the young plants. The subsequent goal was to test the resistance inducing chemicals for activity against smut via the host resistance of a highly susceptible cultivar.

The dissertation is rounded off with a Dissertation Overview that reviews the original goals and whether they were met, and suggestions for future research as a result of the research results and analysis.

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Sugarcane is a tall, thick-stemmed, perennial grass that is grown predominantly throughout the tropical and subtropical regions of the world. In some countries it is the major source of revenue, e.g., in the Dominican Republic, Jamaica, Mauritius and Swaziland (Cadet and Spaull, 2005). The main product of sugarcane is sucrose crystals or sugar. More than 96 000 000 tons of sugar was produced in the 1998/99 season, with the two largest producers being Brazil and India (Cadet and Spaull, 2005). In South Africa the earliest report of sugarcane cultivation was in 1635 near the mouth of the UMzimkhulu River when shipwrecked Portuguese explorers recorded that sugarcane was one of the crops grown by the local inhabitants (Snyman *et al.*, 2008). Sugarcane production in the 2010 was reported at 16 million tons and the area under sugarcane at 317 000 hectares (Singels *et al*., 2011).

Sugarcane is propagated from nodal buds on the stalk of sugarcane cuttings. Within the first few days roots develop from the primordia around the nodes. These initial roots, called sett roots, support initial growth of the primary shoots (Cadet and Spaull, 2005). Subsequently, tillers develop, and along with the primary shoot, they develop shoot roots that rapidly replace the sett roots (Cadet and Spaull, 2005).The first plant crop is usually harvested between 12 to 24 months after planting. After harvest, new shoots develop from axillary buds on the stubble left behind and give rise to the ratoon crop.

Modern cultivars are complex hybrid crosses between *Saccharum spontaneum* (L.) and *S. officinarum* (L.) (Butterfield *et al.*, 2001). The origin of these species is most likely in the region of New Guinea and east Indonesia (Cadet and Spaull, 2005). Sugarcane is susceptible to outbreaks of damaging diseases (Bailey *et al.*, 1994). A contributing factor to this susceptibility is the fact that sugarcane is cultivated over large adjoining areas, which favour disease build-up and spread (Bailey *et al.*, 1994). A range of sugarcane insect pests, e.g., aphids, can be vectors of microbial diseases

(Rybicki and Pietersen, 1999). Stalk borer insects may cause significant yield losses, e.g. *Eldana saccharina* (Walker) and *Sesamia calamistis* Hampson (Smaill, 1978). The most important bacterial disease of sugarcane in South Africa is Ratoon Stunting Disease (RSD) (*Leifsonia xyli* subsp. *xyli*). Important viral diseases include sugarcane mosaic caused by the *Sugarcane mosaic virus* (SCMV) and yellow leaf syndrome (YLS) caused by *Sugarcane yellow leaf virus* (SCYLV) (Goodman *et al*, 1998; Lockhart and Cronje, 2000). The major two fungal diseases in South Africa are currently brown rust (*Puccinia melanocephala* Syd. & P. Syd.,) and sugarcane smut (*Ustilago scitaminea* (Syd.) M. Piepenbr., M. Stoll & Oberw.) (Bailey, 1979). Plant-parasitic nematodes are also major pests of sugarcane in South Africa, especially *Meloidogyne incognita* ((Kofoid and White) Chitwood) and *M. javanica* (Treub) (Cadet and Spaull, 2003).

1.2 Pathogen resistance in plants

In order to protect themselves from microbial pathogens and herbivorous insects, plants have developed an array of mechanisms. In some cases these defence mechanisms are pre-existing, e.g. thorns and hairs. The cell wall serves as a pre-existing physical barrier to pathogen attack (Van der Ent *et al.*, 2008). These pre-existing defence mechanisms may serve to deter pathogens but often this first line of defence is broken. However, plants possess a second line of defence. A wide spectrum of inducible plant defences may be activated to prevent a pathogen from causing further damage. The first step of this second line of defence is plants' ability to detect its attacker and then translate this detection into an effective defence response (Van der Ent *et al.,* 2008). Detection of pathogens involves the recognition of common features present in microbial pathogens, e.g. flagellin, chitin, glycoproteins and lipopolysaccharides. These biochemical signals are referred to as pathogen-associated molecular patterns (PAMPs). PAMPs activate receptors that initiate signalling events that result in the activation of a basal resistance that is called PAMP-triggered immunity.

In addition to PAMP-triggered immunity activated upon attacker-specific recognition, plants are able to activate another line of defence, referred to as induced resistance.

PAMP-triggered immunity is thought to act specifically against a pathogen, whereas induced resistance is thought to act against a broad spectrum of pathogens. Three forms of induced resistance have been identified so far, based on the signal transduction pathways involved: systemic acquired resistance (SAR); induced systemic resistance (ISR) and wound induced resistance. (Kloepper *et al.*, 2004; Bakker *et al.*, 2007; Walters and Heil, 2007; Van der Ent *et al.*, 2008). Induced resistance is also thought to act systemically in regions of the plant distant from the original site of pathogen attack. This allows for protection from subsequent pathogen attack. Activation of systemically induced resistance can occur after a PAMP-triggered immunity response or by other means. SAR is generally activated by necrotising pathogens. ISR is generally activated by biotrophic pathogens, causing little necrosis. Wound induced resistance occurs after tissue damage caused by herbivores, including insects. Broad spectrum resistance can also be achieved through the use of chemicals that trigger one or more of these resistance pathways.

1.2.1 Systemic acquired resistance (SAR)

For more than a 100 years naturalists and scientists have observed than when plants survive pathogen infection they develop an increased resistance to subsequent infections (Ryals *et al*., 1994). In 1933 the term “physiological acquired immunity” was used in a review to describe the above phenomenon (Chester, 1933). At that time it was believed the phenomenon was similar to that of the immune response found in mammals (Ryals *et al*., 1994).

For the next 30 years after the birth of the term, physiological acquired immunity, many studies were published on the topic but these were descriptive studies that reinforced earlier observations (Ryals *et al*., 1994). The first study of systemic acquired resistance was conducted by A. Frank Ross in 1961. Using tobacco plants with tobacco mosaic virus (TMV), he demonstrated that infection with TMV was reduced by a prior infection (Ross, 1961). Resistance conferred by prior infection with TMV was also shown to be effective against tobacco necrosis virus and certain bacteria. The term “systemic acquired resistance” was used to refer to the resistance that developed in the untreated portions of TMV-inoculated plants (Percival, 2001; Ross, 1961). SAR has since been

demonstrated in many plant species against a broad spectrum of pathogens (Ryals *et al*., 1994; Press *et al*., 1997; Mauch-Mani and Metraux, 1998; Pieterse *et al*., 1998; Heil and Ploss, 2006; Stein *et al*., 2008). In 1979, it was shown that salicylic acid (SA) and certain benzoic acid derivatives could induce both resistance and the accumulation of pathogenesis-related (PR) proteins (White, 1979).

Kees Van Loon demonstrated in 1982, that there is a correlation between the accumulation of a group of PR proteins and the onset of SAR (Van Loon and Antoniw, 1982). There are now many well characterised examples of SAR in both monocotyledonous and dicotyledonous plants (Hunt and Ryals, 1996; Schneider *et al*., 1997; Mauch-Mani and Metraux, 1998). Recently, SAR has been defined as a mechanism of induced defence that may confer protection against a broad spectrum of microorganisms that is long-lasting (Francis *et al.*, 2009). Plants employ SAR as a defence mechanism against pathogen attack, which results in resistance and protection in distal tissue (Francis *et al.*, 2009). SAR can be characterised by the accumulation of SA and the expression of PR proteins (Francis *et al*., 2009).

Experiments using NahG-transformed tobacco and *Arabidopsis* show that plants which cannot accumulate SA cannot develop SAR and fail to exhibit PR protein activation following infection (Walters and Heil, 2007). SAR induction and activation of PR proteins is dependent on transduction of the SA signal. Induced or acquired resistance in plants to pathogens can be achieved by inoculation of plants with incompatible or weak pathogens or by the application of chemical inducers such as SA, 2,6-dichloroisonicotinic acid and benzothiadiazole (Oka *et al.*, 1999).

1.2.2 Induced systemic resistance (ISR)

Surrounding the roots of plants is a nutrient-rich habitat called the rhizosphere, which provides a niche for a large and diverse community of microorganisms (Walters and Heil, 2007; Van der Ent *et al.,* 2009). In this community exists a range of parasitic and beneficial microorganisms that either cause disease or enhance plant performance (Walters and Heil, 2007; Van der Ent *et al.*, 2009). Of the beneficial microorganisms

mycorrhizal fungi and *Rhizobium* have received the most attention (Van der Ent *et al.*, 2009). Benefits provided by mycorrhizal fungi include enhancing the root surface for water absorption and mineral nutrients such as phosphates (Van der Ent *et al.*, 2009; Walters and Heil, 2007; Harrison, 2005). *Rhizobium* provides ammonium by fixing nitrogen from the atmosphere (Van der Ent *et al.*, 2009; Spaink, 2000). Another class of soil-borne microbes with beneficial effects are plant growth promoting rhizobacteria (PGPR) and fungi (PGPF). These are non-pathogenic microbes that occur in large numbers in the rhizosphere and can stimulate plant growth by enhancing the plant's photosynthetic capacity, by suppressing plant disease, or by increasing tolerance to abiotic stress (Zhang *et al.*, 2008). PGPR and PGPF can either directly suppress disease by obstructing the growth and development of soil-borne pathogens through secreting antibiotics in the rhizosphere and by competing for nutrients (Van der Ent *el al*., 2009; Bakker *et al*., 2007; De Bruijn *et al*., 2007) or indirectly by eliciting a plant mediated systemic resistance response (Van der Ent *et al.*, 2009; Kloepper *et al.*, 2004; Van Loon *et al.*, 1998).

SAR and ISR produce phenotypically similar effects, such as broad spectrum disease resistance but they are regulated by different signal transduction pathways. The first evidence of this difference was produced from experiments with PGPR WCS417r in radish, where ISR was demonstrated to be effective against *Fusarium* wilt disease. However, this resistance was not associated with the accumulation of PR proteins, which is a characteristic of SAR (Hoffland *et al*., 1995). Induction of ISR in *Arabidopsis* by WCS417r showed no increase in the transcriptional activity of pathogenesis related genes in the leaf tissue (Pieterse *et al*., 1996). Furthermore, treatment of the roots of *Arabidopsis* with WCS417r showed no increase in SA levels (Pieterse *et al*., 1996). Transgenic *Arabidopsis* NahG plants are unable to accumulate SA due to the expression of a bacterial salicylate hydroxylase gene, naHG. These plants demonstrated a similar level of induced disease resistance after colonization of the roots by WCS417r to that of wild type plants, indicating that WCS417r-mediated ISR functions independently of SA (Pieterse *et al*., 1996). Since these experiments, many other examples of SA-independent ISR have been produced in *Arabidopsis* (Van Wees *et al*., 1997; Ryu *et al*., 2003; Lavicoli *et al*., 2003; Ahn *et al*., 2007; Stein *et al*., 2008; Segarra *et al*., 2009) and other plant species such as tomato (Yan *et al*., 2002; Tran *et al*., 2007; Hase *et al*., 2008), cucumber (Press *et al*., 1997), tobacco (Zhang *et al*.,

2002; Press *et al*., 1997) and rice (De Vleesschauwer *et al*., 2008). From these experiments it is appears that the activation of an SA independent pathway of systemic disease resistance is common for beneficial microorganisms and occurs in a broad range of plant species (Van der Ent *et al*., 2009).

Research into the regulation of ISR activated by beneficial microorganisms shows that jasmonic acid and ethylene are key biochemical players (Van der Ent *et al*., 2009). With jasmonic acid-signalling mutants *jar1*, *jin1*, *eds8* and *coi1* of *Arabidopsis*, ISR mediated by WCS417r was blocked (Pieterse *et al*., 1998; Pozo *et al*., 2008). This was also apparent in ethylene-signalling mutants such as *etr1* (*ethylene response 1*) and *ein2* (*ethylene insensitive 2*) (Pieterse *et al*., 1998; Knoester *et al*., 1999).

1.3 Priming

A separate phenomenon associated with resistance to disease and pests in plants, referred to as priming, is similar to immunisation for plants in that it initiates a state of readiness that does not confer resistance *per se* but rather creates a sensitised host capable of responding more quickly and intensely to subsequent challenges (Whan *et al.*, 2009). With induced resistance obtained through the application of elicitors which initiate immediate defence responses, resistance achieved through priming is not likely to disadvantage the plant in terms of growth and yield as premature defence responses are not required until contact with a pathogen or pest occurs (Whan *et al.*, 2009).

1.4 Resistance inducing compounds

The broad-spectrum resistance conferred by ISR and SAR has generated great interest in the development of agrochemical agents capable of mimicking natural inducers of resistance (Walters and Heil, 2007). Molecules released during host-pathogen interactions and those involved in associated signalling pathways have been the main focus of research into agents that can induce resistance (Walters and Heil, 2007).

Cis-jasmone occurs naturally as a component of flower volatiles but can also be produced by damaged plant tissues (Loughrin *et al.*, 1995). It is now known to be a catabolite of stress-induced jasmonic acid, but had previously been considered as only a biological sink for the jasmonate pathway (Koch *et al.*, 1997). There is now evidence that cis-jasmone has a role in plant defences (Birkett *et al.*, 2000), and acts as an external signal, alerting recipient plants when their neighbours are being attacked/damaged by phytophagous insects, thereby enabling them to prepare their own defences prior to insect attack (Chamberlin *et al.*, 2000; Pickett *et al.*, 2001). Practical use of cis-jasmone has initially focussed on the interaction between the grain aphid and wheat. Wheat plants sprayed with low levels of cis-jasmone as an aqueous emulsion are less attractive to aphids but more attractive to their parasitoids in laboratory bioassays (Bruce *et al*., 2003).

Methyl jasmonate is the methyl ester of jasmonic acid and was first identified as a component of the essential oil of several plant species, while jasmonic acid was first obtained from fungal culture filtrate (Creelman and Mullet, 1997). Initial studies showed that exogenous jasmonic acid or methyl jasmonate can promote senescence and act as a growth regulator (Creelman and Mullet, 1997). Further research demonstrated that jasmonic acid specifically alters gene expression and that the application of elicitors and wounding could cause accumulation of jasmonic acid or methyl jasmonate in plants (Creelman and Mullet, 1997). This research implies a role for jasmonate in plant defences. Jasmonate has been described in other research to be involved in vegetative development, pollen viability and fruit development (Creelman and Mullet, 1997).

A benzothiadiazole derivative, benzo-(1,2,3)-thiadiazole-7-carbothioic acid-S-methyl ester (known as acibenzolar-S-methyl, ASM or BTH), has been identified as a chemical inducer of SAR (Perez *et al*., 2003). This compound has been commercially developed as a plant activator and marketed as BION® or ACTIGARD® (Pappu *et al*., 2000; Perez *et al*., 2003; Walters and Heil, 2007). ASM has been reported to be effective at controlling of diseases caused by viruses, bacteria, nematodes and fungi (Perez *et al*., 2003). The spectrum of resistance activation and biochemical changes induced by ASM are usually similar to that of natural biological induction (Heil *et al.*, 2006). ASM is

applied externally to plants and is reported to induce resistance in both monocotyledonous and dicotyledonous plants (Heil *et al.*, 2006).

Imidacloprid is an insecticide used for the control of major insect pests (Gonias *et al*., 2008). There have been suggestions that imidacloprid may cause yield increases in the absence of insect pests (Gonias *et al*., 2008). The imidacloprid molecule has a chloropyridine side chain that has structural resemblance to nicotinamide and nicotinic acid (Gonias *et al*., 2008). Imidacloprid breaks down into 6-chloronicotinic acid, which is an analogue of the SAR-inducer isonicotinic acid (Francis *et al.*, 2009). Thus it is possible for imidacloprid to induce SAR, and has been demonstrated to do so in citrus (Francis *et al*., 2009).

1.5 Pathogenesis - related proteins

Plants respond to pathogen attack or infection by producing a number of proteins that are important in protecting the plant from the negative effects of the pathogen (Ramesh Sundar *et al.*, 2008). These proteins have been named pathogenesis-related proteins (PRP) and are known to be selectively extractible in a buffer of low pH and are highly resistant to proteolytic degradation (Van Loon and Gerritsen, 1989). Many PRPs purified from plant tissues exhibit direct antifungal activity against a broad range of fungal pathogens (Datta *et al.,* 1999). In other experiments it has been shown that genetically engineered over-expression of PRPs can increase resistance in plants (Broglie *et al.,* 1991). It is believed that the activation of defence mechanisms in plants is a result of an initial recognition event where a host plant detects a pathogen's molecular components. These components are known as elicitors (Van't Slot and Knogge, 2002).

Many biotic elicitors, including lipids, peptides, glycoproteins and proteins have been detected in culture fluid, germination fluid and cell walls of many pathogenic fungi (Ebel and Cosio, 1994). These elicitor molecules bind to receptors on the plasma membrane of plant cells and activate signalling events or pathways required to trigger defence responses (Umemoto *et al.,* 1997). A high molecular mass glycoprotein elicitor was

purified from the mycelia walls of *Colletotrichum falcatum* Went, the red rot pathogen of sugarcane, and used to induce phenylpropanoid metabolites and active oxygen species in suspension-cultured sugarcane cells (Ramesh Sundar *et al.,* 2002). This *C. falcatum* elicitor was later used to induce PRPs in sugarcane leaves and suspension cultured cells of red rot resistant and susceptible sugarcane cultivars (Ramesh Sundar *et al.*, 2008). Two PRPs were examined in the study, chitinase and β-1,3-glucanase. The activity of chitinase significantly increased in the leaves of both a red rot resistant and susceptible sugarcane cultivar 24 h after elicitor treatment (Ramesh Sundar *et al.*, 2008). It was found that the activity of chitinase increased over the experimental time period of 72 h. The red rot resistant sugarcane cultivar showed a more rapid activation of chitinase than the susceptible cultivar (Ramesh Sundar *et al.,* 2008). Similar results were observed when examining β-1,3-glucanase activity, as a rapid increase by 24 h after elicitor treatment was observed with the resistant cultivar showing an elevated response in comparison to the susceptible cultivar (Ramesh Sundar *et al.,* 2008).

Chitinases belong to the PR-3 group of PR proteins that catalyze the hydrolysis of β-1,4 linkages of the N-acetyl-D-glucosamine polymer, chitin. Chitin is a major component of cell walls of many fungi (Ramesh Sundar *et al.,* 2008). Chitin is also present in many insect tissues, as part of the exoskeletal material and is found in the peritrophic membrane. It has been proposed that chitinases might interfere with insect digestion (Falco *et al.*, 2001). Chitinases are often studied for their anti-fungal properties but there is an increasing interest in chitinases as a protective agent against insects (Falco *et al.*, 2001). Several purified plant chitinases have exhibited antifungal activity *in vitro* (Ramesh Sundar *et al.,* 2008). Experimentation with transgenic plants has demonstrated that constitutive over-expression of chitinase can enhance disease resistance (Ramesh Sundar *et al.,* 2008). The rapid increase in chitinase activity in a resistant cultivar of sugarcane after elicitor treatment suggests a possible role for chitinases in the defence mechanism of sugarcane against red rot (Ramesh Sundar *et al.,* 2008).

β-1,3-glucanase has been proposed to have a direct role in defence of plants against pathogens as the substrate for the enzyme, β-1,3-glucan is a major component of the cell walls of many fungi (Ramesh Sundar *et al*., 2008). In the leaves of tomato lines

infected with *Alternaria solani* (E. and M.) Jones and Grout a correlation between constitutive β-1,3-glucanase levels and resistance was detected (Ramesh Sundar *et al.,* 2008). In musk melon infected with *Fusarium*, evidence of higher levels of β-1,3-glucanase activity were observed in resistant plants than in susceptible plants (Ramesh Sundar *et al.,* 2008).

There is a high level of expression of polyphenol oxidase (PPO) in sugarcane apical meristems (Falco *et al.*, 2001). Polyphenol oxidase enzymes are responsible for the typical browning of plant extracts and damaged tissue caused by spontaneous polymerization and cross-linking of *o*-quinones (Falco *et al.*, 2001). Most fruit contain large amounts of PPOs. The physiological function of PPOs in fruit and in other organs of healthy plants is uncertain, but it has been proposed that there is a role for PPO enzymes in defence against leaf eating insects (Falco *et al.*, 2001). When an insect feeds, the mixing of PPOs and phenolic substrates generates o-quinones and these highly reactive compounds are then able to co-valently modify free amino acids and sulfhydryl groups in the dietary proteins within the mouth and gut of the insect (Falco *et al.*, 2001). The resulting phenolic compounds prevent the efficient digestion and absorption alkylated amino acids and thus reduces the nutritional value of plant protein (Falco *et al.*, 2001). Experiments aimed at investigating variation in PPO activity in the stems of alfalfa, showed that activity of the enzyme was much higher in insect resistant than susceptible alfalfa. This suggests a possible role for PPO in resistance to biotic stress (Falco *et al.*, 2001).

1.6 Nematodes

Plant-parasitic nematodes are found in all sugarcane fields throughout the world, and on poor sandy soils they can cause significant yield losses (Cadet and Spaull, 2003). The diversity of nematodes in sugarcane is greater than in most other crops. There are more than 310 species of 48 genera of endoparasitic and ectoparasitic nematodes recorded from sugarcane roots and the rhizosphere of sugarcane (Cadet and Spaull, 2005). Some genera are particularly wide-spread in sugarcane fields, e.g., *Pratylenchus* with at least 20 species reported worldwide, *Helicotylenchus* with 35 spp. and *Tylenchorhynchus* with 36 spp. (Cadet and Spaull, 2005). *Meloidogyne javanica*

and *M. incognita* are considered to be the most serious nematode pests of sugarcane in South Africa (Cadet and Spaull, 2003), along with *P. zeae* and *Xiphinema elongatum* Schuurmans Stekhoven &. Teunissen (Berry *et al.*, 2008). Yield losses due to *M. javanica* have been demonstrated to be 15 tonnes of sugarcane/hectare/annum under South African conditions. This corresponds to approximately 25% of the production capacity under rain-fed conditions (Berry *et al.*, 2008).

Symptoms of *Pratylenchus* damage in sugarcane consist of red, reddish-purple or brown lesions on the roots (Cadet and Spaull, 2005). These lesions become necrotic and change to a purplish-black colour. With this there is an associated reduction in shoot and root mass and stalk length, as well as yellowing of the leaves (Cadet and Spaull, 2005). The symptoms of *Meloidogyne* damage are usually distinct but in the case of sugarcane, they are less easily diagnosed than in other crops (Cadet and Spaull, 2005). The galls that are formed by *M. incognita* and *M. javanica* develop on the tips of sett roots and young shoot roots (Cadet and Spaull, 2005). These galls are often very small and difficult to detect.

Control of nematodes is becoming increasingly difficult due to the withdrawal of nematicides and soil fumigants from the market, e.g., methyl bromide (Oka *et al.*, 1999). Sugarcane farmers in South Africa commonly use the nematicide, aldicarb (Temik®), for the control of nematodes. Aldicarb will be withdrawn from the market at the end of 2016 (Berry and Leslie, 2011). The availability of Aldicarb is predicted to end sooner than expected with the closure of a Bayer plant in the USA which is responsible for the manufacture of methyl isocyanate, a precursor for the production of aldicarb (Berry and Leslie, 2011). This is an important problem for sugarcane growers with nematode problems. Alternative nematicides are available, however. All nematicides registered for sugarcane production fall into Hazard Group 1 (very toxic), and are likely to be banned in the future (Berry and Leslie, 2011).

The use of chemical inducers of SAR have been tested for the control of nematode damage in tomato plants (Oka *et al.*, 1999). Treatments consisted of a foliar spray and a soil drench with either DL-β-amino-*n*-butyric acid (BABA), α- amino-*n*-butyric acid,

jasmonic acid, methyl jasmonate and SA. BABA reduced root-galling and the number of *M. javanica* eggs. However, the other treatments were phytotoxic to the tomato plant or did not improve the control of root-knot nematodes (Oka *et al.*, 1999).

1.7 Sugarcane smut (*Ustilago scitaminea*)

1.7.1 Spread

Smut (*U. scitaminea)* is a major disease of sugarcane worldwide. The first reported outbreak was in the province of Natal, South Africa in 1877 (McMartin 1945; Albert and Schenck 1996; Croft *et al.*, 2008). It is has remained an important disease problem in many African sugar industries and is also a serious problem in Asian countries. The first appearance of the disease in the west was in Argentina in 1940 (Croft *et al.*, 2008). The next report of significant spread came in 1972 when the disease was identified in Hawaii (Croft *et al.*, 2008). Then in 1974 the disease was found in the Caribbean, and spread throughout North America, Central America and the northern countries of South America between 1974 and 1980 (Croft *et al.*, 2008). The second outbreak of sugarcane smut in South Africa occurred between 1945 and 1955 when the cultivar Co301 was severely infected (Bailey, 1979). Then in the 1960s a third outbreak occurred in the northern sugarcane producing regions of South Africa, when the cultivar NCo310 was severely infected (Bailey, 1979). In the 1980s the cultivar NCo376 was heavily infected, this being the fourth major outbreak of the disease in South Africa (Bailey, 1995). In October 2007 and March 2008 severe smut infections were reported on Mpumalanga farms, where seedcane from 70% of the inspected farms had to be ploughed in (van den Berg *et al.*, 2008). A number of significant smut infections have been observed in Mpumalanga since 2008 (Singels *et al.*, 2010).

Smut has been described as one of the most easily diagnosable diseases of sugarcane because its symptoms are so distinctive. The most characteristic symptom of smut infection is a brown whip-like fungal sorus that develops from the apex of the stem of the sugarcane plant (Figure 1.1). This whip-like structure can range in length from 20 mm to over 1 m in length. The whip is composed of a central core of pithy plant cells

that is surrounded by a layer of fungal tissue that produces large amounts of brown teliospores (Croft *et al.*, 2008). Infection of sugarcane plants by teliospores occurs through the buds on stalks or shoots when they are less than 100 mm in length (Croft *et al.*, 2008).

Figure 1.1. Sugarcane smut whip

1.7.2 Life cycle

The life cycle of *U. scitaminea* resembles that of other *Ustilago* species. From whips, airborne diploid teliospores are produced and serve to spread the fungus from plant to plant. On wet plant surfaces teliospores germinate to form a promycelium that in turn

produces haploid sporidia when meiosis takes place. Of the four initial sporidia, two are plus and two minus mating types. Single haploid sporidia continue to multiply by budding in a yeast-like manner on the plant surface and sporidia of complementary mating types can fuse to form a dikaryotic mycelium. The dikaryon, unlike sporidia, can systemically infect sugarcane through the buds at each node on the cane stalk (Albert and Schenck, 1996). The hyphae of the fungus are responsible for the penetration of the basal portion of the bud scales and invade the meristematic tissues (Croft *et al.*, 2008). The fungus grows with the meristematic tissue until it stimulates the plant to produce a sorus (Croft *et al.*, 2008).

1.7.3 Resistance

Resistance to smut is quantitative, thus field trials are used to rate for varietal resistance on a scale of 1 (highly resistant) to 9 (highly susceptible). Sugarcane cultivars are tested by dipping setts in a suspension of teliospores before planting and evaluating resistance by scoring plants based on observations of the number of whips, and at the speed with which whips form (Albert and Schenck, 1996). There are reports of infected plants that do not develop whips during the first season, so they are grown a second season and again scored for smut whip development (Albert and Schenck, 1996). Sugarcane stalks are often shipped between countries to enhance breeding programmes. However, these setts may carry smut mycelia as an asymptomatic infection. This poses a serious threat to countries that are currently free of smut disease (Albert and Schenck, 1996).

Sugarcane smut resistance mechanisms have been separated into bud resistance (infection resistance) and inner tissue resistance (colonisation resistance) (Dean, 1982; Elston and Simmons, 1988; da Gloria *et al*., 1995). An example of the two types of resistance was seen in Brazil with the cultivar SP70-1143, which is known for its high smut resistance in the field. This has been attributed to infection resistance, but a high incidence of smut infection was observed when injured buds of this cultivar were inoculated (da Gloria *et al*., 1995). The structural characteristics of the buds of a susceptible (NA56-79) and resistant (SP70-1143) sugarcane cultivar to smut was investigated, and an association between bud characteristics and resistance was found

(da Gloria *et al*., 1994). The resistant cultivar had an average of 5,5 scales per bud whereas the susceptible cultivar had an average of 4,4 scales per bud (da Gloria *et al*., 1994).

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CHAPTER TWO

RESISTANCE INDUCING CHEMICALS – THEIR EFFECT ON PATHOGENESIS-RELATED PROTEINS IN SUGARCANE LEAVES

Five potential resistance inducing chemicals were applied to two sugarcane varieties (N12 and N27) in a pot trial, with the aim of achieving increased pathogenesis-related protein activity, a marker of induced resistance. BION® (acibenzolar-S-methyl), methyl jasmonate, cis-jasmone and 2,6-dichloroisonicotinic acid (INA) were applied as foliar sprays and suSCon Maxi® (imidacloprid) was applied to the soil. All chemicals were tested at two rates. The activity of chitinase, β-1,3-glucanase, peroxidase and polyphenol oxidase in sugarcane leaves were examined. Methyl jasmonate produced significant increases in chitinase, β-1,3-glucanase and peroxidase activity relative to the Control. No significant increases in polyphenol oxidase activity relative to the Control was observed for any of the treatments. Chitinase and β-1,3-glucanase activity was similar in both varieties. Polyphenol oxidase and peroxidase activity was higher in the N12 variety.

2.1 Introduction

Plants respond to pathogen attack or infection by producing a number of proteins that are part of a biochemical cascade that results in the release of chemicals such as phytoalexins that protect plants from the negative effects of the pathogen (Ramesh Sundar *et al.*, 2008). These proteins have been named pathogenesis-related (PR) proteins and are known to be selectively extractible in a buffer of low pH, and are highly resistant to proteolytic degradation (Van Loon and Gerritsen, 1989). Many PR proteins

purified from plant tissues exhibit direct antifungal activity against a broad range of fungal pathogens (Datta *et al.,* 1999).

Insect herbivores induce PR proteins in host plants as a result of their feeding on plant tissues (Stout *et al*., 1994). In tomatoes, various insects have been reported to induce PR proteins, among these the tomato fruit worm (*Helicoverpa zea* Boddie) and russet mites (*Aculops lycopersici* Massee). The subsequent insect resistance is related to elevated levels of the PR proteins polyphenol oxidase, peroxidase and lipoxygenase (Stout *et al*., 1994). The induction of plant defense systems can also be achieved with abiotic elicitors. These compounds act as signals that stimulate the production of natural plant products, phytoalexins and PR proteins that serve to reduce pathogen damage (Inbar *et al*., 1998). Treatment of tomato plants with BION® (acibenzolar-S-methyl) produced significant increases in foliar chitinase and β-1,3-glucanase activity (Bokshi *et al*., 2003). Similarly, foliar application of BION® to potato plants resulted in increased β-1,3-glucanase activity in the leaves, stems, tubers and stolons but not in the roots (Bokshi *et al*., 2003).

Systemic acquired resistance (SAR) is a defense mechanism employed by plants against pathogen attack. Characteristics of SAR include accumulation of salicylic acid (SA) protection in distal tissue, resistance against a broad spectrum of pathogens and PR protein expression (Van der Ent *et al*., 2008; Francis *et al*., 2009). Increased expression of PR proteins has been used as conformation of SAR (Schneider *et al*., 1997; Oka *et al*., 1999; Walters and Heil, 2007).

Induction of PR proteins in sugarcane leaves has been achieved using a glycoprotein elicitor isolated from *Colletotrichum falcatum* Went (the red rod pathogen of sugarcane). Elicitor treatment resulted in increased chitinase and β-1,3-glucanase activity in two cultivars (Ramesh Sundar *et al.*, 2008). The aim of this study was to investigate the effects of several chemical elicitors, known to be inducers of plant resistance, on the activity of PR proteins in the leaves of two sugarcane cultivars in a pot trial.

2.2 Materials and methods

2.2.1 Plant material and treatments

Two sugarcane cultivars where selected, N12 (nematode-tolerant) and N27 (nematode-susceptible). Single-budded sets where planted in pots containing approximately 8 kg of sandy soil (<10% clay), collected from a nearby sugarcane field in UMdloti, KwaZulu-Natal. The pots where housed in a rain shelter facility, watered three times weekly and fertilized with Hygrotech® (NPK 3.1.5(38)) seedling fertilizer once weekly. A randomised complete block design was used to lay out the pots. Five elicitor treatments were selected along with a water only treatment as the Control (Table 2.1). Two concentrations for each elicitor treatment where used with five replicates for each treatment and concentration. All treatments contained 1 ml ℓ^{-1} BREAK-THRU® (Polyether- polymethylsiloxane - copolymer1000g / 1) solution and 2 ml ℓ^{-1} of ethanol as the adjuvant and solvent, respectively.

Table 2.1. Resistance inducing chemicals and application concentrations

Elicitor	Low concentration	High concentration
BION® (acibenzolar-S-methyl)	0.1 g ℓ^{-1}	1 g ℓ^{-1}
cis-jasmone	150 µM	1.5 mM
2,6-dichloroisonicotinic acid	1 mM	10 mM
methyl jasmonate	150 µM	1.5 mM
suSCon® max*i* (imidacloprid)	1.5 g/m^2	4 g/m^2
H_20 (Control)	-	-

For each elicitor application, pots where removed from the rainshelter and grouped according to the type of elicitor treatment and concentration of elicitor to be applied. Foliar application of elicitors was conducted using “Down to Earth” spray bottles, until run off from the leaves. Twenty four hours post application, the pots where returned to the rainshelter and put back in their original positions. The granular insecticide suSCon® max*i* was directly applied to the soil during planting.

2.2.2 Enzyme extraction

One week after foliar applications, leaf disks (5mm in diameter) were collected from a single leaf adjacent to the spindle of each plant. Disks where cut from both sides of the midrib, halfway along the length of the leaf. Disks where quickly frozen in liquid nitrogen and stored at -80°C until required for enzyme extraction. Leaf disks were ground into a fine powder with a plastic plunger in a 1.5ml tube with liquid nitrogen. To extract protein from the ground leaf disks, 500µl of cold 0.015M sodium phosphate buffer (pH 6.0) was added and tubes centrifuged at 10 000 x g for 10min at 4˚C. Total protein concentration for each extract was determined, using the Bio-Rad Protein Assay (http://www.bio-rad.com/), with bovine serum albumin (BSA) as a standard.

2.2.3 β-1,3-glucanase assay

β-1,3-glucanase activity was assessed using the supernatant from the protein extraction. To 400µl of 50mM potassium acetate buffer (pH 5.0) was added 100µl of supernatant in a 1. 5ml tube and allowed to equilibrate for 5min at 30˚C. The reaction was then initiated by the addition of 100µl of substrate. The substrate for the reaction was azurine-crosslinked pachyman in a tablet form (Megazyme - http://www.megazyme.com/). The tablet was added to 1 ml of sterile deionised water and homogenised by vortexing. The reaction products were incubated for 20min at 30˚C. Centrifugation at 12 000 x g for 5min was used to terminate the reaction and to precipitate non-degraded substrate. From the resulting supernatant, a 300µl sample was taken to read the absorbance at 595nm on a spectrophotometer (maker and address). Results were expressed as $\Delta OD_{595}\, mg^{-1}$ protein min.

2.2.4 Chitinase assay

Chitinase activity was assessed by adding 100µl of protein extract to 200µl of 50mM potassium acetate buffer (pH5.0) in a 1.5ml tube and allowed to equilibrate for 5min at 30˚C. The reaction was initiated by the addition of 100µl of carboxy-methyl chitin,

linked with the dye Remazol Brilliant Violet 5R (CM-Chitin-RBV www.sigmaaldrich.com). Tubes were kept at 30˚C for 10min and the reaction terminated and all non-degraded product precipitated by the addition of 2N HCℓ. Tubes were cooled on ice for 10min followed by centrifugation for 5min at 12 000 x g. The absorbance of the resulting supernatant was read at 540nm and results expressed as $\Delta OD_{595}\, mg^{-1}$ protein min.

2.2.5 Peroxidase assay

The peroxidase assay used here was based on a measurable colour change that occurs from guaiacol oxidation in the presence of hydrogen peroxide. A 96-well plate was used and 230µl of 100mM sodium phosphate buffer (pH 6.0), with 10µl of 0.02M guaiacol added to each well. Enzyme extract (10µl) was added and finally 50µl of 0.38 M H_2O_2. The optical density was read at 470nm every 10sec for 120sec. Results were expressed as $\Delta OD_{595}\, mg^{-1}$ protein min.

2.2.6 Polyphenol oxidase assay

Using a 96-well plate, 240µl of 100mM sodium phosphate buffer (pH 6.0) was added, with 10µl of enzyme extract and incubated for at room temperature for 5min. The reaction was initiated by the addition of 50µl of 0.1M chlorogenic acid. The change in optical density at 420nm was recorded every 10sec for 120sec. Two blanks were used, one containing buffer and enzyme extract without chlorogenic acid, and another with buffer and chlorogenic acid without enzyme extract. The sum of the blanks was subtracted from the reaction values. Results are expressed as $\Delta OD_{595}\, mg^{-1}$ protein min.

2.2.7 Statistical analysis

Results were analysed using the Restricted Maximum Likelihood (REML) procedure (Genstat ver.11). Significant differences between treatment means were tested using the Holm-Sidak all-pairwise multiple comparison test at the 5% level of significance (Genstat ver.11).

2.3 Results

2.3.1 β-1,3-glucanase assay

β-1,3-glucanase activity in both varieties increased with plant age, particularly in treatments where the high rates had been applied, as well as in the Control treatment (Table 2.2). This was not evident in the sugarcane treated with the low rates of the chemicals. There were few differences between the mean β-1,3-glucanase activity in Control plants of N12 and N27, the mean activity in N12 plants being 0.237 and in N27 plants being 0.255. Only on two occasions did any treatment cause a significant increase in activity relative to the Control plants. Those treatments were methyl jasmonate at a low dose at 9 weeks on the N12 variety and methyl jasmonate at a high dose at 11 weeks on the same variety (Table 2.2). No significant increases relative to the Control occurred in the N27 variety. For both varieties at 7 and 9 weeks the low dose of all treatments caused higher enzyme activity than the high dose treatments. However, this trend was reversed at 11 weeks.

Table 2.2a. β-1,3-glucanase activity in the leaves of N12 and N27 sugarcane plants subjected to foliar treatment with various chemicals. Plants where sprayed prior to harvest and harvested at three different ages (7, 9 and 11 weeks). β-1,3-glucanase activity is expressed as ΔOD_{595} mg^{-1} protein min.

N12

TREATMENT	7 weeks*		9 weeks*		11 weeks*	
BION® High (acibenzolar-S-methyl)	0.079	**ab**	0.260	**ab**	0.249	**a**
BION® Low (acibenzolar-S-methyl)	0.349	**bc**	0.415	**abcd**	0.212	**a**
cis jasmone High	0.051	**a**	0.307	**abc**	0.459	**a**
cis jasmone Low	0.420	**c**	0.420	**bcd**	0.314	**a**
2,6-dichloroisonicotinic acid High	0.042	**a**	0.261	**ab**	0.521	**ab**
2,6-dichloroisonicotinic acid Low	0.434	**c**	0.385	**abcd**	0.289	**a**
methyl jasmonate High	0.113	**ab**	0.438	**cd**	0.801	**b**
methyl jasmonate Low	0.479	**c**	0.522	**d**	0.417	**a**
suSCon® max*i* High (imidacloprid)	0.051	**a**	0.252	**a**	0.492	**ab**
suSCon® max*i* Low (imidacloprid)	0.492	**c**	0.368	**abcd**	0.316	**a**
Control	0.237	**abc**	0.331	**abc**	0.406	**a**

N27

TREATMENT	7 weeks*		9 weeks*		11 weeks*	
BION® High (acibenzolar-S-methyl)	0.119	**ab**	0.295	**a**	0.522	**a**
BION® Low (acibenzolar-S-methyl)	0.505	**c**	0.507	**ab**	0.480	**a**
cis jasmone High	0.093	**a**	0.345	**a**	0.582	**a**
cis jasmone Low	0.441	**abc**	0.500	**ab**	0.461	**a**
2,6-dichloroisonicotinic acid High	0.144	**ab**	0.402	**a**	0.656	**a**
2,6-dichloroisonicotinic acid Low	0.454	**bc**	0.441	**ab**	0.428	**a**
methyl jasmonate High	0.104	**ab**	0.393	**a**	0.671	**a**
methyl jasmonate Low	0.533	**c**	0.714	**b**	0.382	**a**
suSCon® max*i* High (imidacloprid)	0.217	**abc**	0.420	**a**	0.539	**a**
suSCon® max*i* Low (imidacloprid)	0.526	**c**	0.508	**ab**	0.344	**a**
Control	0.255	**abc**	0.434	**ab**	0.445	**a**

*Different letters following values in columns representing each time point indicate significant differences between treatments ($p<0.05$; Holm-Sidak test).

Figure 2.1 shows the combined results for both varieties and the results for high and low doses of each treatment in order to determine the effect each chemical on sugarcane. Methyl jasmonate application resulted in a significant increase in β-1,3-glucanase activity relative to all other treatments as well as the Control. No significant differences between other treatments were observed.

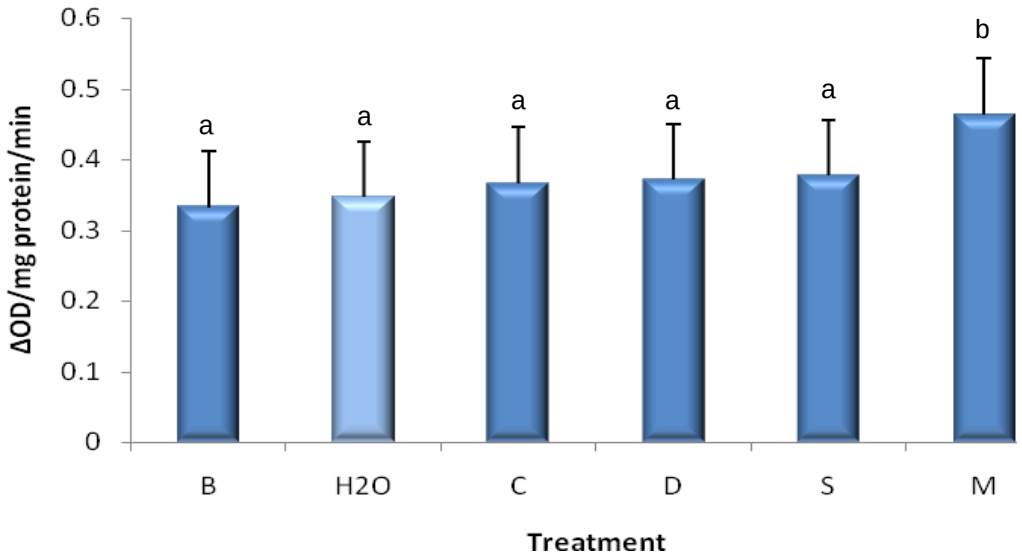

Figure 2.1. Average β-1,3-glucanase activity in sugarcane leaves of both N12 and N27 varieties treated with chemical elicitors of resistance. Activity is expressed as change in optical density at 595 nm per total protein added per minute. Vertical bars represent Holm-Sidak statistics at the 5% level of significance.
B: BION® (acibenzolar-S-methyl)
C: cis jasmone
D: 2,6-dichloroisonicotinic acid
H_2O: water control
M: methyl jasmonate
S: suSCon® max*i* (imidacloprid)

2.3.2 Chitinase activity

As with β-1,3-glucanase, chitinase activity tended to increase over time, particularly in the sugarcane sprayed with the higher concentrations (Table 2.3). However, there were no significant changes in chitinase activity for any treatments or dosage for N27. The only treatments to increased chitinase activity significantly relative to the Control were cis jasmone (low rate) and methyl jasmonate (at both the high and low rates) in the N12 variety at 9 weeks (Table 2.3).

Table 2.3. Chitinase activity in the leaves of N12 and N27 sugarcane plants subjected to foliar treatment with various chemicals. Plants where sprayed prior to harvest and harvested at three different ages. Chitinase activity is expressed as change in optical density at 540 nm ΔOD_{540}/mg protein/min.

N12

TREATMENT	7 weeks*		9 weeks*		11 weeks*	
BION® High (acibenzolar-S-methyl)	0.725	**ab**	0.742	**abcde**	0.932	**ab**
BION® Low (acibenzolar-S-methyl)	0.786	**ab**	0.743	**abcde**	0.896	**ab**
cis jasmone High	0.596	**a**	0.799	**bcde**	0.932	**ab**
cis jasmone Low	0.847	**ab**	0.951	**e**	0.827	**ab**
2,6-dichloroisonicotinic acid High	0.560	**a**	0.617	**abcd**	1.139	**ab**
2,6-dichloroisonicotinic acid Low	0.860	**ab**	0.893	**cde**	0.870	**ab**
methyl jasmonate High	1.232	**b**	1.058	**e**	1.214	**b**
methyl jasmonate Low	0.705	**ab**	0.925	**de**	1.145	**ab**
suSCon® max*i* High (imidacloprid)	0.593	**a**	0.457	**a**	1.099	**ab**
suSCon® max*i* Low (imidacloprid)	0.696	**ab**	0.549	**ab**	0.584	**a**
Control	0.858	**ab**	0.589	**abc**	0.864	**ab**

N27

TREATMENT	7 weeks*		9 weeks*		11 weeks*	
BION® High (acibenzolar-S-methyl)	0.936	**ab**	0.721	**ab**	1.104	**ab**
BION® Low (acibenzolar-S-methyl)	0.983	**ab**	0.874	**ab**	0.98	**ab**
cis jasmone High	0.506	**ab**	0.847	**ab**	1.425	**b**
cis jasmone Low	0.723	**ab**	0.890	**ab**	0.888	**ab**
2,6-dichloroisonicotinic acid High	0.710	**ab**	0.721	**ab**	1.477	**b**
2,6-dichloroisonicotinic acid Low	1.060	**b**	0.960	**b**	0.918	**ab**
methyl jasmonate High	0.727	**ab**	0.829	**ab**	1.195	**ab**
methyl jasmonate Low	1.015	**ab**	0.865	**ab**	0.864	**ab**
suSCon® max*i* High (imidacloprid)	0.371	**a**	0.449	**a**	0.959	**ab**
suSCon® max*i* Low (imidacloprid)	0.878	**ab**	0.411	**a**	0.623	**a**
Control	0.698	**ab**	0.654	**ab**	0.864	**ab**

*Different letters following values in columns representing each time point indicate significant differences between treatments ($p<0.05$; Holm-Sidak test).

Combining results for both varieties, all time points and high and low dose for each treatment demonstrated that methyl jasmonate and 2,6-dichloroisonicotinic increased chitinase activity significantly in sugarcane leaves relative to the Control and suSCon® max*i* treatment (Figure 2.2).

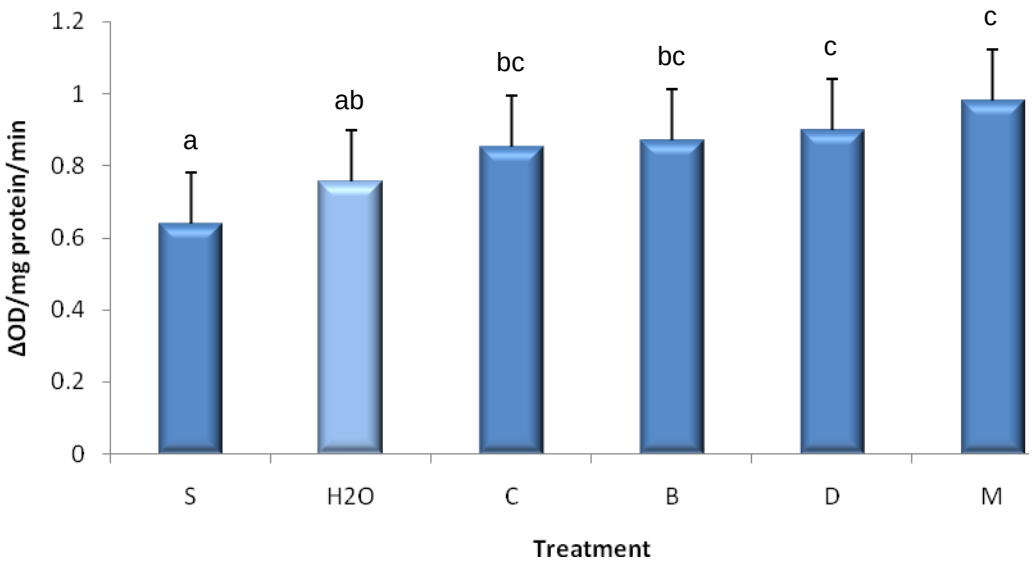

Figure 2.2. Average chitinase activity in sugarcane leaves of both N12 and N27 varieties treated with chemical elicitors of resistance. Activity is expressed as change in optical density at 540 nm per total protein added per minute. Vertical bars represent Holm-Sidak statistics at the 5% level of significance.
B: BION® (acibenzolar-S-methyl)
C: cis jasmone
D: 2,6-dichloroisonicotinic acid
H_2O: Water control
M: methyl jasmonate
S: suSCon® max*i* (imidacloprid)

2.3.3 Peroxidase activity

In the Control plants, the peroxidase activity at all three time points was higher in N12 than it was in N27 (Table 2.4). The methyl jasmonate (high rate) treatment resulted in a significant increase in peroxidase activty relative to the Control in N12 sugarcane leaves at 11 weeks (Table 2.4). In N27, a significant increase in peroxidase activity was observed at 9 weeks in plants treated with BION® at the low concentration (Table 2.4).

Table 2.4. Peroxidase activity in the leaves of N12 and N27 sugarcane plants subjected to foliar treatment with various chemicals. Plants where sprayed prior to harvest and harvested at three different ages. Chitinase activity is expressed as change in optical density at 470 nm ΔOD_{470}/mg protein/min.

N12

TREATMENT	7 weeks*		9 weeks*		11 weeks*	
BION® High (acibenzolar-S-methyl)	11.43	**a**	16.85	**ab**	20.75	**abc**
BION® Low (acibenzolar-S-methyl)	5.84	**a**	25.69	**b**	13.79	**ab**
cis jasmone High	12.76	**a**	16.31	**ab**	15.58	**ab**
cis jasmone Low	7.10	**a**	20.19	**ab**	14.87	**ab**
2,6-dichloroisonicotinic acid High	8.80	**a**	16.2	**ab**	23.09	**bc**
2,6-dichloroisonicotinic acid Low	6.81	**a**	23.32	**b**	17.94	**abc**
methyl jasmonate High	13.36	**a**	22.92	**b**	29.44	**c**
methyl jasmonate Low	6.88	**a**	15.21	**ab**	13.42	**ab**
suSCon® max*i* High (imidacloprid)	4.94	**a**	5.14	**a**	15.27	**ab**
suSCon® max*i* Low (imidacloprid)	3.79	**a**	10.64	**ab**	8.94	**a**
Control	7.83	**a**	11.93	**ab**	14.19	**ab**

N27

TREATMENT	7 weeks*		9 weeks*		11 weeks*	
BION® High (acibenzolar-S-methyl)	10.63	**a**	10.23	**ab**	13.91	**c**
BION® Low (acibenzolar-S-methyl)	6.72	**a**	22.89	**b**	11.68	**abc**
cis jasmone High	4.91	**a**	15.02	**ab**	15.58	**c**
cis jasmone Low	4.31	**a**	16.43	**ab**	10.19	**abc**
2,6-dichloroisonicotinic acid High	7.28	**a**	9.91	**ab**	11.7	**abc**
2,6-dichloroisonicotinic acid Low	5.35	**a**	18.61	**ab**	11.7	**abc**
methyl jasmonate High	8.40	**a**	15.65	**ab**	13.15	**bc**
methyl jasmonate Low	3.46	**a**	17.77	**ab**	10.28	**abc**
suSCon® max*i* High (imidacloprid)	3.92	**a**	5.99	**a**	6.02	**ab**
suSCon® max*i* Low (imidacloprid)	2.67	**a**	7.58	**a**	4.22	**a**
Control	4.37	**a**	8.26	**a**	8.59	**abc**

*Different letters following values in columns representing each time point indicate significant differences between treatments ($p<0.05$; Holm-Sidak test).

Combined results for varieties, all time points and, high and low dose showed that methyl jasmonate significantly increased peroxidase activity relative to the Control and suSCon® max*i* treatments (Figure 2.3).

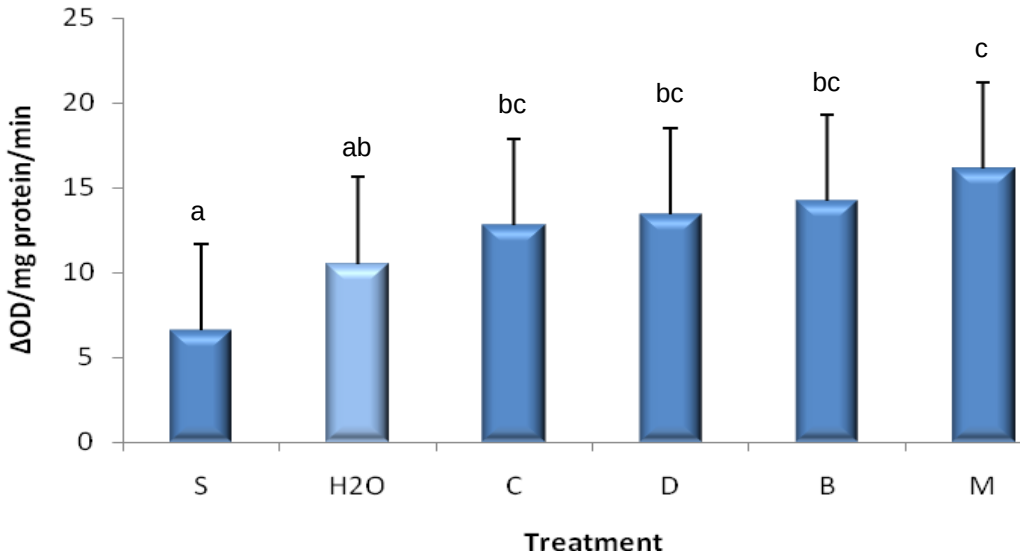

Figure 2.3. Average peroxidase activity in sugarcane leaves of both N12 and N27 varieties treated with chemical elicitors of resistance. Activity is expressed as change in optical density at 470 nm per total protein added per minute. Vertical bars represent Holm-Sidak statistics at the 5% level of significance.
B: BION® (acibenzolar-S-methyl)
C: cis jasmone
D: 2,6-dichloroisonicotinic acid
H_2O: Water control
M: methyl jasmonate
S: suSCon® max*i* (imidacloprid)

2.3.4 Polyphenol oxidase activity

There were no significant differences in polyphenol oxidase activity for any treatment and dose at all three time points for N27 (Table 2.5), nor was there for N12 at 7 and 9 weeks. However, at 11 weeks both of the suSCon® max*i* treatments (high and low rates) significantly decreased polyphenol oxidase activity relative to the Control (Table 2.5).

Table 2.5. Polyphenol oxidase activity in the leaves of N12 and N27 sugarcane plants subjected to foliar treatment with various chemicals. Plants where sprayed prior to harvest and harvested at three different ages. Chitinase activity is expressed as change in optical density at 420 nm ΔOD_{420}/mg protein/min.

N12

TREATMENT	7 weeks*		9 weeks*		11 weeks*	
BION® High (acibenzolar-S-methyl)	40.11	**a**	40.13	**a**	28.73	**ab**
BION® Low (acibenzolar-S-methyl)	61.52	**a**	28.5	**a**	35.79	**abc**
cis jasmone High	53.05	**a**	33.07	**a**	23.23	**ab**
cis jasmone Low	45.42	**a**	43.22	**a**	32.59	**ab**
2,6-dichloroisonicotinic acid High	52.26	**a**	47.43	**a**	58.85	**c**
2,6-dichloroisonicotinic acid Low	34.58	**a**	37.44	**a**	27.51	**ab**
methyl jasmonate High	48.03	**a**	51.02	**a**	46.54	**bc**
methyl jasmonate Low	46.96	**a**	41.38	**a**	35.4	**abc**
suSCon® max*i* High (imidacloprid)	29.99	**a**	16.47	**a**	12.67	**a**
suSCon® max*i* Low (imidacloprid)	31.21	**a**	23.2	**a**	13.06	**a**
Control	48.32	**a**	49.93	**a**	46.36	**bc**

N27

TREATMENT	7 weeks*		9 weeks*		11 weeks*	
BION® High (acibenzolar-S-methyl)	41.41	**ab**	40.04	**a**	28.46	**ab**
BION® Low (acibenzolar-S-methyl)	84.63	**b**	41.31	**a**	28.11	**ab**
cis jasmone High	38.74	**ab**	54.3	**a**	41.42	**ab**
cis jasmone Low	64.64	**ab**	39.03	**a**	41.7	**b**
2,6-dichloroisonicotinic acid High	52.92	**ab**	30.69	**a**	34.3	**ab**
2,6-dichloroisonicotinic acid Low	60.02	**ab**	40.8	**a**	29.34	**ab**
methyl jasmonate High	49.41	**ab**	42.7	**a**	42	**b**
methyl jasmonate Low	44.09	**ab**	43.65	**a**	27.91	**ab**
suSCon® max*i* High (imidacloprid)	14.16	**a**	19.66	**a**	11.66	**a**
suSCon® max*i* Low (imidacloprid)	12.71	**a**	26.81	**a**	15.33	**ab**
Control	38.24	**ab**	46.65	**a**	35.06	**ab**

*Different letters following values in columns representing each time point indicate significant differences between treatments ($p<0.05$; Holm-Sidak test).

Combining treatment rates, time points and varieties again showed no significant differences between treatments and the Control, except for suSCon® max*i*, which exhibited significantly lower activity than all of the other treatments (Figure 2.4)

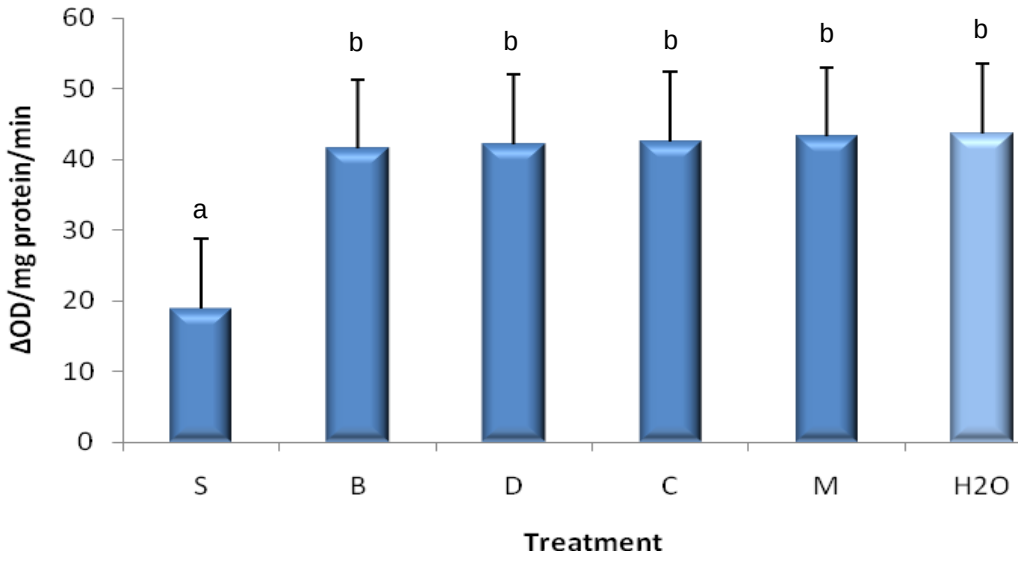

Figure 2.4. Average polyphenol oxidase activity in sugarcane leaves of both N12 and N27 varieties treated with chemical elicitors of resistance. Activity is expressed as change in optical density at 420 nm per total protein added per minute. Vertical bars represent Holm-Sidak statistics at the 5% level of significance.
B: BION® (acibenzolar-S-methyl)
C: cis jasmone
D: 2,6-dichloroisonicotinic acid
H_2O: water Control
M: methyl jasmonate
S: suSCon® max*i* (imidacloprid)

2.4 Discussion

One of the defense mechanisms that plants employ against pathogen attack is SAR. SAR is characterized by broad spectrum disease resistance and accumulation of PR proteins (Francis *et al*., 2009). Four PR proteins in sugarcane leaves were investigated

after elicitor application in this study. Increased activity of these PR proteins in the leaves of treated sugarcane plants would indicate induced resistance.

Examining enzyme activity in the leaves of each variety at each time point after elicitor application at one of two rates, none of the treatments consistently increased enzyme activity. However, when results for each time point, concentration and variety were pooled and examined, the treatments with methyl jasmonate produced a significant increase in activity of β-1,3-glucanase, chitinase, and peroxidase (Figures 2.1; 2.2; 2.3 respectively). Methyl jasmonate has been applied previously to sugarcane roots through a soil drench treatment and transcriptional responses were examined (Bower *et al*., 2005). Homologues of genes encoding PR-10 proteins and lipoxygenase were induced by methyl jasmonate (Bower *et al*., 2005).

The SA analogues used (BION®, 2,6-dichloroisonicotinic acid and suSCon® max*i*) have all be shown to cause increases in β-1,3-glucanase activity in other plants species. Increases in β-1,3-glucanase activity have been observed in cotton plants when treated with BION® (Whan *et al*., 2009). Application of BION® resulted in increased β-1,3-glucanase activity and disease resistance in potato (Bokshi *et al*., 2003). BION® and isonicotinic acid application in citrus demonstrated increased β-1,3-glucanase expression (Francis *et al*., 2009). However, in our work, the SA analogues caused small variations of β-1,3-glucanase activity relative to the Control in the leaves of both sugarcane varieties across the three time points, but none were significant increases. Methyl jasmonate was the only treatment to cause a significant increase in β-1,3-glucanase activity relative to the Control treatment at any stage. However, this significant increase by methyl jasmonate occurred only in the N12 variety, with no significant increase for the N27 variety.

Chitinases hydrolyzes chitin, the major component of most fungal cell walls (Viswanthan *et al*., 2005). Chitinases have been identified as key role-players in active defense responses in plants. Chitinases alone and in combination with β-1,3-glucanase have been demonstrated to inhibit the growth of many fungi *in vitro* (Mauch *et al*., 1988, Viswanthan *et al*., 2005). Few PR proteins are constitutively expressed in plants at low levels. Instead, the majority of PR proteins are turned on in response to pathogen

attack (Viswanthan *et al*., 2005). Induction of PR proteins is a result of the activation of plant defensive pathways. This induction limits the entry or further spread of a pathogen (Viswanthan *et al*., 2005). It has been proposed that in resistant cultivars, hydrolytic enzymes act on germlings immediately after pathogen penetration, leading to reduced disease development. In susceptible cultivars, the pathogen may penetrate and colonize host tissue before induction of PR proteins to a required level to prevent colonization (Viswanthan *et al*., 2005). For sugarcane, only 2,6-dichloroisonicotinic acid and methyl jasmonate had any effect on chitinase activity and this was only for one variety (N12).

Polyphenol oxidase activity in treated plants was generally lower than in the Control plants. Polyphenol oxidase enzymes are involved in the typical browning of damaged tissue caused by spontaneous polymerization and cross-linking of *o*-quinones (Falco *et al*., 2001). There is uncertainty as to the physiological function of PPO enzymes in fruit and organs of healthy plants. However, a role for foliar PPO enzymes has been proposed and documented (Constabel *et al*., 1995). Upon insect feeding the mixing of PPO and phenolic substrates generates o-quinones and these highly reactive compounds covalently modify free amino and sulfhydryl groups in dietary proteins within the mouth and gut of insects (Falco *et al*., 2001; Constabel *et al*., 2000). The end result is to reduce the nutrient value of the consumed protein.

In most cases, PR protein activity did not increase in sugarcane leaves as a result of applying SAR elicitors. This was a surprising result, given the many positive reports on the effectiveness of this group of chemicals.

The largest increases were observed with methyl jasmonate application, making this elicitor the most promising for application to sugarcane to induce resistance against leaf pathogens of sugarcane. However, caution should be used in this approach because it was evident that the two different sugarcane cultivars responded differently to the different elicitors, and that the elicitors were relatively ineffective.

2.5 References

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CHAPTER THREE

RESISTANCE INDUCING CHEMICALS – THEIR EFFECT ON SUGARCANE BIOMASS AND NEMATODE INFESTATION

Five potential resistance-inducing chemicals were applied to two sugarcane varieties (N12 and N27) in a pot trial with the aim of reducing *Pratylenchus* and *Meloidogyne* nematode infestation of roots. BION® (acibenzolar-S-methyl), methyl jasmonate, cis-jasmone and 2,6-dichloroisonicotinic acid (INA) were applied as foliar spray and suSCon® max*i* (imidacloprid) was applied to the soil. All chemicals were tested at two rates. Plants were harvested at 7, 9 and 11 weeks and root and shoot dried biomass recorded. Root and shoot dried biomass was not significantly increased ($p<0.05$; Holm-Sidak test). However, root and shoot dried biomass was highest in the N12 variety treated by suSCon® max*i*. The N27 variety responded better to chemical treatment than did N12 (the more nematode tolerant variety). No significant reduction in nematode infestation was achieved by any one treatment ($p<0.05$; Holm-Sidak test). This may have been due to nematode infestation prior to the chemical treatment. Achieving induced resistance before nematode challenge may prove more successful if applied as pre-planting treatments. This would also reduce the dependency upon the systemic nature of induced resistance required by foliar application for induced resistance to nematodes. Overall, the chemical treatments that were applied provided the two sugar cane cultivars with little or no protection from nematode infestation that was significant, consistent and measureable.

3.1 Introduction

Plant-parasitic nematodes are found in all sugarcane fields throughout the world, and on poor sandy soils they can cause significant yield losses (Cadet and Spaull, 2003).

Current methods used to combat nematode damage involve selecting resistant varieties and application of nematicides (Cadet and Spaull, 2003). *Meloidogyne javanica* Treub and *M. incognita* (Kofoid and White) Chitwood, are considered to be the most serious nematode pests of sugarcane in South Africa (Cadet and Spaull, 2003), along with *Pratylenchus zeae* Graham (Berry *et al*., 2008).

Control of nematodes is becoming increasingly difficult due to the withdrawal of nematicides and soil fumigants from the market, e.g., methyl bromide (Oka *et al.*, 1999) and Temik® (aldicarb). Sugarcane farmers in South Africa commonly use the nematicide Temik for the control of nematodes. Temik was to be withdrawn from the market at the end of 2016 (Berry and Leslie, 2011). However, it has been withdrawn sooner than expected because of the closure of the Bayer plant in the United States of America, which was responsible for the manufacture of methyl isocyanate, a precursor for the production of Temik (Berry and Leslie, 2011). Alternative nematicides are available, however. All the nematicides registered for sugarcane production fall into the hazardous Group 1 (very toxic) category (Berry and Leslie, 2011), and all of them face loss of their registration status due to the risks they pose to humans and the environment. Consequently, there is an increasing need for new nematode management techniques, and using chemical treatment to induce resistance could be one alternative control strategy for nematodes in sugarcane. Chemicals that induce resistance in crops are less toxic and are therefore considered to be more environmentally friendly than traditional pesticides (Sonnemann *et al*., 2002).

Induced resistance is a plant defence mechanism that can be triggered by pathogen challenge or by application of chemical inducers (Mauch-Mani and Metraux, 1998; Oka and Cohen, 2001; Kloepper *et al*., 2004; Bakker *et al*., 2007; Van der Ent *et al*., 2008). Induced resistance works systemically and protects plants from a broad spectrum of pathogens (Oka and Cohen, 2001). The systemic nature of induced resistance can prove advantageous to the application method of chemical inducers, e.g., foliar application can result in resistance in distal tissue such as the roots (Oka and Cohen, 2001). Foliar application of methyl jasmonate prior to nematode challenge can result in reduced root-knot-nematode infection after challenge (Sonnemann *et al*., 2002).

In this study, five chemical inducers of resistance were investigated for their ability to induce resistance in sugarcane plants to *Meloidogyne* and *Pratylenchus* nematodes. Each chemical inducer was applied at two different rates to two sugarcane varieties (N12 and N27) and their effect on nematode infestation of roots and on root and shoot mass was investigated.

3.2 Materials and Methods

3.2.1 Plant material and treatments

Two sugarcane varieties were selected, N12 (nematode-tolerant) and N27 (nematode-susceptible). Sugarcane stalks were collected from commercial seedcane trials and then cut into single budded sets (with one node each) and planted into pots containing approximately 8 kg of sandy soil (<10% clay), collected from a nearby sugarcane field in Umdloti, KwaZulu-Natal. The pots where housed in a rain shelter facility, watered three times weekly and fertilized with Hygrotech® seedling fertilizer once weekly. A randomised complete block design was used to lay out the pots. Five elicitor treatments were selected along with a water-only treatment as the Control treatment (Table 3.1). Two concentrations for each elicitor treatment where used, with five replicates for each treatment and concentration. All treatments contained 1 ml ℓ^{-1} Breakthru solution and 2 ml ℓ^{-1} ethanol as an adjuvant and solvent, respectively. Plants were sprayed 1 week before each harvest interval.

Table 3.1. Resistance inducing chemicals and application concentrations

Elicitor	Low concentration	High concentration
BION® (acibenzolar-S-methyl)	0.1 g ℓ^{-1}	1 g ℓ^{-1}
cis-jasmone	150 µM	1.5 mM
2,6-dichloroisonicotinic acid	1 mM	10 mM
methyl jasmonate	150 µM	1.5 mM
suSCon® max*i* (imidacloprid)	1.5 g m^{-2}	4 g m^{-2}
H_20 (control)	-	-

For each elicitor application, pots where removed from the rainshelter and grouped according to type of elicitor treatment and concentration of elicitor to be applied. Foliar application of elicitors was conducted using “Down to Earth” spray bottles, until run off from the leaves. Twenty four hours post-application, the pots were then returned to the rainshelter and put back into their original positions. The granular insecticide suSCon® max*i* was applied directly to the soil during planting and the leaves sprayed with water containing 1mlℓ^{-1} Breakthru solution and 2 ml ℓ^{-1} ethanol.

3.2.2 Measurement of nematode damage

Roots of the two sugarcane varieties (N12 and N27) where analyzed at 7, 9 and 11 weeks for *Pratylenchus* and *Meloidogyne* infestation. Nematodes were extracted from 10 to 50g fresh weight of roots using the mist chamber technique (Seinhorst, 1950). All samples were then forwarded to trained technicians in the nematology laboratory at SASRI for identification and counting of nematodes. At 7 weeks only sett roots where analyzed for nematodes due to the lack of shoot roots at this early stage of the plants development. For the second (9 week) and third (11 week) harvests only shoot roots where analyzed for nematodes. All root material was collected, dried and weighed and results recorded.

3.2.3 Statistical analysis

All data was analyzed using the Restricted Maximum Likelihood (REML) procedure (Genstat ver.11). Significant differences between treatment means were tested using the Holm-Sidak all-pairwise multiple comparison test at the 5% level of significance (Genstat ver.11). Nematode counts of *Pratylenchus* and *Meloidogyne* were divided by root dry weight to get the counts of *Pratylenchus* nematodes per gram of root and *Meloidogyne* per gram of root. The nematode count per gram of root for each treatment was then divided by the count per gram of root of the Control in order to obtain a percentage for each treatment relative to the Control.

3.3 Results

3.3.1 Nematodes in the roots

Results at 7 weeks for N12 sugarcane, showed that two elicitors, methyl jasmonate and BION®, both at the Low concentrations, caused an increase in *Pratylenchus* numbers by 47% and 26%, respectively (Table 3.2). In contrast, both methyl jasmonate and BION® at the High concentration caused the lowest number of *Pratylenchus* nematodes, reductions of 62% and 42%, respectively (Table 3.2). All other treatments caused reduced *Pratylenchus* numbers in sett roots at 7 weeks.

At 9 weeks, only two treatments caused a reduction in *Pratylenchus* numbers, cis-jasmone and 2,6 dichloroisonicotinc acid, both at their High concentration (Table 3.2). All other treatments caused increased numbers of *Pratylenchus* in the shoot roots.

At 11 weeks, BION® at the Low concentration caused significantly reduced *Pratylenchus* infestation in shoot roots, at 64% less than the water Control (Table 3.2). Conversely, high concentrations of cis-jasmone and methyl jasmonate caused 62% and 39% higher *Pratylenchus* infestations, respectively, than the Control treatment at 11 weeks. The only treatment to consistently cause a reduction in *Pratylenchus* infestations at all three time points was 2,6-dichloroisonicotinic acid at the High concentration, however, this reduction was not statistically significant (Table 3.2).

Table 3.2. *Pratylenchus* infestations in the roots of N12 sugarcane as a percentage of the water Control. Plants were treated with five different treatments, each at two concentrations. Nematodes in the roots were counted at 7, 9 and 11 weeks.

	Plant age		
Treatment	7 weeks	9 weeks	11 weeks
BION® High (acibenzolar-S-methyl)	-42 bc	45 abc	-59 abc
BION® Low (acibenzolar-S-methyl)	26 c	286 bc	-64 a
cis-jasmone High	-27 bc	-16 abc	62 bc
cis-jasmone Low	-27 bc	145 bc	-13 bc
methyl jasmonate High	-62 abc	130 bc	39 abc
methyl jasmonate Low	47 c	51 bc	-3 bc
2,6-dichloroisonicotinic acid High	-39 bc	-58 ab	-25 bc
2,6-dichloroisonicotinic acid Low	-3 bc	80 bc	-50 abc
suSCon® max*i* High (imidacloprid)	-6 c	82 bc	-29 bc
suSCon® max*i* Low (imidacloprid)	-37 bc	214 bc	-9 bc
Control	100 bc	100 abc	100 bc

All values are presented as a percentage relative to the Control. Negative percentages showing reduced infestation relative to the Control are shaded green. Positive percentages showing increased infestation relative to the Control are shaded red. Different letters following percentages in columns representing each time point indicate significant differences between treatments ($p<0.05$; Holm-Sidak test).

Table 3.3. *Pratylenchus* infestation in the roots of N27 sugarcane as a percentage of the water Control. Plants were treated with five different treatments, each at two concentrations. Nematodes in the roots were counted at 7, 9 and 11 weeks.

	Plant age		
Treatment	7 weeks	9 weeks	11 weeks
BION® High (acibenzolar-S-methyl)	-47 ab	16 ab	51 ab
BION® Low (acibenzolar-S-methyl)	23 b	153 ab	129 ab
cis-jasmone High	-26 ab	53 ab	-63 ab
cis-jasmone Low	30 b	11 ab	-18 ab
methyl jasmonate High	-30 ab	51 ab	-64 ab
methyl jasmonate Low	-36 ab	20 ab	88 ab
2,6-dichloroisonicotinic acid High	-16 ab	30 ab	-26 ab
2,6-dichloroisonicotinic acid Low	-48 ab	-1 ab	275 ab
suSCon® max*i* High (imidacloprid)	-55 ab	-35 a	-78 ab
suSCon® max*i* Low (imidacloprid)	-66 ab	-1 ab	220 ab
Control	100 ab	100 ab	100 ab

All values are presented as a percentage relative to the Control. Negative percentages showing reduced infestation relative to the Control are shaded green. Positive percentages showing increased infestation relative to the Control are shaded red. Different letters following percentages in columns representing each time point indicate significant differences between treatments ($p<0.05$; Holm-Sidak test).

Results for the N27 sugarcane variety are shown in Table 3.3. At 7 weeks, two treatments caused an increase of *Pratylenchus* infestations. Cis-jasmone at the Low concentration (30%) and BION® at the Low concentration (23%). All other treatments caused reduced *Pratylenchus* nematode numbers relative to the water Control. suSCon® max*i* treated cane showed the lowest *Pratylenchus* numbers, at 55% less than the Control at the High concentration and 66% less at the Low concentration (Table 3.3).

At 9 weeks, suSCon® max*i* at the High concentration reduced *Pratylenchus* numbers by 35% relative to the Control. All other treatments caused increases in *Pratylenchus* numbers, with BION® at a Low concentration causing the greatest increase of 153% more than that of the Control, a remarkable result.

At 11 weeks, suSCon® max*i* at the High concentration reduced the number of *Pratylenchus* nematodes in N27 sugarcane by 78% relative to the Control (Table 3.3). Conversely, BION® treatments at both concentrations resulted in increased *Pratylenchus* numbers. The only treatment to consistently reduce *Pratylenchus* numbers at all three sampling dates was suSCon® max*i* at the High concentration (Table 3.3).

A second important nematode, *Meloidogyne,* was counted in the roots of both sugarcane varieties (Table 3.4 and Table 3.5). As previously mentioned, at 7 weeks only sett roots where examined. At 7 weeks, for the N12 variety, all treatments except 2,6-dichloroisonicotinic acid at the Low concentration and methyl jasmonate at the High concentration, reduced the number of *Meloidogyne* nematodes (Table 3.4). The greatest reduction at 7 weeks relative to the Control treatment was seen with suSCon® max*i* at the Low concentration, with 96% less *Meloidogyne* (Table 3.4).

At 9 weeks, when shoot roots were processed, four treatments caused decreased *Meloidogyne* numbers relative to the Control, with methyl jasmonate at the High concentration causing the greatest decrease of 49% (Table 3.4). Cis-jasmone at both concentrations reduced *Meloidogyne* numbers relative to the Control treatment. The largest increase in *Meloidogyne* numbers was observed where N12 sugarcane was treated with 2,6-dichloroisonicotinic at the Low concentration (Table 3.4).

At 11 weeks, 2,6-dichloroisonicotinic at the Low concentration caused a reduction of *Meloidogyne* numbers by 21% relative to the Control (Table 3.4). The largest increase in *Meloidogyne* numbers was as a result of the cis-jasmone treatment at the High concentration, with an increase of 138% relative to the Control treatment.

Table 3.4. *Meloidogyne* infestations in the roots of N12 sugarcane as a percentage of the water Control. Plants were treated with five different treatments, each at two concentrations. Nematodes in the roots were counted at 7, 9 and 11 weeks.

	Plant age		
Treatment	7 weeks	9 weeks	11 weeks
BION® High (acibenzolar-S-methyl)	-46 a	36 a	-11 a
BION® Low (acibenzolar-S-methyl)	-38 a	14 a	46 a
cis-jasmone High	-55 a	-30 a	138 a
cis-jasmone Low	-23 a	-39 a	-18 a
methyl jasmonate High	152 a	-49 a	-12 a
methyl jasmonate Low	-68 a	19 a	77 a
2,6-dichloroisonicotinic acid High	-64 a	21 a	-7 a
2,6-dichloroisonicotinic acid Low	260 a	102 a	-21 a
suSCon® max*i* High (imidacloprid)	-32 a	-43 a	47 a
suSCon® max*i* Low (imidacloprid)	-96 a	21 a	110 a
Control	100 a	100 a	100 a

All values are presented as a percentage relative to the Control. Negative percentages showing reduced infestation relative to the Control are shaded green. Positive percentages showing increased infestation relative to the Control are shaded red. Different letters following percentages in columns representing each time point indicate significant differences between treatments ($p<0.05$; Holm-Sidak test).

Table 3.5. *Meloidogyne* infestation in the roots of N27 sugarcane as a percentage of the water Control. Plants were treated with five different treatments, each at two concentrations. Nematodes in the roots were counted at 7, 9 and 11 weeks.

	Plant age		
Treatment	7 weeks	9 weeks	11 weeks
BION® High (acibenzolar-S-methyl)	-55 a	-65 a	-92 a
BION® Low (acibenzolar-S-methyl)	-41 a	36 a	-15 a
cis-jasmone High	143 a	-36 a	-84 a
cis-jasmone Low	-20 a	-45 a	-79 a
methyl jasmonate High	60 a	35 a	-94 a
methyl jasmonate Low	-18 a	-57 a	-28 a
2,6-dichloroisonicotinic acid High	3 a	-96 a	-86 a
2,6-dichloroisonicotinic acid Low	-65 a	-51 a	-3 a
suSCon® max*i* High (imidacloprid)	-19 a	-44 a	-93 a
suSCon® max*i* Low (imidacloprid)	31 a	13 a	266 a
Control	100 a	100 a	100 a

All values are presented as a percentage relative to the Control. Negative percentages showing reduced infestation relative to the Control are shaded green. Positive percentages showing increased infestation relative to the Control are shaded red. Different letters following percentages in columns representing each time point indicate significant differences between treatments ($p<0.05$; Holm-Sidak test). None of the treatments were significantly different from each other or the Control.

For N27, at 7 weeks, all treatments at one or both rates caused reduced *Meloidogyne* infestation (Table 3.5). BION® was the only treatment to cause a reduction in the counts of *Meloidogyne* at both rates in sett roots. The largest reduction of *Meloidogyne* in sett roots of N27 sugarcane was seen with the 2,6-dichloroisonicotinic acid treatment at the Low concentration (Table 3.5).

In the shoot roots of N27 sugarcane at 9 weeks of age, *Meloidogyne* numbers were reduced by all treatments at one (or both) rates (Table 3.5). Cis-jasmone and 2,6-dichloroisonicotinic acid at both rates reduced *Meloidogyne* numbers. The largest reduction, 96% less *Meloidogyne* than the Control, was achieved by 2,6-dichloroisonicotinic acid treatment at the High concentration (Table 3.5).

All treatments except suSCon® max*i* at the Low rate resulted in reduced *Meloidogyne* infestation in shoot roots of 11 week old N27 sugarcane (Table 3.5). Reduced *Meloidogyne* nematodes greater than 90% relative to the Control was observed as a result of treatments with BION®, methyl jasmonate and suSCon® max*i*, all at the High dose.

3.3.2 Biomass

The effect of each treatment and rate on the root mass was examined at 7, 9 and 11 weeks (Figures 3.1. A-E). Using the Holm-Sidak all-pairwise multiple comparison test at the 5% level of significance revealed that there was no significant difference between treatments after 11 weeks of growth for either dried shoot or root mass.

Figure 3.1 A. shows the mean dried root mass of N12 sugarcane treated with BION®. Slightly reduced dried root mass was seen with BION® application at the Low concentration (Figure 3.1 A). Cis-jasmone caused similar results to the Control in N12 sugarcane (Figure 3.1 B). suSCon® max*i* was the only treatment that resulted in a marginal increase in root mass relative to the Control in N12 sugarcane (Figure 3.1 E). This increased root mass in suSCon® max*i* treated plants was seen at 9 weeks and to a greater extent at 11 weeks. Methyl jasmonate treatment at both concentrations resulted in reduced root mass relative to the Control (Figure 3.1 D).

With N27 sugarcane both concentrations of BION® resulted in a root mass less than the Control at 11 weeks (Figure 3.2 A). suSCon® max*i* at the High concentration was the only treatment that did not result in root mass less than the Control at 11 weeks in N27 sugarcane (Figure 3.2). Plants treated with 2,6-dichloroisonicotinic acid performed poorest of all (Figure 3.2 C).

Dried shoot mass was also recorded for each treatment, time point and variety. The results of dried shoot mass mirror those of the root mass (Figure 3.3 and 3.4).

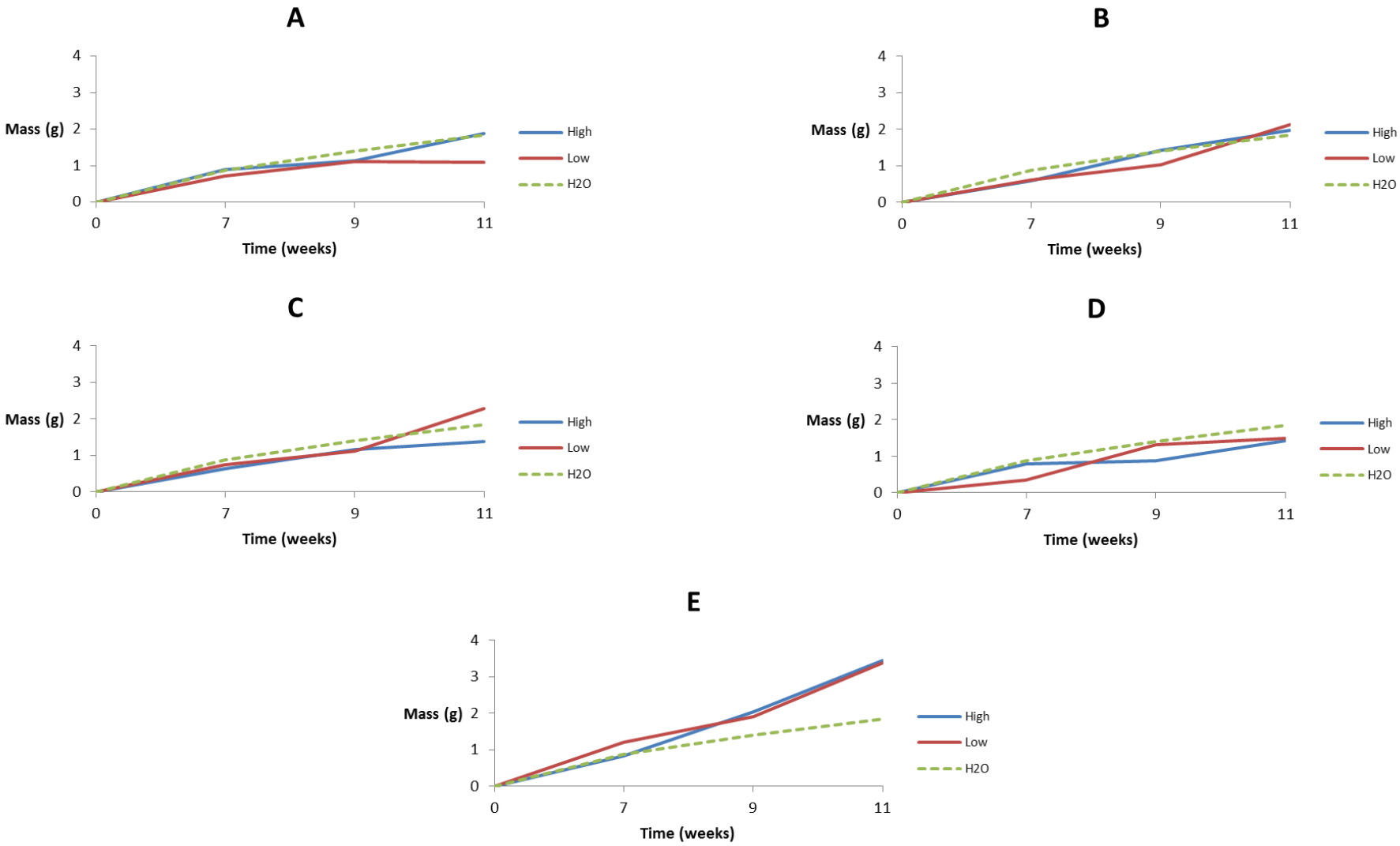

Figure 3.1. Root mass of N12 sugarcane treated with elicitors of induced resistance. **A** – BION®, **B** – cis-jasmone, **C** – 2,6-dichloroisonicotinic acid, **D** – methyl jasmonate, **E** – suSCon® max*i*. No significant difference exists between treatments ($p<0.05$; Holm-Sidak test).

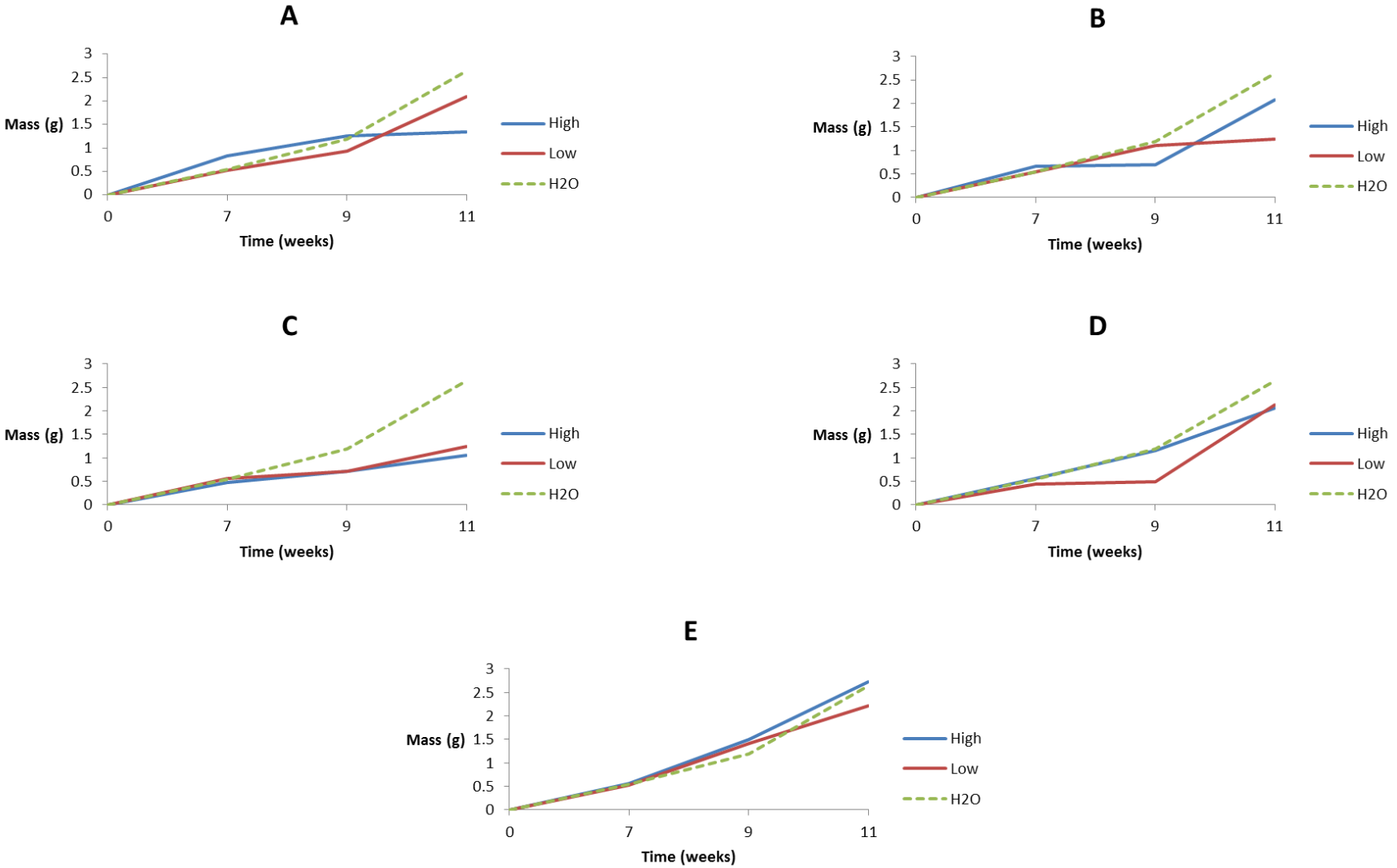

Figure 3.2. Root mass of N27 sugarcane treated with elicitors of induced resistance. **A** – BION®, **B** – cis-jasmone, **C** – 2,6-dichloroisonicotinic acid, **D** – methyl jasmonate, **E** – suSCon® max*i*. No significant difference exists between treatments ($p<0.05$; Holm-Sidak test).

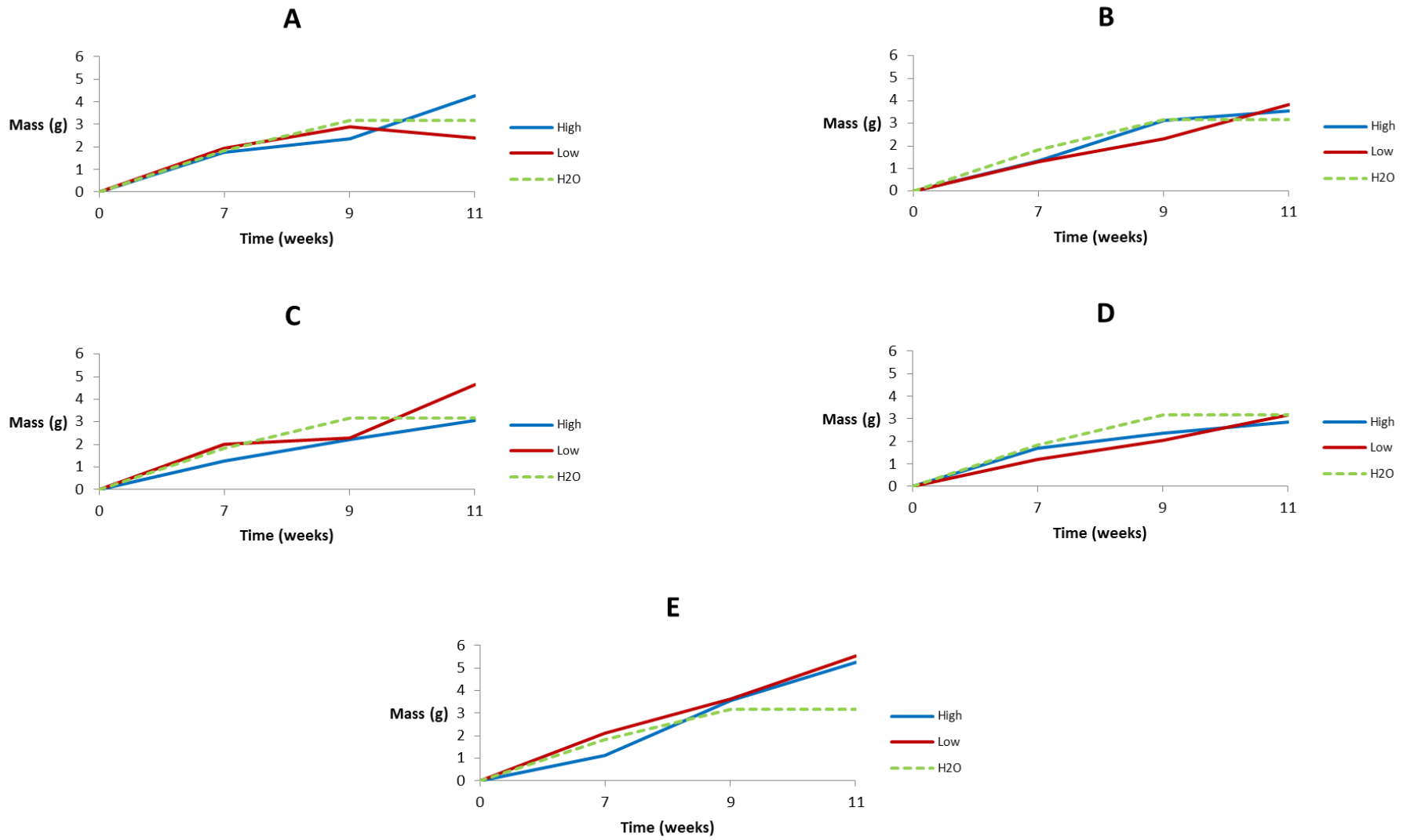

Figure 3.3. Shoot mass of N12 sugarcane treated with elicitors of induced resistance. **A** – BION®, **B** – cis-jasmone, **C** – 2,6-dichloroisonicotinic acid, **D** – methyl jasmonate, **E** – suSCon® max*i*. No significant difference exists between treatments ($p<0.05$; Holm-Sidak test).

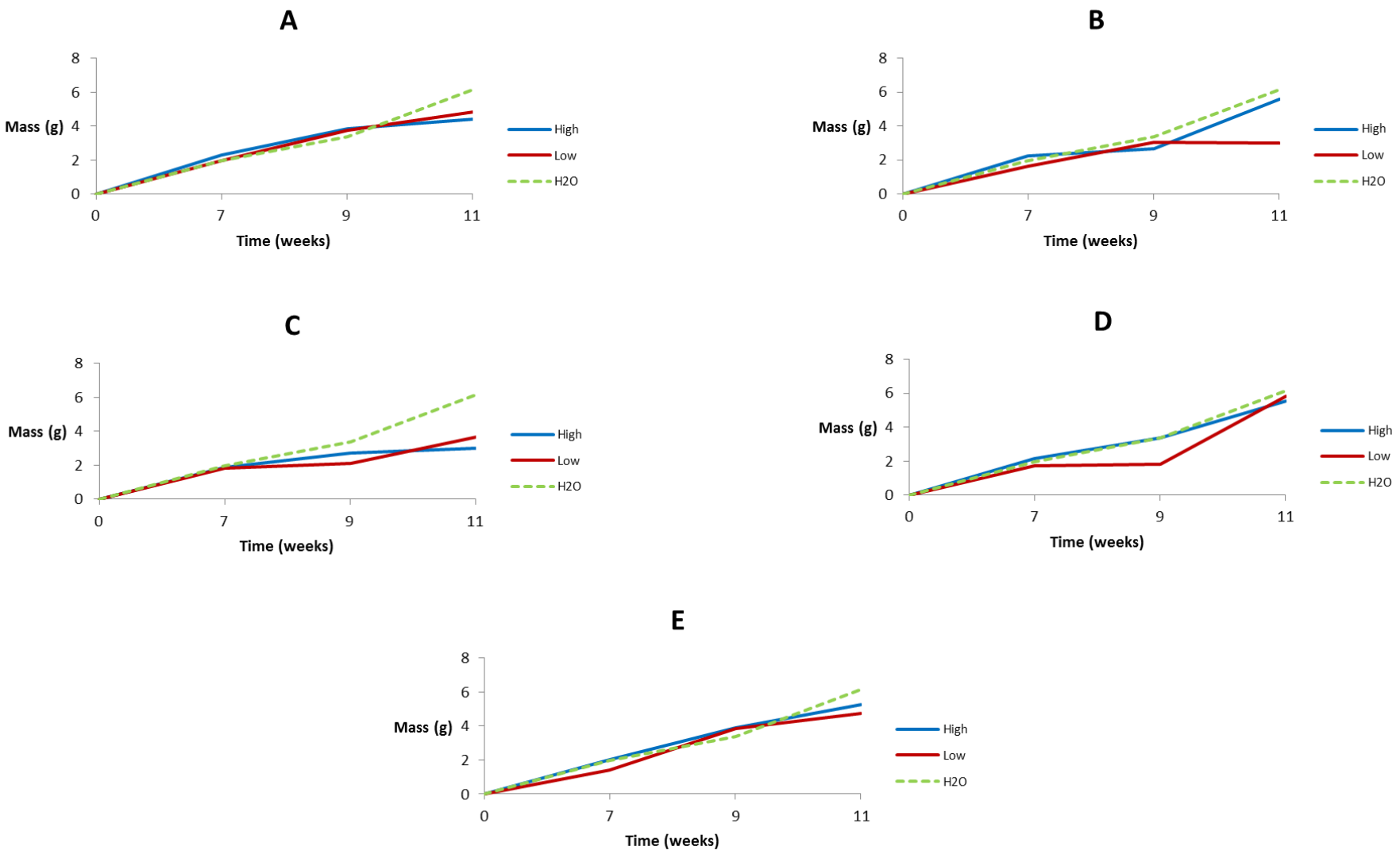

Figure 3.4. Shoot mass of N27 sugarcane treated with elicitors of induced resistance. **A** – BION®, **B** – cis-jasmone, **C** – 2,6-dichloroisonicotinic acid, **D** – methyl jasmonate, **E** – suSCon® max*i*. No significant difference exists between treatments ($p<0.05$; Holm-Sidak test).

3.4 Discussion

Few studies have been published on induced resistance in sugarcane. One study investigated the transcriptional response of sugarcane roots to methyl jasmonate treatment in order to identify up-regulated genes (Bower *et al*., 2005). In other work, acibenzolar-S-methyl, the active ingredient in BION® was applied to sugarcane as a soil drench in order to induce resistance to red rot disease caused by the fungus *Colletotrichum falcatum* Went. The authors reported reduced pathogen colonization in stalk tissues pretreated with acibenzolar-S-methyl. They also used a glycoprotein elicitor isolated from *C. falcatum* to treat sugarcane leaves, resulting in increased activities of chitinase and β-1,3-glucanase (Ramesh Sundar *et al*., 2008).

To our knowledge no studies investigating induced resistance to nematodes in sugarcane have been reported. Studies have been conducted on nematodes of other crops, the majority having concentrated mainly on *Meloidogyne* (Ogallo and McClure, 1995; Oka and Cohen, 2001; Siddiqui and Shaukat, 2004; Cooper *et al*., 2005; Molinari and Baser, 2010). Our research on sugarcane involved both *Meloidogyne* and *Pratylenchus*. Many studies investigated the effect of a single chemical treatment, whereas we examined the effects of five treatments at two rates each.

In South and West Africa, the density of nematodes has been found to be greatest in the sett roots (Cadet and Spaull, 1985). Sett roots are first to emerge from the nodes after planting. They are relatively thin and branched (van Antwerpen, 1999). Sett roots serve the plant until the shoot produces a shoot root, between 8 to 10 weeks after planting (van Antwerpen, 1999). The shoot root is relatively thick, white and less branched than the sett roots (van Antwerpen, 1999). Due to the fact that the first chemical sprays only began at 6 weeks (to ensure sufficient leaf foliage for spraying), it is likely that nematode infestation of sett roots would have already occurred. Attempting to induce resistance at this stage may not have the potential to reduce nematode numbers curatively in sett roots as effectively as earlier preventative treatment. Performing a seed soak, in this case of sugarcane, a sett soak, may induce resistance prior to nematode contact and thus reduce nematode infestation. However, this assumes that the chemical treatment will induce a resistance response that will last the

life of the sett roots. Seed soaks and soil drench applications of resistance inducing chemicals is common (Molinari and Baser, 2010; Oka and Cohen, 2001; Oka *et al*., 1999; Kataria *et al*., 1997). Root dip application has proved to be successful in tomato plants where acibenzolar-S-methyl was applied prior to challenging the plants with *Meloidogyne* nematodes, and reduced nematode infestation was observed (Molinari and Baser, 2010).

The active ingredient in suSCon® max*i* is the insecticide imidacloprid, which breaks down in plants into 6-chloronicotinic acid, a compound that is capable of inducing systemic acquired resistance (Francis *et al*., 2009). Imidacloprid has been shown to increase cotton, oat and wheat yields in the absence of pathogens without any clear explanation (Gonias *et al*., 2008). Some authors have suggested that this active ingredient has growth promoting properties (Francis *et al*., 2009; Gonias *et al*., 2008; Gourmet *et al*., 1996). In our experiments, we found that suSCon® max*i* treated N12 sugarcane showed increased root mass relative to the Control. However, this increase was not statistically significant. The difference was greatest at 11 weeks. This may be similar to the increased growth responses seen in cotton, oat and wheat (Gourmet *et al*., 1996; Gonias *et al*., 2008). In terms of suSCon® max*i* inducing resistance to nematodes; the number of *Pratylenchus* nematodes in the roots of N12 sugarcane treated with suSCon® max*i* was reduced at 11 weeks. The reduced nematode numbers could be considered a contributing factor towards the increased root mass seen at 11 weeks. However, at 9 weeks there was increased root mass in the N12 variety along with increased numbers of *Pratylenchus* nematodes in the roots relative to the Control, which indicated that there was no correlation between *Pratylenchus* nematode numbers and root mass.

Induction of systemic acquired resistance without pathogen challenge can result in “fitness costs” (Heil *et al*., 2000). Experiments using the chemical inducer BION® on wheat plants were conducted in the absence of pathogen challenge and the results showed reduced biomass relative to Control plants, and that plants developed fewer shoots and ears (Heil *et al*., 2000). It was suggested that differences between BION® treated plants and controls was due to allocation costs, resulting from metabolic competition between processes involved in the synthesis of defense-related

compounds and plant growth (Heil *et al*., 2000). In our experiments 2,6-dichloroisonicotinic acid reduced root and shoot mass to a large extent in the N27 variety, along with inconsistent reductions in nematode numbers (Figure 3.2 C and Figure 3.4 C). Treatment with 2,6-dichloroisonicotinic acid may have been phytotoxic to the sugarcane plant at both concentrations used in this case. Reduced biomass in tomato plants have been attributed to chemical inducers being phytotoxic (Oka *et al*., 1999).

Different concentration for each treatment returned contrasting results, e.g., in the N12 variety *Pratylenchus* infestation was reduced by the High concentration of cis-jasmone but was increased by the Low concentration at 9 weeks (Table 3.2). At 11 weeks for the same treatment the High concentration resulted in increased *Pratylenchus* infestation, whereas the Low concentration resulted in reduced infestation relative to the Control treatment (Table 3.2). In most cases the nematode count increased as often as it decreased with chemical treatment. The treatments used in this study were unable to provide any resistance to nematodes in sugarcane and the use of a positive control like Temik would have clearly demonstrated this.

Overall, the chemical treatments that were applied provided the two sugar cane cultivars little or no control that was significant, consistent and measureable.

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CHAPTER FOUR

INDUCED RESISTANCE TO SUGARCANE SMUT

(*Ustilago scitaminea*)

Four artificial inoculation methods were tested for infecting NCo376 sugarcane with *Ustilago scitaminea*. The method that provided the most consistent results was one that incorporated germinating sugarcane setts at 30˚C for 24 hours, puncturing the bud with a sterile toothpick and immersing it into a suspension of $1x10^8$ smut spores per ml of sterile distilled water for 30min. This inoculation technique proved to be 90% effective at infecting NCo376 sugarcane, as determined by staining meristematic tissue with Lactophenol Cotton Blue Solution, followed by microscopic examination. Fresh smut spores germinated at the frequencies of 81%, 80% and 65% when they were germinated on a 1% water agar medium, and water agar with 0.075ml ℓ^{-1} of Gaucho® (imidacloprid) and 0.075g ℓ^{-1} BION® (acibenzolar-S-methyl), respectively. The concentrations of these two chemicals did not significantly affect NCo376 plant growth over 4 weeks. Treatment with Gaucho® or BION® as a sett soak aimed at inducing resistance to smut, resulted in 80% and 75% of NCo376 plants being infected, respectively. In contrast an untreated resistant variety (N29) developed only 60% infection.

4.1 Introduction

Sugarcane smut is caused by *Ustilago scitaminea* and is one of the main diseases that affects the development of sugarcane (da Gloria *et al*., 1995; Millanes *et al*., 2005; de Armas *et al*., 2007). The first reported outbreak was in the province of Natal, South

Africa in 1877 (McMartin 1945; Albert and Schenck 1996; Croft *et al.*, 2008). In the 1980s the cultivar NCo376 was frequently heavily infected with smut, this being the fourth major outbreak of the disease in South Africa (Bailey, 1995). In October 2007 and March 2008 severe smut infections where reported on Mpumalanga farms, where seedcane from 70% of the inspected farms had to be ploughed out (van den Berg *et al.*, 2008). A slight decrease in smut infections has been observed in the 2 years following 2008 but, significant occurrences have been observed in Mpumalanga subsequently (Singels *et al.*, 2010).

Spore germination occurs on the internode surface and entry into the meristem in the bud occurs between 6 and 36 hours after inoculation (de Armas *et al*., 2007). Hyphal growth occurs throughout the infected plant (de Armas *et al*., 2007). In the parenchyma cells of the lower internodes hyphal growth is most prevalent, whereas in the upper internodes hyphal growth concludes with the formation of the whip (de Armas *et al*., 2007). Scale leaves prevent hyphae from penetrating and thus, buds tightly enclosed within scale leaves may escape infection. Based on this it has been proposed that smut resistance is determined by morphological features of buds (Waller 1970). However, other research suggests that resistance may be associated with chemical properties rather than bud morphology alone (da Gloria *et al*., 1995; Borrás-Hidalgo et al., 2005; de Armas *et al*., 2007; Santiago *et al*., 2009).

Induced resistance provides systemic resistance against a broad spectrum of pathogens. Associated with induced resistance is the accumulation of pathogenesis related proteins, e.g., chitinase and β-1,3-glucanase. These enzymes have shown to possess anti-fungal activity *in vitro*.

Four methods of inoculating sugarcane with smut were tested and a single method was selected to investigate induced resistance against smut. Two resistance inducing chemical were selected and used to treat sugarcane setts prior to smut inoculation with the goal of reducing smut infection.

4.2 Materials and methods

4.2.1 Smut spore collection and storage

Sugarcane smut spores were collected from infected NCo376 sugarcane plants displaying smut whips. Spores were removed from whips and stored in a desiccator. Spore germination was tested on 1% agar plates alone or containing either Gaucho® (imidacloprid) or BION® (acibenzolar-S-methyl) at various concentrations.

4.2.2 Smut inoculation

Four inoculation procedures were tested.

1. Single budded setts were immersed in d.H_2O containing smut spores at a concentration of 1x10^8 spores per ml of sterile distilled water for 30 min (Figure 4.3).
2. Single budded setts were allowed to germinate for 24hrs at 30°C (Figure 4.1) before being immersed in d.H_2O containing smut spores at a concentration of 1x10^8 spores per ml of sterile distilled water for 30 min.
3. The bud of each sett was punctured using a sterile toothpick (Figure 4.2) and immersed in d.H_2O containing smut spores at a concentration of 1x10^8 spores per ml of sterile distilled water for 30 min.
4. Single budded setts were allowed to geminate for 24hrs at 30°C before being punctured and immersed in d.H_2O containing smut spores at a concentration of 1x10^8 spores per ml of sterile distilled water for 30 min.

After each inoculation procedure plants were planted in pots and kept under glasshouse conditions at 30°C.

Figure 4.1. Single budded NCo376 sugarcane in plastic containers for 24 hour germination at 30˚C.

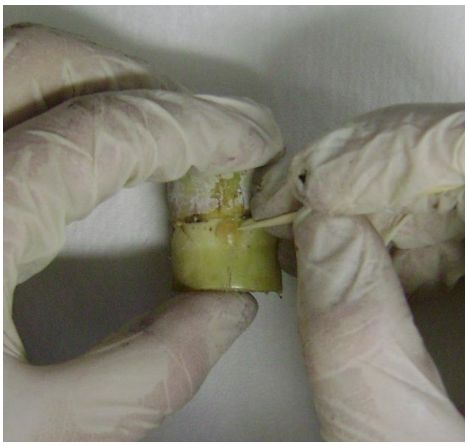

Figure 4.2. Single budded NCo376 sugarcane punctured with a sterile toothpick.

Figure 4.3. Single budded NCo376 sugarcane setts immersed in $1x10^8$ smut spores per ml.

4.2.3 Lactophenol Cotton Blue staining

Sugarcane buds were cut longitudinally through the meristem (Figure 4.4). Cut sections were placed into 1.5 ml microcentrifuge tubes and 5% KOH added to cover the plant tissue. Tubes were heated in a 65˚C waterbath for 30 min to leach pigments. The 5% KOH solution was removed and the plant tissue rinsed with 1% HCL followed by d.H_2O. Tubes were filled with the stain solution (0.05% Cotton Blue in lactophenol) and heated in a 65˚C waterbath for 30 min. The stain solution was removed and replaced with destain solution (lactophenol) and tubes were heated in a 65˚C water bath for 30 min. Wet mount slides of the stained tissue were prepared using a lactic acid-glycerol medium (1:1) and examined under a light microscope.

Figure 4.4. Cutting of longitudinal sections through the meristem of NCo376 sugarcane and leaching of pigments with potassium hydroxide.

4.2.4 BION® and Gaucho® application

BION® was dissolved in d.H_2O at concentrations of 7.5g ℓ^{-1}, 0.75g ℓ^{-1} and 0.075g ℓ^{-1}. Gaucho® was diluted in d.H_2O and used at 7.5ml ℓ^{-1}, 0.75ml ℓ^{-1} and 0.075ml ℓ^{-1}. Sugarcane setts were soaked in 25mℓ per sett of each chemical solution.

4.2.5 NCo376 germination and growth

NCo376 sugarcane single node cuttings were treated with three concentrations of Gaucho® or BION® at either 20˚C or 50˚C and planted in seedling trays (Figure 4.5). The number of plants that germinated and the Top visible dewlap (TVD) height of each

plant was recorded on a weekly basis for 4 weeks. For each treatment, concentration and temperature a total of 60 replicates was planted.

Figure 4.5. Planting of NCo376 sugarcane setts after treatment with Gaucho® or BION®.

4.3 Results

4.3.1 Smut spore and NCo376 sugarcane germination and growth

Gaucho® and BION® were tested at three concentrations each for their effect on smut spore germination on 1% agar medium. The number of spores that germinated for each treatment is presented as a percentage in Table 5.1.

Table 4.1. Smut spore germination on 1% agar medium containing water, BION® or Gaucho® after 6 hours

Treatment	Concentration	Smut spore germination (%)	Germ spore length relative to spore diameter
1% water agar	0	81	2.98 x
BION®	7.5g ℓ^{-1}	0	0 x
BION®	0.75g ℓ^{-1}	55	1.52 x
BION®	0.075g ℓ^{-1}	65	2.12 x
Gaucho®	7.5ml ℓ^{-1}	55	1.36 x
Gaucho®	0.75ml ℓ^{-1}	75	2.34 x
Gaucho®	0.075ml ℓ^{-1}	80	2.7 x

On water agar 81% of smut spores germinated after 6 hours The presence of BION® or Gaucho® in agar medium reduced the germination of smut spores, as well as the length of the germ spore relative to the spore diameter (Table 5.1), especially at their higher concentrations. Photographs of the smut spores on 1% agar for each treatment are presented in Figure 4.6 It is clearly visible that BION® at 7.5g ℓ^{-1} prevented smut spore germination completely at 6 hours (Figure 4.6).

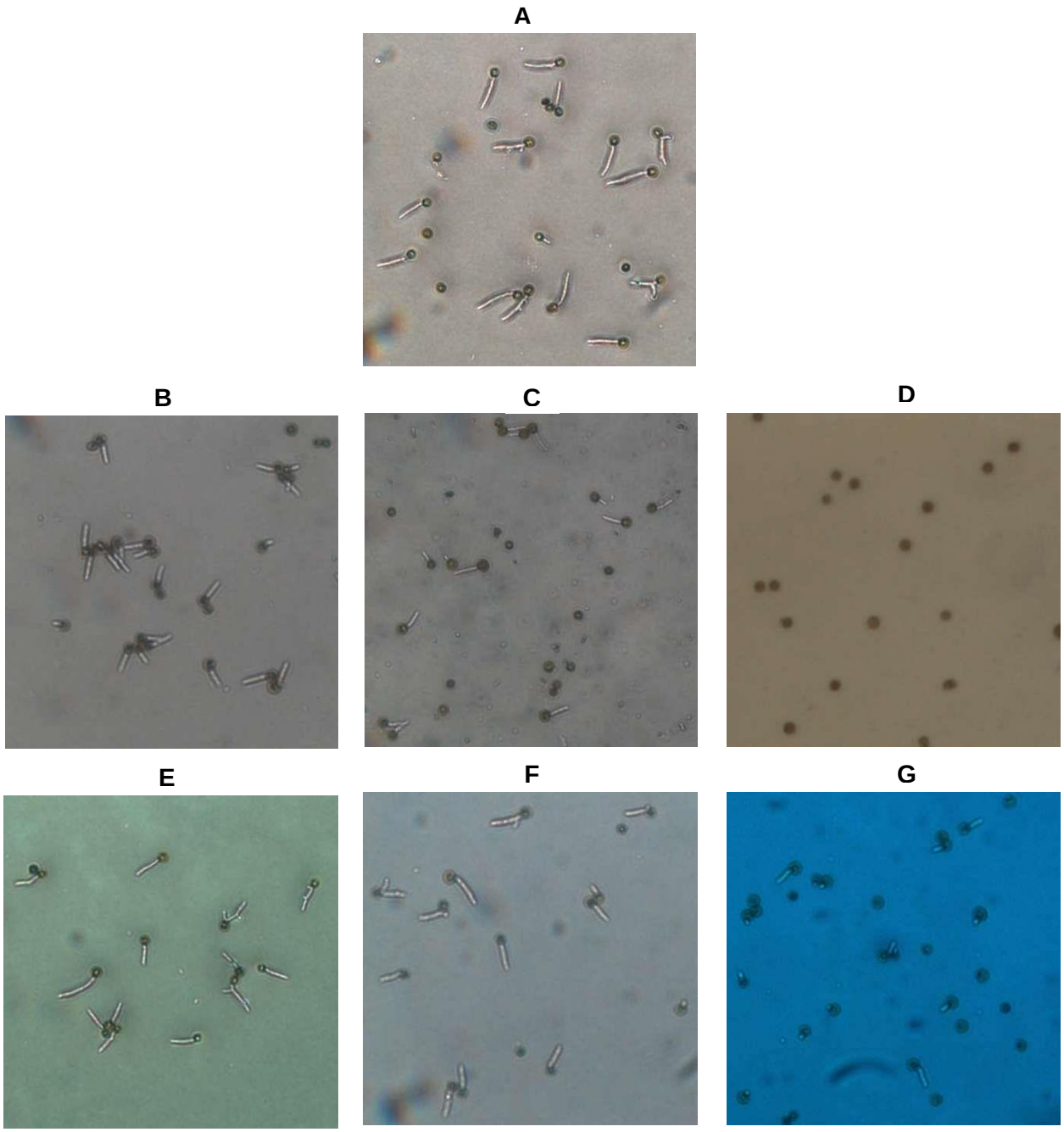

Figure 4.6. Smut spore germination on 1% agar after 6 hours

A: Water agar only

B: BION® 0.075g ℓ^{-1} C: BION® 0.75 g ℓ^{-1} D: BION® 7.5g ℓ^{-1}

E: Gaucho® 0.075ml ℓ^{-1} F: Gaucho® 0.75ml ℓ^{-1} G: Gaucho® 7.5ml ℓ^{-1}

The same concentrations of BION® and Gaucho® were used to treat single budded setts of NCo376 sugarcane at 20°C and 50°C. The height of each plant was recorded by measuring the top visible dewlap (TVD). After 4 weeks of growth there were significant differences in the mean TVD height between plants treated at 20°C and 50°C (Figure 4.7). Most plants treated at 50°C had a higher TVD height than those treated at 20°C. Notably, BION® treatment at 7.5g ℓ^{-1} and 0.75g ℓ^{-1} resulted in significant decreases in TVD height after 4 weeks of growth (Figure 4.8).

Based on this result further experimentation with treating sugarcane setts with BION® and Gaucho® to achieve induced resistance was conducted at 50°C.

Based on their affect on smut spore germination and TVD height BION® at 0.075g ℓ^{-1} and Gaucho® at 0.75 ml ℓ^{-1} were selected to treat sugarcane plants with the aim of inducing resistance to sugarcane smut.

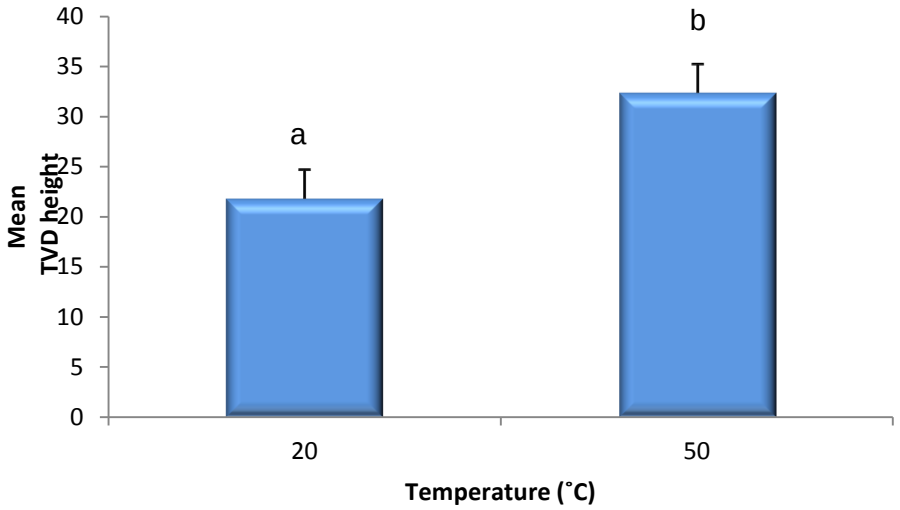

Figure 4.7. Mean TVD height of NCo376 sugarcane plants at 4 weeks after treatment at either 20 or 50˚C. For each temperature 420 plants were used.

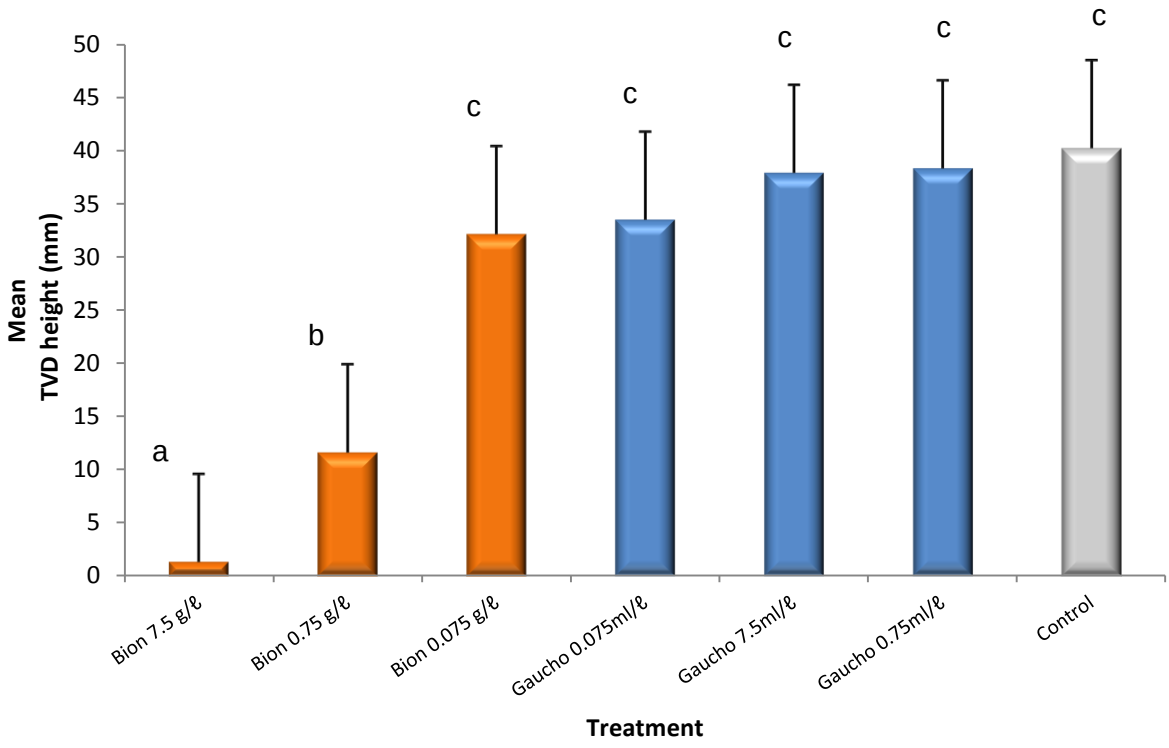

Figure 4.8. Mean TVD height of 4 week old NCo376 sugarcane after various pre-planting treatments. For each treatment 120 plants were used.

4.3.2 Sugarcane smut inoculation

Four methods of smut inoculation were tested. Two methods involved puncturing the sugarcane bud to overcome any morphological resistance. The method that involved allowing the bud to swell for 24 hours followed by puncturing the bud proved to be the most consistent for infecting NCo376 sugarcane with smut (Table 4.2). This method resulted in 36 out of 40 plants being successfully infected with smut. This was determined by staining with the plant tissue with lactophenol Cotton Blue solution and making a microscope examination looking for fungal mycelium (Figure 4.9). This method was used to investigate if induced resistance to smut could be achieved in the susceptible variety NCo376.

Table 4.2. Comparison of smut inoculation methods

Method	Method description	Number of plants treated	Number of plants infected
1	inoculate with smut spores	40	19
2	germinate 24 hrs, inoculate with smut spores	40	20
3	puncture bud, inoculate with smut spores	40	32
4	germinate 24 hrs, puncture bud, inoculate with smut spores	40	36

Plants were examined for smut presence via Cotton Blue staining and microscopy, four weeks after inoculation.

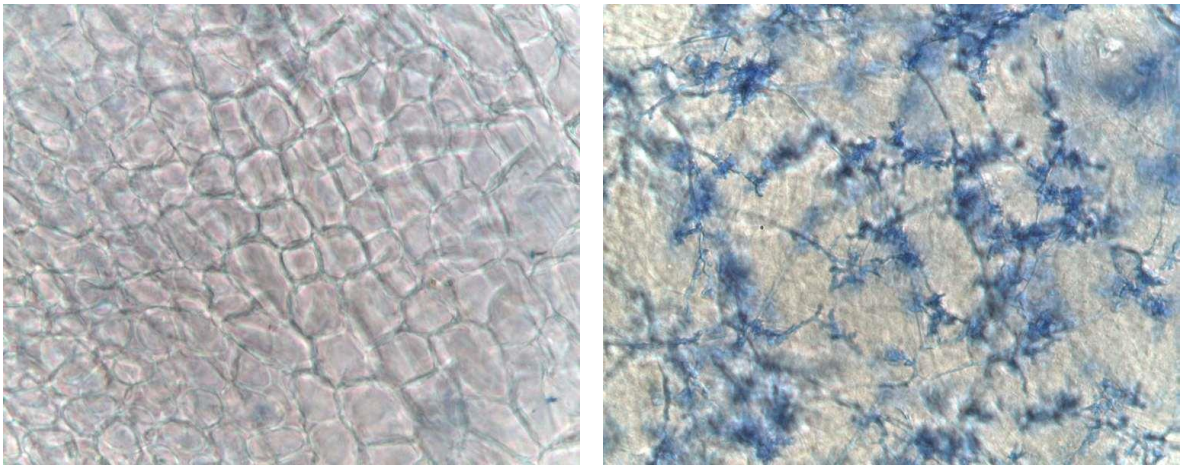

Figure 4.9. Longitudinal sections stained with lactophenol Cotton Blue solution of NCo376 sugarcane non-inoculated and inoculated with smut (*Ustilago scitaminea*). Intercellular hyphae are stained blue in inoculated tissue.

Of the plants treated with BION® at 0.075g ℓ^{-1} 30 out of 40 (75%) were infected with smut. With a treatment of Gaucho® at 0.75ml ℓ^{-1} 32 out of 40 (80%) of the plants were infected with smut.. Both treatments resulted in less plants being infected relative to the water Control where 35 out of 40 (87.5%) plants were infected. A water treated sample of the resistant variety N29 developed the fewest infected plants, with 24 out of 40 (60%) (Table 4.3).

Table 4.3. Effects of BION® and Gaucho® and treatments on sugarcane smut infection

Treatment	Variety	Number of Test Plants	Infected with Smut
Gaucho® 0.75ml ℓ^{-1}	NCo376	40	32 (80%)
BION® 0.075g ℓ^{-1}	NCo376	40	30 (75%)
Water	NCo376	40	35 (87.5%)
Water	N29 (resistant)	40	24 (60%)

Plants were treated with BION®, Gaucho®, or water at 50˚C for 30 min, and then allowed to germinate at 30˚C for 24 hours. Buds were punctured with a sterile toothpick and immersed in 1x 10^8 smut spores per ml of water. After four weeks, longitudinal sections through the meristem of each plant were cut and stained with Cotton Blue, followed by microscopic examination for the presence of smut.

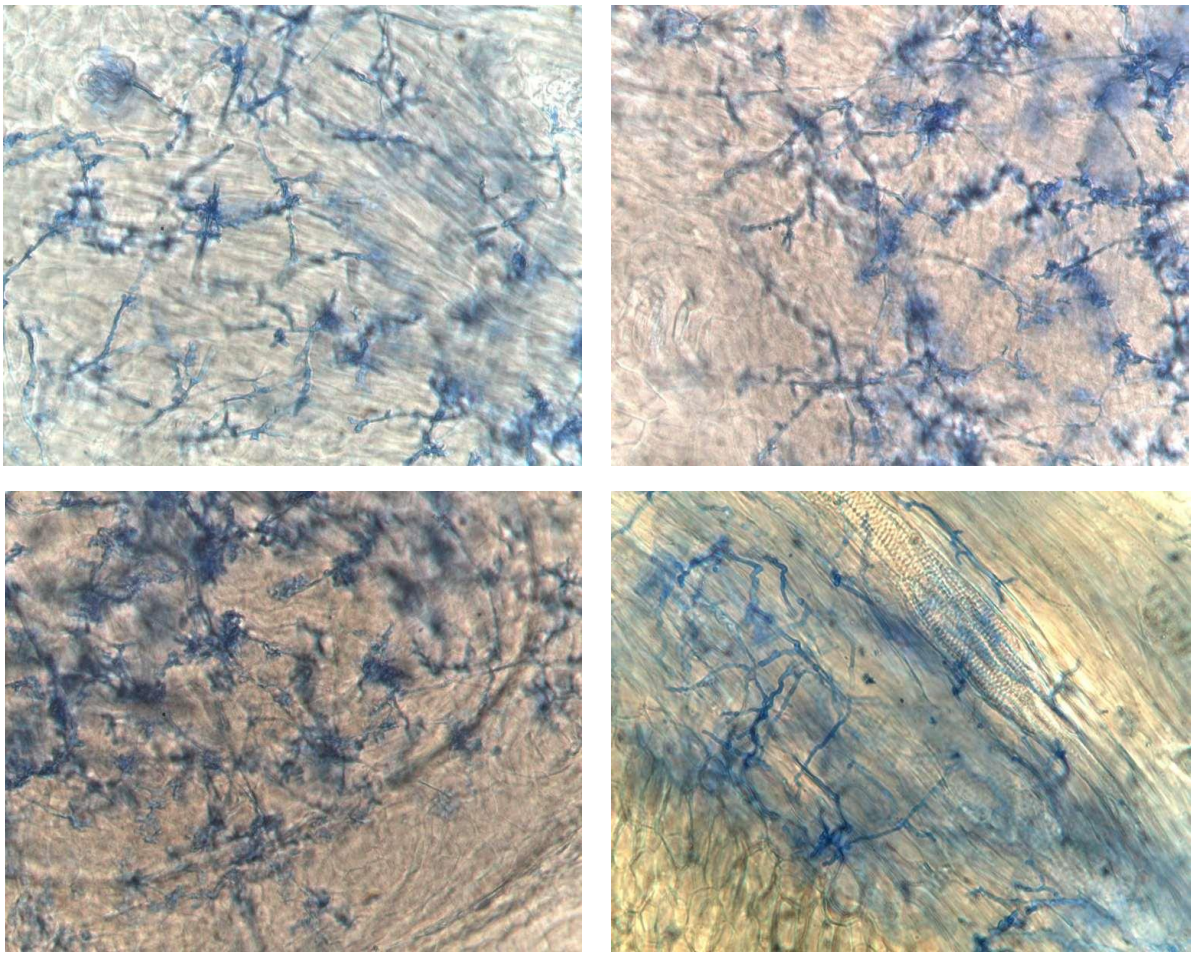

Figure 4.10. Longitudinal sections of NCo376 sugarcane meristematic tissue stained with Cotton Blue in lactophenol

4.4 Discussion

It is evident that high levels of BION® and Gaucho® negatively affect smut spore germination and growth. Sugarcane growth is also negatively affected by high levels of BION®. However, Gaucho treatment of sugarcane showed no significant effect at the concentrations used. Pre-treatment of sugarcane setts at 50˚C resulted in increased growth of plants on average after four weeks. In order to investigate whether or not induced resistance to sugarcane smut could be achieved through pre-planting

treatment with BION® and Gaucho®, concentrations of these two treatments that do not severely affect smut germination or sugarcane germination and growth were required. This would allow any reduction in smut infection to be attributed to induced resistance provided by the plant's defence mechanisms.

The concentrations of BION® and Gaucho® used to investigate induced resistance were 0.075g ℓ^{-1} and 0.75ml ℓ^{-1}, respectively. Smut spore germination on 1% agar alone was 81%, treatment with BION® and Gaucho® resulted in spore germinations of 65% and 80%, respectively. Both these concentrations of BION® and Gaucho® had no significant effect on sugarcane growth. Higher germination of smut spores on agar plates containing BION® would be more desirable. However, 65% was the highest smut spore germination achieved with any of the BION® treatments.

Staining of fungal elements can be achieved with lactophenol Cotton Blue solution. This proved to be successful for detecting sugarcane smut present in the tissue of sugarcane plants. The lactophenol Cotton Blue solution used binds to both plant cellulose, the chitin present in the fungal cell walls of sugarcane smut. This resulted in heavy background staining of the cellulosic plant cells, thus multiple rounds of destaining were required, to remove the stain from the cellulose. The chitin of the intercellular hyphae remained stained blue and were clearly visible in sugarcane tissue (Figure 4.10).

Longitudinal sections through sugarcane buds were cut by hand using a scalpel. This proved to be a challenging procedure because cutting sections of appropriate thickness was difficult. Thin sections are desirable for the viewing of clear single cells and stained hyphae. However, sections that were too thin were easily damaged, and disintegrated during the staining procedure. A better approach to the cutting sections of consistent thickness would have been to use a microtome.

Using an inoculation procedure that incorporates the puncturing of the sugarcane bud allows for easy penetration of germinating smut spores. This practice circumvents the

morphological resistance provided by the bud and its physical features, i.e., number of bud scales, scale leaves and trichomes (da Gloria *et al*., 1994). The method used here to puncture the sugarcane bud involves the use of a sterile toothpick, a cheap and easy alternative to the Hamilton syringe used by others (Santiago *et al*., 2009). The Hamilton syringe allows for injection of spores directly into plant tissue. However, using a sterile toothpick and soaking in smut spores proved to be successful in inoculating sugarcane plants.

An association between sugarcane smut resistance and the structural characteristics of the bud has been shown (da Gloria *et al*., 1994). This was done by examining the number of scales of buds and number of trichomes per square millimetre of the outer scales of resistant and susceptible sugarcane varieties (da Gloria *et al*., 1994). Using the inoculation procedure presented in this report made it possible to investigate the biochemical (non-structural) resistance to smut. Thus both aspects of smut resistance can be assessed in breeding programs, which should allow for a far more rapid evaluation of smut resistance than traditional methods. Traditional methods involve artificial smut infection but evaluation is done on the number of whips produced per ratoon (de Armas *et al*., 2007). Using lactophenol Cotton Blue solution as a stain followed by microscopic examination is a far more rapid method for smut resistance screening.

Of the 40 untreated and inoculated plants of the resistant cultivar N29, 24 plants became infected, compared to 35 out of 40 plants of the susceptible cultivar NCo376. Treatment with BION® and Gaucho® did not reduce smut infection even to the level of the unprotected, untreated resistant variety N29. This suggests that treatment with BION® or Gaucho® may only marginally reduce smut infection and cannot be recommended for practical use as treatments to control smut in sugarcane.

4.5 References

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DISSERTATION OVERVIEW

Only two studies have been published on the use of induced resistance in sugarcane. The transcriptional response of sugarcane roots to methyl jasmonate has been investigated and homologues of genes encoding PR-10 proteins and lipoxygenase were induced. Application of acibenzolar-S-methyl as a soil drench reduced stalk colonization of sugarcane plants by *Colletotrichum falcatum* Went (Ramesh Sundar *et al*., 2001). However, sugarcane has many pathogens and the broad spectrum resistance provided by induced resistance could be beneficial. Therefore, the aim of this study was to investigate the potential of a number of different chemical treatments to induce nematode and smut resistance in sugarcane. A number of resistance inducing chemicals have shown positive results in other crops. Some of these were selected for sugarcane treatment in this study, namely: cis-jasmone, methyl jasmonate, BION® (acibenzolar-S-methyl), suSCon® max*i* (imidacloprid) 2,6-dichloroisonicotinic acid and Gaucho® (imidacloprid) The most common method for application of resistance inducing chemicals is as a foliar spray. However, soil drenches, root dips and seed soak applications have also been used.

One metabolic response associated with induced resistance is elevated levels of pathogenesis related proteins (PR proteins). In order to investigate whether foliar application of resistance inducing chemicals and their effects on PR proteins in sugarcane leaves, four enzyme assays were used for β-1,3-glucanase, chitinase, polyphenol oxidase and peroxidase. Of the five resistance inducing chemicals applied to sugarcane plants of the varieties N12 and N27, methyl jasmonate treatment caused significant increases in β-1,3-glucanase, chitinase and peroxidase activity when time, variety and concentration data was pooled. Examining the non pooled data methyl jasmonate caused both increased and decreased enzyme activity. All other elicitor treatments caused non-significant changes in enzyme activity relative to the Control even when the data was pooled. Polyphenol oxidase activity was generally lower in treated plants than in Control plants, which was contrary to expectations. Significant increases in PR protein activity resulting from treatment with methyl jasmonate treatment show that one of the characteristics of induced resistance can be triggered in

the sugarcane plant. However, the other chemicals all failed to deliver on their alleged potential to induce elevated levels of PR proteins and the associated increase in induced resistance.

In Chapter Three, resistance inducing chemicals tested for their ability to induce resistance to two important genera of sugarcane nematodes, *Pratylenchus* and *Meloidogyne*, in both sett roots and shoot roots. None of the chemicals caused a significant reduction in nematode counts. On a number of occasions small differences in nematodes counts were seen, but these included both increased and diminished nematode counts. Furthermore, there was no discernible pattern of nematode counts increasing or decreasing over time as a function of any of the chemical treatments. Treating the plant at an earlier stage with a pre-planting treatment might have allowed for induced resistance prior to nematode challenge. Treatment with resistance inducing chemicals took place at six weeks, at which stage nematode infestation of sett roots would have occurred. Using a sett soak application would provide a means to induce resistance prior to nematode infestation.

The active ingredient in suSCon® max*i* is imidacloprid, which has been described as having growth promoting properties. suSCon® max*i* treatment of the N12 variety resulted in the most biomass. However, none of the chemical treatments significantly increased the biomass of sugarcane shoots and roots in 11 weeks old plants. The conclusion is that it did not affect the growth of sugarcane plants at an early stage of growth, in the presence of nematodes.

In terms of induced resistance to pests and disease, only nematodes and smut were examined in this study. Induced resistance is said to be systemic, long lasting and to provide broad spectrum resistance. Investigating induced resistance in sugarcane to other pests and disease may reveal positive outcomes. The fact that methyl jasmonate increased the activity of in β-1,3-glucanase, chitinase and peroxidase in sugarcane leaves may be an indicator that induced resistance can be triggered against leaf pathogens such as sugarcane rust (*Puccinia melanocephela* Syd. & P. Syd) and insect pests. Sugarcane rust is a fungal pathogen and chitinase alone and in combination with β-1,3-glucanase have been demonstrated to inhibit the growth of many fungi *in vitro*.

However, most of the trials were equivocal, and the results were non-significant, which does not give grounds for optimism that this class of resistance inducing chemicals will control pests or diseases of sugarcane.

There is little to no evidence within this study that suggest induced resistance in sugarcane can be achieved in order to combat pathogens such as nematodes and sugarcane smut.