RAPID MEANS OF SCREENING FOR RESISTANCE TO PESTS IN A SUGARCANE PLANT BREEDING SELECTION PROGRAMME

By

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DISSERTATION SUMMARY

Chilo partellus (Lepidoptera: Crambidae) and *Chilo sacchariphagus* (Lepidoptera: Crambidae) are two stem borers which pose a threat to the South African sugar industry at present. The reliable supply of good quality insects for host-plant resistant studies is vital. The techniques used at the South African Sugar Research Institute (SASRI) for establishing and maintaining *C. partellus* colonies were described because these insects are vital in host-plant resistance research. Sugarcane agro-ecosystems in KwaZulu-Natal were surveyed for *C. partellus*, and species confirmation took place using cytochrome oxidase I subunit barcoding. A neighbor-joining tree showing *Chilo* phylogeny supported the concept of using *C. partellus* as a surrogate insect for *C. sacchariphagus* for host-plant resistant screening studies in South Africa. Artificial diets were developed to optimize insect growth and reproduction and to meet or exceed the nutritional requirements of the target insect. Experiments were conducted to test different diets, with the incorporation of various ingredients, and the use of different inoculation and rearing methods. Vials that were inoculated with two neonate larvae each gave greater mean larval weights and larval survival percentages compared to the multicell trays and plastic jars. An improved artificial diet for rearing *C. partellus* was established incorporating non-fat milk powder (2.35% m/v) and whole egg powder (1.75% m/v). This diet gave higher mean larval survival percentages and mean larval weights than other diets tested. A version of this diet was developed with an increased content of cane leaf powder (from 2.5% to 6.5% m/v), so that better discrimination between leaf powders from different sugarcane genotypes would become possible in diet incorporation bioassays.

Stalk borers can have detrimental effects on crops such as sugarcane, maize and sorghum in sub-Saharan Africa. *C. partellus* and *C. sacchariphagus* are serious pests on a number of hosts and pose a serious threat to the sugarcane industry in South Africa. The use of host-plant resistance is extremely important in controlling these pests, and the breeding of new and improved varieties are important in maintaining good yields. Constitutive antibiosis resistance to *C. partellus* larvae was explored in a diverse collection of 20 sugarcane varieties, by incorporating crushed

dried leaf whorl powder into an artificial diet. There were significant differences in larval weight, total *C. partellus* weight and larval survival in diets incorporating leaf powder from different sugarcane varieties. Varieties M1135/64 and N24 gave consistently lower larval weights and larval survival, whereas varieties M1025/70, R573 and N25 gave higher larval weights and larval survival when incorporated into the diet, which suggests that they have little or no constitutive resistance against *C. partellus*. The concept of insect surrogacy was also explored, whereby known field resistance ratings of specific sugarcane varieties to *C. sacchariphagus* were compared to the results obtained for resistance to *C. partellus* from this study. Some correlations were observed for specific sugarcane varieties, such as N25 and R570, with respect to *C. partellus* and *C. sacchariphagus* resistance. However, further investigations will be required using different resistance screening methods to determine the different components of resistance of sugarcane varieties.

Chilo partellus was used as a surrogate insect for *C. sacchariphagus* in ovipositing studies on sugarcane because *C. sacchariphagus* is not yet present in South Africa. Both pests belong to the same family and have the same feeding mechanisms, therefore similar defense mechanisms in plants may operate against them. The concept of ovipositional antixenosis behaviour of insects is based on the theory that female insects will choose their hosts in a hierarchal manner, laying most of their eggs on the preferred plant. This could be due to characteristics of the plant that either fail to provide ovipositing behaviour-including stimuli (attractants), or contain ovipositional-inhibiting stimuli (repellents). In this study, differences with respect to ovipositing by *C. partellus* moths were investigated on 20 selected sugarcane varieties. Two experiments were conducted, whereby the 20 sugarcane varieties were planted into 98 well seedling trays in a completely randomized design, replicated five and ten times for Experiments One and Two, respectively. Individual trays were placed into BugDorm® rearing tents when plants were still in their seedling stage, and moths were put into the tents for ovipositing to take place. No statistically significant differences were found between sugarcane varieties for both egg and batch number for both experiments (F pr > 0.05). A direct correlation was seen between egg number and batch number, with R^2 values of 0.696 and 0.899 for Experiments One and Two, respectively.

C. partellus and *C. sacchariphagus* initially feed on the leaf whorl of their hosts before boring into the stalk, which results in destruction of the growing point and extensive stalk damage. Host-plant resistance plays a pivotal role in controlling such pests, and therefore it is important to identify sugarcane varieties that could potentially have resistance against them. Glasshouse trials conducted in pots were used to compare 21 sugarcane varieties for their resistance against *C. partellus*. The whorls of the plants were inoculated with 10 neonate larvae, and after 30 days sugarcane varieties were assessed for various damage parameters. Results from these trials give preliminary indications as to whether *Fulmekiola serrata* (Thysanoptera: Thripidae) and *Chilo sacchariphagus* resistances are correlated; whether *C. partellus* or *F. serrata* could serve as surrogates in assessing resistance to *C. sacchariphagus*; and whether *C. partellus* itself poses a threat to sugarcane varieties. There was a significant difference between sugarcane varieties for the mean number of shotholes/lesions, mean number of borings, and mean number of larvae recovered. Sugarcane varieties N32 and M1135/64 showed the highest levels of resistance against *C. partellus*, which concurs with *C. sacchariphagus* ratings obtained from previous studies. With respect to *F. serrata* numbers, sugarcane varieties N21, R568, R574 and R572 had the highest *F. serrata* numbers in a trial conducted by the South African Sugar Research Institute. All four of these varieties were shown to be susceptible to *C. partellus* in the pot trials conducted in this study.

Eldana saccharina Walker (Lepidoptera: Pyralidae) and *F. serrata* are considered serious pests of sugarcane in South Africa. The potential for an invasion by the borer *C. sacchariphagus* from Mozambique poses a great risk to the South African sugarcane industry, and *C. partellus* may represent a threat similar to the one once posed by *E. saccharina* before it adapted to feed on sugarcane. *F. serrata*, *C. partellus*, and *C. sacchariphagus* all feed on the whorl of their hosts, and therefore similar plant resistance mechanisms may act against them. Rating of sugarcane clones for damage caused by these pests and the selection of resistant genotypes can be difficult and expensive, and it can take up to 15 years before new varieties are released. A study was made to develop a rapid, non-destructive, on-site technique for predicting sugarcane resistance to pests such as *Chilo* spp. and *F. serrata.* The technique was based on near infrared reflectance spectroscopy (NIRS), which can also be used to examine the interaction between sugarcane and its attackers.

Differences in resistance of sugarcane varieties to these pests may be in part due to biochemical and structural differences within the leaf. NIRS can penetrate up to 2.5 mm into plant material which infers that NIR spectra should represent the constitutive structural and chemical composition of the leaf that could be related to sugarcane resistance to pests such as *C. partellus* and *F. serrata*. Therefore, spectral data was obtained from intact leaves of 21 selected sugarcane varieties using a portable NIR spectrometer. Correlations between NIR spectral data and reference data obtained for *C. partellus* and *F. serrata* were developed using partial least squares (PLS) regression with full cross validation. Validation plots were useful in discriminating between sugarcane varieties for either constitutive or induced resistance based on predicted and actual values of reference data. Test validation was conducted on selected reference material using a validation set of five samples. Test validations gave better results than cross validations, with the best predictive model for the mean number of shotholes per variety (R^2 of 0.75, SEP of 8.1). Larger calibration and validation sample sets, with equal numbers of resistant, intermediate and susceptible varieties, are required for models to have an improved predictive capability. Reference data such as the number of shotholes per variety, which are directly related to leaf characteristics, gave better model performance than reference parameters not directly related to the scanned leaves, such as boring length in the stalk.

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCES STUDENT DECLARATION

I, **Cindy Moon,** Student Number: **206502582** declare that:

1. The research contained in this dissertation, except where otherwise indicated, is my original research conducted at the South African Sugar Research Institute (SASRI), under the supervision of Dr R.S. Rutherford and Prof. M.D. Laing (UKZN).

2. This dissertation has not been submitted for any other degree or examination at any other University.

3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been re-written but the general information attributed to them has been referenced.

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Signed at…….....on the.....day of…….2014

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STUDENT DECLARATION

I, **Cindy Moon** Student Number: **206502582** declare that:

The research reported in this dissertation, except where otherwise indicated is the result of my own activities at the South African Sugar Research Institute (SASRI, Mount Edgecombe), under the supervision of Dr R . S. Rutherford and Prof. Mark Laing (UKZN).

Signed at……..on the……. day of ……..... 2013

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SIGNATURE

CONFERENCE CONTRIBUTIONS FROM THIS RESEARCH

2013 Moon, C.M., Mhora, T.T., Rutherford, R.S., Sabatier, R.D., and Laing, M.D. (2013). Near infrared reflectance (NIR) spectroscopy – Behind the scenes. South African Sugar Technologists Association Congress, International Convention Centre, Durban, South Africa. 6 August – 8 August 2013.

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TABLE OF CONTENTS

DISSERTATION INTRODUCTION

Background and motivation

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous plant, cultivated in tropical and subtropical areas worldwide for its sugar-rich stalks (Sampietro *et al*., 2007). South Africa currently produces approximately 16 million tons of sugarcane per annum from a total area of 373,000 hectares (Singels *et al*., 2011). It is a leading producer worldwide, and the industry plays an important economic role in the country because it provides employment and contributes toward sustainable development and the national economy (Maloa, 2001). The industry provides employment to approximate 439,000 people and generates an estimated eight billion rand towards the economy (South African Sugar Industry Directory, 2012). Furthermore, there are approximately 35,300 registered cane growers in South Africa (Anon, 2009).

There are over 1,500 species of insects that attack sugarcane worldwide resulting in yield losses in all sugar industries (Ul-Hussnain *et al*., 2007). *Eldana saccharina* Walker (Lepidoptera: Pyralidae) and *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae) are two of the most serious pests to sugarcane production in South Africa (Singels *et al*., 2011). Stalk borers are significant pests of sugarcane because they feed directly on the vegetative tissue in which sucrose is stored, effecting both yield and quality of the crop (Vercambre *et al*., 2001). The spotted stem borer *Chilo sacchariphagus* Bojer (Lepidoptera: Crambidae) causes serious damage to sugarcane in Mozambique (Way *et al*., 2011). Although this borer is not yet present in South Africa, it does pose a risk to sugar industries in countries bordering Mozambique (Way *et al*., 2011). The rise in trade and the change in global climate have resulted in the easier spread and establishment of insects and diseases in previously unaffected areas (Way *et al*., 2011). In addition, the related stem borer *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) has adapted to sugarcane in North Africa (Assefa and Conlong, 2009). It is present in the South African sugarcane agro-ecosystem feeding on maize, sorghum and indigenous grasses (Hutchison *et al*., 2008). *C. partellus* may represent a threat similar to the one previously posed by

E. saccharina before it fully adapted to feeding on sugarcane in the early 1970s (Atkinson *et al*., 1981).

The use of insecticides for controlling pests raises environmental concerns, and can have a negative impact on beneficial insects and natural enemies of pests (Broekgaarden *et al*., 2011). The use of host plant resistance has a number of advantages over other control methods, and is one of the most effective means of controlling insects (Broekgaarden *et al*., 2011). Resistant varieties do not harm the environment, and in some cases, plant resistance is the only effective method for controlling certain pests (Kfir *et al*., 2002). Breeding for resistance to insects in sugarcane lags behind other crops, and this is most likely due to its complex genome and the inheritance of polygenic traits (White *et al*., 2010). Screening for resistance to pests and diseases is presently limited to later selection stages within the sugarcane breeding programme (Rutherford, 1998). The development of new varieties can take up to 15 years, and is a resource intensive process (Purcell *et al*., 2010b). Applications of new screening tools at earlier selection stages will reduce costs, increase productivity, and increase the number of resistant clones progressing to later selection stages (Purcell *et al*., 2005). With an increasing number of potential pests of sugarcane in South Africa, the need for rapid and less costly methods to screen sugarcane varieties increases in importance.

Near infrared reflectance spectroscopy (NIRS) is a rapid, non-invasive, and reliable technique which has the potential to examine the interaction between sugarcane and its related pests. Use of NIRS might allow for the earlier screening of varieties and a reduction in the need for field trials, which will in turn allow for better resource management (Purcell *et al*., 2005). Calibration of near-infrared spectrometers involves acquiring spectra of representative samples, reference analysis of samples using laboratory or traditional methods, and model building using chemometrics (Blanco and Villarroya, 2002; Chen *et al*., 2002).

Description of area of research

Globalisation and climate change are likely to lead to the increased emergence and spread of new pests (Way *et al*., 2011). While these pests are currently absent it is desirable to find alternative methods of determining the level of risk to current sugarcane varieties. To this end, surrogate insects can be used, and was explored. *C. partellus* was used as a surrogate for *C. sacchariphagus*, since both initially feed on the whorl of the plant and then become top borers (Way and Turner, 1999; Tefera and Pringle, 2004). There is also a possibility that *F. serrata* (thrips), which also feeds on the whorl, could act as a surrogate for *Chilo* spp. resistance screening. Among the few South African varieties with known resistance or susceptibility to *C. sacchariphagus*, there appears to be a correlation between resistance rankings to *F. serrata* and *C. sacchariphagus* (shown in this thesis). Near infrared (NIR) reflectance spectra obtained from intact surfaces reflect biochemical and structural differences within the leaf, since NIR can penetrate up to 2.5 mm into plant material (Purcell *et al*., 2010a). Building on research conducted by the South African Sugar Research Institute (SASRI) from the early 1990s, the Bureau of Sugar Experiment Stations (BSES) in Australia have successfully employed NIR to scan intact nodal buds for the prediction of constitutive smut resistance (Purcell *et al*., 2010b). BSES has also used NIRS for scanning intact undamaged sugarcane leaves in order to predict for constitutive resistance to Fiji Leaf Gall Virus (Purcell *et al*., 2009). In this case the model was shown to actually predict varietal preference of the insect vector *Perkinsiella saccharicida* Kirkaldy (Hemiptera: Delphacidae) suggesting that a similar approach could be developed for *F. serrata* and top borer lepidopteran species.

Hypothesis

A. *C. partellus* and *F. serrata* can be used as surrogates for *C. sacchariphagus* in screening sugarcane varieties for resistance.
B. Fibre optic NIR methods can be used to predict constitutive resistance of sugarcane varieties to *C. partellus* and *F. serrata* using reflectance spectra from intact leaf surfaces.

Objectives

A. To develop rearing methods for *C. partellus* and to develop methods for screening sugarcane varieties for resistance to sugarcane borers. In this study *C. partellus* and *C.sacchariphagus* were the stem borers of interest.
B. To screen a collection of sugarcane varieties for overall resistance to *C. partellus*
C. To obtain preliminary indications as to whether *F. serrata* and *Chilo* resistances of sugarcane varieties are correlated; whether *C. partellus* or *F. serrata* could serve as surrogates in assessing resistance to *C. sacchariphagus*, and whether *C. partellus* might itself pose a threat to certain sugarcane varieties.
D. To develop and validate predictive models for constitutive resistance traits against *F. serrata* and *Chilo* spp. Overall resistance ratings are already known for *F. serrata* but need to be determined for *Chilo*.
E. To identify NIR 'outlier' sugarcane varieties which predict as being susceptible in terms of constitutive resistance, but are in fact resistant. Such sugarcane varieties are likely to possess strong physiologically reactive resistance (induced resistance).
F. To identify a set of potentially *C. sacchariphagus* resistant and susceptible sugarcane varieties that in the future could be sent to Mafambisse in Mozambique for confirmation screening in another project.

Dissertation referencing and format style

The system used for referencing in the chapters of this dissertation is based on the Harvard system of referencing (De Montfort University), and follows the style used in "Journal of Biocontrol Science and Technology".

The dissertation is laid out in discrete research chapters, each one following the format of a stand-alone research paper (whether or not the chapter has already been published). This is the dominant dissertation format adopted by the University of KwaZulu-Natal because it facilitates the publishing of research out of theses far more

than the older monograph form of dissertation. As such, there is some unavoidable repetition of references and some introductory information between chapters.

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CHAPTER 1

Literature review

1.1 Background information on sugarcane

1.1.1 Origin and importance

Sugarcane varieties belong to the genus *Saccharum* L., of the Poaceae family (Blackburn, 1984). Sugarcane is a monocotyledonous crop that is grown mainly in the tropics and subtropics for its stalks that contain a high level of sucrose (Anon, 2004; Dillon *et al*., 2007). It is the source for 75% of the world's sugar supply and its fully grown stem can store up to 12-16% of its fresh weight, and about 50% of its dry weight as sucrose (Bull and Glasziou, 1963). It is believed that sugarcane has its origin in New Guinea where it was grown as a native garden crop used for its chewing purposes (Barnes, 1974). *S. sinense* Roxb. and *S. barberi* Jesw. have been grown for years in China and India respectively, but the increased use of *S. officinarum* L. resulted in the growth of the sugar industry in the tropics and subtropics (Blackburn, 1984). The cross between S. *officinarum* L. and S. *spontaneum* L. and in some lineal descents, S. *sinense*, or S. *barberi* resulted in the modern sugarcane hybrid (*Saccharum* spp.) that is cultivated today (Dillon *et al*., 2007).

Sugarcane is produced in large quantities worldwide, with over 1,000 million tons harvested per year (Henry and Kole, 2010). In many countries sugarcane provides a number of useful raw materials to a range of industries (Hunsigi, 2001). The by-products of sugarcane, such as molasses, bagasse and filter mud, are also used in a number of ways (Almazan *et al*., 1998; Anon, 2004). Sugar is the most commonly used carbohydrate source globally, particularly for those with a below average income (Almazan *et al*., 1998).

1.1.2 The sugarcane industry in South Africa

South Africa currently produces approximately 16 million tons of sugarcane per annum from a total area of 373,000 hectares (Singels *et al*., 2011). The three major

areas of sugarcane production in South Africa are the coastal belt and midlands areas in KwaZulu-Natal, the Mpumalanga Lowveld and Northern Pondoland in the Eastern Cape (Figure 1.1) (Lewis, 1990). There are an estimated 35,300 registered cane growers, with the majority farming in KwaZulu-Natal (Anon, 2009b). The South African sugarcane industry started in Natal in 1847 where it proved a great success (Lewis, 1990). It is a leading producer worldwide and the industry plays an important role in the country as it provides employment and contributes toward sustainable development and the national economy (Maloa, 2001). The industry provides employment to approximately 439,000 people and generates an estimated R8 billion towards the economy (South African Sugar Industry Directory, 2012). In South Africa, molasses and sugar are produced at 14 mills for the local and foreign markets. The industry focuses on the production of sugar that is of good quality and that is sold at a good price, together with sustainable development (Anon, 2009b).

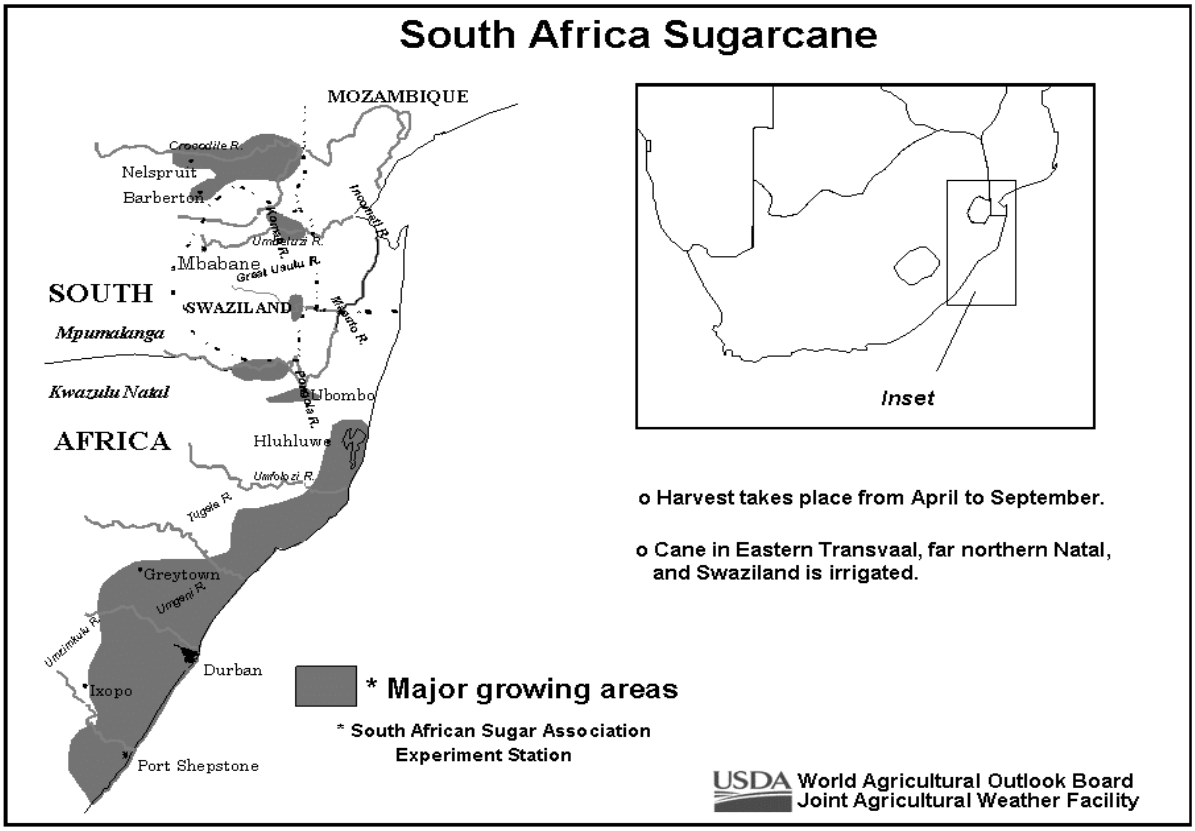

Figure 1.1 Distribution of sugarcane and its major growing areas in South Africa (www.usda.gov)

1.2 Sugarcane insect pests

1.2.1 Introduction

There are over 1500 species of insects that attack sugarcane worldwide (Ul-Hussnain *et al*., 2007). They belong to a range of orders, including Lepidoptera, Homoptera, Coleoptera, Hemiptera, Orthoptera and Isoptera (Assefa and Conlong, 2009). In South Africa, insects which have a major effect on sugarcane production are *Eldana saccharina* Walker (Lepidoptera: Pyrialidae), *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae) (thrips), *Schizonycha affinis* Boheman and *Hypopholis sommeri* Burm. (Coleoptera: Scarabaeidae) (white grubs), and *Petamella prosternalis* (Acrididae: Orthoptera) (grasshopper) (Way *et al*., 2011; Bam and Conlong, 2012). *E. saccharina* and *F. serrata* are the most serious and extensive insects in sugarcane production (Singels *et al*., 2011). *Chilo sacchariphagus* Bojer (the spotted sugarcane stalk borer) and *Chilo partellus* (Swinhoe) (the spotted maize stem borer) belong to the insect order Lepidoptera (butterflies and moths) and family Crambidae (Arabjafari and Jalali, 2007; Goebel and Way, 2009). Stalk borers are one of the most serious insect pests of crops such as sugarcane, maize, and sorghum in sub-Saharan Africa (Kfir *et al*., 2002). Insect damage can result in the total loss of a crop in a relatively short time. This loss can be seen by poor stands, poor growth, decreased yields, and poor quality of the crop (Bezuidenhout *et al*., 2008; Goebel and Way, 2009).

Alien invasive species are those that are brought into a country either accidentally or intentionally, which invade their new home and cause a threat to ecosystems, habitats, biological diversity and humans (Chenje and Mohamed-Katerere, 2008). Invasive alien species are a threat to South African agriculture (Way *et al*., 2011). Extreme changes in climate and increasing global trade results in the spread of pests and diseases occurring more easily and establishing in new, previously unaffected countries (Goebel and Sallam, 2011). Invasion of alien species can be extremely harmful to important agricultural crops as they are expensive to control; can result in the prevention of export programmes; and cause disturbances in ecosystems which can lead to loss of biodiversity (Chenje and Mohamed-Katerere, 2008; Westphal *et al*., 2008).

1.2.2 *Chilo partellus* (Swinhoe) and *Chilo sacchariphagus* Bojer (Lepidoptera: Crambidae)

1.2.2.1 Background of *Chilo partellus* (Swinhoe)

Chilo partellus (Swinhoe) is a serious pest in Asia and southern African countries (Arabjafari and Jalali, 2007). It invaded Africa prior to 1930 when it was first noted in Malawi; however it was not reported for the next 20 years until it was recorded in Tanzania (Kfir *et al*., 2002). In Africa it is in: Botswana, Cameroon, Ethiopia, Kenya, Malawi, Mozambique, Somalia, Sudan, Lesotho and South Africa (Hutchison *et al*., 2008). Host crops of *C. partellus* include sorghum (*Sorghum bicolor L. Moench*), rice (*Oryza sativa* L.), maize (*Zea mays* L.), pearl millet (*Pennisetum glaucum* L.), Job's tears (*Coix lacryma-jobi* L.) and a number of grasses such as Sudan grass (*Sorghum x drummondii*) and Napier grass (*Pennisetum purpureum* Schumach.) (Arabjafari and Jalali, 2007; Hutchison *et al*., 2008).

C. partellus is extremely competitive and has been found to displace other native stem borers in eastern and southern Africa, making it one of the most harmful and serious stem borers of crops (Kfir *et al*., 2002). An example is given by Kfir *et al*. (2002) of the Eastern Province of Kenya, where *C. partellus* has almost completely displaced the local stem borer, *Busseola fusca* Fuller (Lepidoptera: Noctuidae). This could be clearly seen in sorghum where the *C. partellus* population was found in higher proportions than other borers, rising from 3% in 1986 to 91% in 1992. The reason for the higher competitiveness of *C. partellus* compared to other borers such as *B. fusca* and *Chilo orichalcociliellus* Strand (Lepidoptera: Crambidae) could be due to its shorter generation time and its capability to end diapause quickly, which allows for it to inhabit its host plants first (Kfir *et al*., 2002). The damage caused by *C. partellus* has been reported to result in losses ranging from 24-75% (Arabjafari and Jalali, 2007).

The life cycle of *C. partellus* shows complete metamorphosis, with an egg, larval, pupal, and adult stage (Figure 1.2a-d) (Hutchison *et al*., 2008). The borer can undergo one or more generations per year, but this depends on host plant availability and regional conditions (Hutchison *et al*., 2008). The larval stage can undergo diapause depending on conditions such as altitude and climate, and when in areas with sufficient crop and grass host plants, the borer will repeat its life cycle

throughout the year (Kfir *et al*., 2002). The female moths tend to show preference for the whorl stage of the plants for ovipositing. However, their eggs are laid on both the upper and under sides of the leaves, as well as on the stem. They are laid in clusters of approximately 100 eggs per female (Hutchison *et al*., 2008). Eggs hatch in seven to ten days and the hatched larvae move to the top of the plant where they continue feeding on the leaf whorls (Hutchison *et al*., 2008). The larva of this borer feed on the leaves of the plants from 15 minutes to eight hours and eventually bore into the stem which leads to the plant's deterioration (Figure 1.3b-c) (Sarup *et al*., 1985). The feeding of the newly hatched larvae on the leaves results in lesions and give a "shothole" appearance to the leaf (Figure 1.3a) (Tefera and Pringle, 2004). Larva also cause 'dead-hearts' by feeding and tunnelling into the growing points of plants (Midega *et al*., 2011; Tefera and Pringle, 2004). The extensive damage caused by the borer weakens the plant, reduces yields, and makes it more vulnerable to pathogenic infections (Tefera and Pringle, 2004). Once the larvae enter the stalk, and after feeding for two to three weeks, the pupal stage commences for five to twelve days, and the adult stage for about two to five days (Anon, 2011). The entire life cycle lasts for 25 to 50 days, depending on environmental conditions (Hutchison *et al*., 2008; Uys, 2009).

The larvae of *C. partellus* are cream to yellow in colour and have dark brown spots along the top surface of their bodies (Hutchison *et al*., 2008). The head capsule is reddish-brown and the larvae reach up to 25mm when fully mature (Anon, 2011).

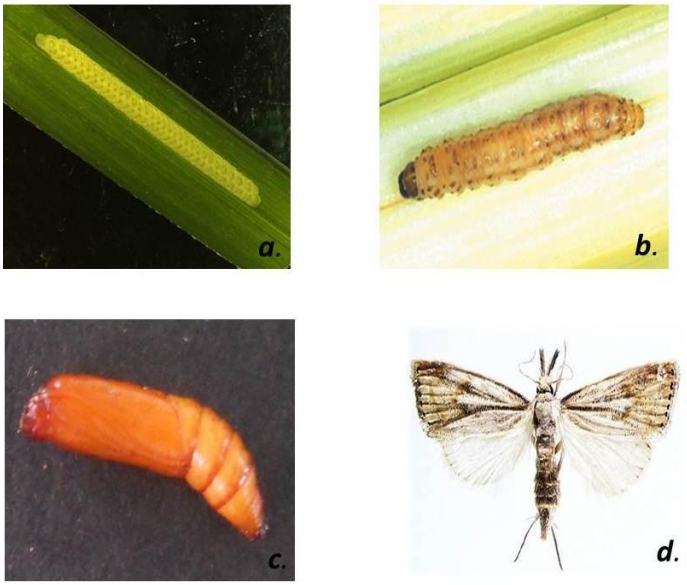

Figure 1.2 Life stages of *Chilo partellus* (a) the egg stage; (b) The larval stage (photos from http://keys.lucidcentral.org) (c) The pupal stage; (d) the adult stage (photos from http://www.plantwise.org)

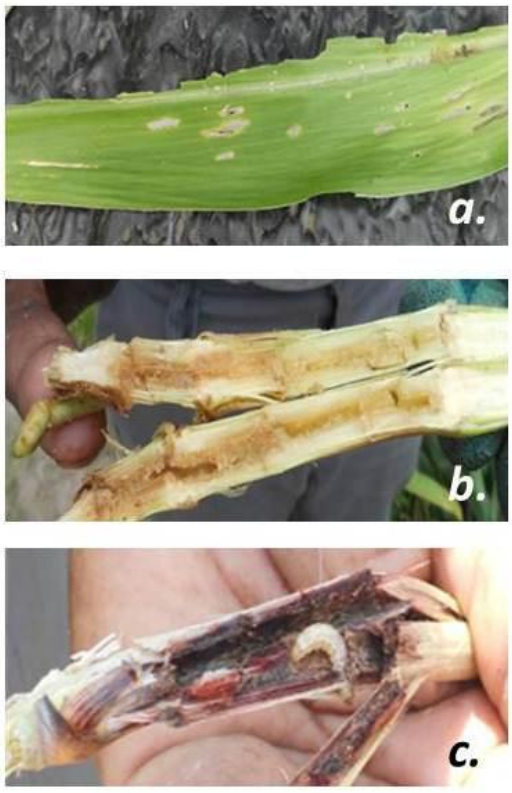

Figure 1.3 Damage due to *Chilo partellus* (a) on the leaves of Job's tears (*Coix lacryma-jobi* L.*);* (b) in the stalk of Job's tears; (c) in the stalk of wild sorghum (*Sorghum halepense* (L.) Pers.)

1.2.2.2 Background of *Chilo sacchariphagus* Bojer

Chilo sacchariphagus Bojer originated in southeast Asia (Conlong and Goebel, 2002). It has been a serious insect pest of sugarcane since the 19th century in Reunion, Mauritius, and Madagascar (Rochat *et al*., 2001). In 1998 *C. sacchariphagus* was found in Mozambique, and today it is a great threat to sugarcane production of sugar estates in the Companhia de Sena (Marromeu or Sena) and in the north at the Açucareira de Mozambique (Mafambisse) estate (Way *et al*., 2011). There have been no records yet of *C. sacchariphagus* in neighbouring countries of Mozambique, including South Africa (Way and Turner, 1999). In Mauritius, *C. sacchariphagus* is the most important borer of sugarcane, whereas borers such as *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) (the pink borer) and *Tetramoera schistaceana* Snellen (Lepidoptera: Tortricidae) (the white borer) are not as important (Soma and Ganeshan, 1998). Sugar cane is the primary host of *C. sacchariphagus*; however, it can occasionally attack maize and sorghum (Williams, 1983).

Like *C. partellus*, *C. sacchariphagus* can breed through the whole year with up to 4 generations (Conlong and Goebel, 2002). Eggs are laid on the top and bottom of the leaf blades, along the midribs in clusters of approximately 20 to 40 eggs, with an estimated fecundity of 300 to 850 eggs in total per female (Goebel, 2006). This process occurs at night after the adults have emerged and mated (Way and Turner, 1999). Unlike *E. saccharina*, the female of *C. sacchariphagus* emits pheromones and not the male (Goebel, 2006). First instar larvae feed on the leaves or within the rolled up spindle (leaf whorl). This feeding habit results in shotholes in the leaves when they fully emerge (Way and Turner, 1999). In younger cane, older larvae feed on the meristematic region which can cause “dead hearts” (Way and Turner, 1999). In mature cane the larvae feed just below the growing tip, which results in side shooting, and if widespread tunnelling occurs, stalks become seriously damaged (Way and Turner, 1999). Larvae usually attack cane that is three to seven months old (relatively young) and therefore loss in cane weight is of more importance than reduced sucrose content (Goebel, 2006; Bezuidenhout *et al*., 2008).

Larvae are cream to white in colour with a spotted exterior and may also have stripes running along the body (Figure 1.4b) (Anon, 2009a). Pupation generally takes place

on the inside of the leaf sheath in an undefined cocoon (Uys, 2009). Populations of *C. sacchariphagus* seem to prosper when the temperatures are greater than 20^{o}C, with an optimum temperature of 26^{o}C for their development (Rochat *et al*., 2001).

Goebel and Way (2009) investigated the impact of stem borers *E. saccharina* and *C. sacchariphagus* on sugarcane yield and quality. They found that these borers both have an effect on both sugarcane quality (sucrose) and biomass (yield). The larvae of these borers feeding on sugarcane resulted in a decreased size and mass of sugarcane stalks and also in a reduction in the amount of juice. The levels of gums and non-sugar juice constituents in the stalks increased in infested stalks as compared to healthy stalks.

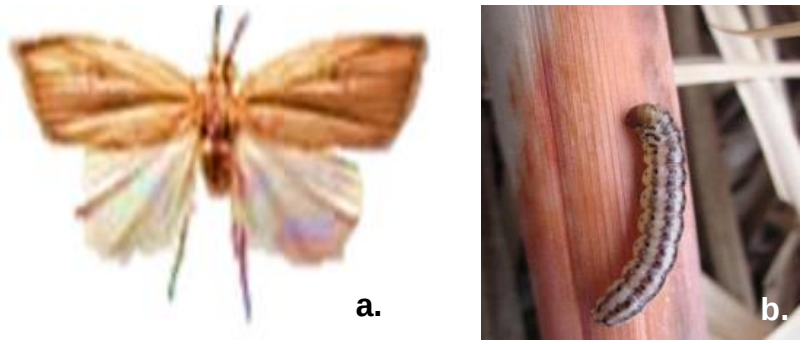

Figure 1.4 Appearance of *Chilo sacchariphagus* (a) adult and (b) larva (Hutchison *et al*., 2008)

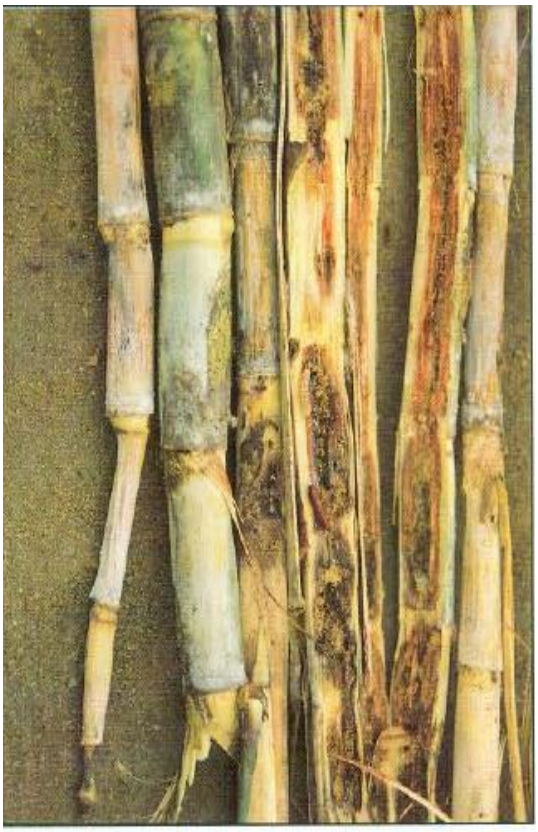

Figure 1.5 Larva and pupa of *Chilo sacchariphagus* on infested sugarcane stalks (Way, 1999)

1.2.2.3 Economic importance and threat of *Chilo sacchariphagus* and *Chilo partellus* to the sugarcane industry in South Africa

The high likelihood of *C. sacchariphagus* moving from Mozambique into South Africa poses a great risk to the South African sugarcane industry. Although it has not yet entered South African territory, studies on climatic conditions suited for the pest show that the coastline of KwaZulu-Natal and neighbouring river valleys, particularly in the north, are suitable for the pest's establishment (Goebel, 2006; Bezuidenhout *et al*., 2008). Temperature thresholds have been determined for the different life cycle stages of *C. sacchariphagus*, and these can be used to determine whether certain sugarcane producing areas are vulnerable to attack by this pest (Goebel, 2006). However, in order for such predictions to be accurate, an accurate history of records of temperature readings is required (Bezuidenhout *et al*., 2008).

Bezuidenhout *et al*. (2008) conducted a study using the temperature thresholds known for *C. sacchariphagus* to put together a series of maps which show the potential spread of this borer into regions of South Africa and Swaziland, based on long term temperature data sets of these areas. The maps were developed to show the distribution of *C. sacchariphagus* based on mortality, maintenance and mating indices. Maps (for comparison purposes) were also put together for La Mare, Reunion Island, where survival conditions for *C. sacchariphagus* are ideal. It was found that the north-eastern areas of KwaZulu-Natal are the most prone to *C. sacchariphagus,* and although the mating and maintenance indices are high for Limpopo and Mpumalanga, their mortality index is also significantly higher. Although the north-eastern areas of KwaZulu-Natal have the highest chance of survival for *C. Sacchariphagus*, the index values are not the same as that of La Mare, particularly for mortality index (49 days versus one day per annum) and mating index (3275 versus 4300 hours per annum). Therefore, this may indicate that there could be a climate barrier for the spread of *C. sacchariphagus* into South Africa. However, these predictions will need to be backed up by further studies.

Losses due to *C. sacchariphagus* in Réunion are approximately eight to ten million Euros per annum, but this value can differ according to the area planted with the more susceptible variety R579 (Goebel and Way, 2009). The larvae can result in huge crop losses due to the damage that they cause, namely, damage to growing points, disruption of metabolite and nutrient translocation, stunting, lodging, and ultimately death (Hutchison *et al*., 2008). In South Africa *C. partellus* can cause yield losses of 50% to maize and sorghum crops (Hutchison *et al*., 2008). In Maputo, the Limpopo Valley, and in Southern Mozambique there have been records of 100% of plant infestations in maize (Kfir *et al*., 2002). Way (1999) reported losses of 40% at the Mafambisse Estate in Mozambique in a sugarcane crop infested with *C. sacchariphagus*.

According to Way *et al*. (2011), the natural spread of *C. sacchariphagus* from Mozambique into other countries is not likely, because the sugar estates are isolated and surrounded by vegetation over which the moths are unable to fly. The most likely means by which this borer could spread is through movement of infested sugarcane setts between sugar industries. Travellers may either bring infested material

innocently or deliberately from Mozambique into South Africa because private vehicles are not checked as efficiently as they should be (Way *et al*., 2011).

Although *C. sacchariphagus* has not yet spread outside Mozambique into adjacent countries, this borer is still very important in terms of biosecurity (Bezuidenhout *et al*., 2008). *C. partellus* has adapted to sugarcane in North Africa and is present in the South African sugarcane agroecosystem (Assefa and Conlong, 2009; Hutchison *et al*., 2008). *C. partellus* therefore represents a threat similar to the one once posed by *E. saccharina* before it adapted to feeding on sugarcane. The remarkably fast spread of *C. partellus* from Malawi, and the associated crop losses and damage due to this borer, prove how serious these exotic pests can be if left uncontrolled (Conlong and Goebel, 2002).

1.2.3 *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae)

1.2.3.1 Background of *Fulmekiola serrata*

Fulmekiola serrata Kobus (Thysanoptera: Thripidae) is a recent incursion in southern Africa suspected of causing significant yield losses in sugarcane (Way *et al*., 2010). Its origin is in the Oriental region where it has been recorded in Java, Taiwan, Japan, China, and India (Way *et al*., 2006b). It has now spread to other countries such as Mauritius, Reunion and Madagascar (Way *et al*., 2006b). *F. serrata* was first found on sugarcane in Africa in 2004 (Abdel-Rahman *et al*., 2008). It is not known how the pest was introduced into Africa, but it is thought that it entered through contaminated plant material or via wind from Mauritius (Way *et al*., 2006b). The increase in *F. serrata* populations in South Africa could be due to increased temperatures, drought, and periods of wind which makes for a favourable environment for the fast multiplication and spread of the pest (Way *et al*., 2006b). Species of thrips that attack sugarcane include *F. serrata*, *Haplothrips aculeatus* Fabricius, *Anaphothrips sudanensis* Trybom (Way *et al*., 2006b) and more recently the Oriental rice thrips *Stenchaetothrips biformis* Bagnall (Thysanoptera: Thripidae) in Australia (Sallam *et al*., 2013).

In *F. serrata*, all life stages live in the folded leaf spindles at the top of the plant where they feed on plant sap by piercing the leaf tissues using their mouthparts (Way *et al*.,

2006a; Way *et al*., 2010). This enclosed environment provides the insect with ideal humid conditions and also provides for some protection from predators (Way *et al*., 2006a). Approximately 80 eggs are laid by the female inside the leaf spindles and the nymphs cause most of the crop damage (Leslie, 2006). Eggs hatch about four to five days after being laid, and this occurs quicker when temperatures are higher (Alleyne, 1981). The minute larvae are initially white in colour and later turn yellow, with red coloured eyes (Way *et al*., 2006b). They develop quite quickly, with two distinct stages having been identified. There is a pre-pupal and pupal stage as well, where both are usually inactive. The pupae become active when disturbed (Alleyne, 1981). The adult insects (Figure 1.6) are very small, being 2-3 mm long, black in colour, and have well developed wings (Alleyne, 1981). When one opens the young leaf spindles, which exposes the insects, they move in erratic circles on the leaf, which could indicate that *F. serrata* is very photosensitive (Way *et al*., 2006a). It is possible that it is thigmotactic, meaning that it inhabits small spaces, which allow for its close contact with a surface (Way *et al*., 2006b).

Figure 1.6 The adult insect of *Fulmekiola serrata*, approximately 2 mm in length (Leslie, 2006)

The damage caused by *F. serrata* can be serious because all its life stages feed on the leaves (Way *et al*., 2006a). The spindle leaf is usually where *F. serrata* thrives and where damage is most noticeable (Leslie, 2006). In young cane, *F. serrata* infestations result in the leaves turning yellow and eventual death of the tips of leaves (Way *et al*., 2006b). The leaves in turn, remain unrolled and join at the tips (Leslie, 2006). In older cane, unrolled leaves have streaks of yellow with brown chlorotic

lesions and silver leaf margins, and the leaf tips also become brown and withered (Way *et al*., 2006a; Way *et al*., 2006b).

Figure 1.7 *Fulmekiola serrata* (a) on a sugarcane leaf and (b) in the leaf spindle (Anon, 2007; Leslie, 2005)

1.2.3.2 Economic importance of *Fulmekiola serrata*

There have been only a few yield loss trials on *F. serrata*, and the information obtained from them is unclear, possibly due to confounding plant growth promoting effects of the neonicotinoid insecticides effective against them (Thielert, 2006; Gonias *et al*., 2008; Way *et al*., 2010). In Mauritius, the economic loss due to this pest has not been quantified, even though it does attack sugarcane from time to time (Way *et al*., 2006b). In the 1950s in Taiwan, approximately 20,000 hectares of sugarcane was seriously infested with *F. serrata* causing large amounts of damage (Anon, 2013b). It is presumed that *F. serrata* does not cause a decrease in cane quality, because the stalks, which are the storage sites for sucrose, are not directly attacked, and so the damage is more likely to cause a decrease in yield because it stunts the overall growth of the plants by affecting leaves and hence photosynthetic activity (Way *et al*., 2006b). In China, there have been yield losses of up to 15% due to thrips (Way *et al*., 2006b).

1.2.4 Control of sugarcane pests

Biological control, cultural practices, chemical control and host plant resistance are the methods used to control borers such as *C. sacchariphagus* and *C. partellus* (James, 2004). In Mauritius, cultural and chemical control methods are not considered feasible in controlling *C. sacchariphagus* because it was found that the cultural practice of burning sugarcane before and after harvest actually had a negative impact on the natural enemies of the borer, whereas the larvae and pupae living inside stalks remained unaffected (Rochat *et al*., 2001). Classical biological control is the favoured method of control whereby a number of natural parasitoids have been introduced from other countries to control the borers (Way and Turner, 1999). Since *C. sacchariphagus* is not indigenous to Africa, and mainly attacks sugarcane, it should fit the profile for being a target for biological control (Conlong and Goebel, 2002). Rochat *et al*. (2001) reported that approximately 17 species of natural enemies of *C. sacchariphagus* have been brought into Reunion from different countries, but only a third of them survived and none were able to reduce the levels of *C. sacchariphagus.* The control of *C. partellus* and *C. sacchariphagus* is particularly difficult, because once the larvae enter the plant tissue; it is difficult for natural enemies and insecticides to reach the target (Afzal *et al*., 2009). Since *C. sacchariphagus* has a larger impact on cane weight than on sucrose content due to its early attack of sugarcane, control methods should focus on early infestations, with the parasites being released when the crop is still young. In turn this will prevent the insect populations from increasing, which could potentially reduce yield losses (Goebel and Way, 2009).

Way *et al*. (2011) made recommendations for the control of *C. sacchariphagus*, including the establishment of a breeding programme for developing resistant varieties. Resistant varieties have a number of advantages over other control methods. Resistant varieties are not influenced by changing weather conditions, do not harm the environment, and in some cases, are the only effective method for controlling certain pests (Kfir *et al*., 2002). Breeding for resistance to insects in sugarcane lags behind other crops, and this is most likely due to its complex genome and the inheritance of polygenic traits (White *et al*., 2010).

A number of studies have been published reporting on the evaluation of sugarcane varieties for their resistance to sub-species of *C. sacchariphagus*. However, none of the data provides information on the resistance status of Reunion sugarcane varieties, and the gain that could be achieved by improving sugarcane varieties (Nibouche and Tibere, 2009). The two main sugarcane varieties of sugarcane grown in Reunion are R570 and R579, which have different susceptibilities to *C. sacchariphagus* (Nibouche and Tibere, 2010). It was shown that R570 is one of the most resistant sugarcane varieties to *C. sacchariphagus* when compared to other sugarcane varieties (Nibouche and Tibere, 2010). Conlong *et al*. (2004) reported on differences in Southern African sugarcane varieties grown in Mozambique with respect to damage caused by *C. sacchariphagus*. The development of thrips resistant varieties is still in its initial stages. In onion, wheat, and cabbage some resistant varieties have been developed. Some of this resistance is based on morphological characters of the plant such as having round or flat leaves, hairy leaves, and an open plant design (Parrella and Lewis, 1997). In sugarcane it has been shown that those varieties that have a slow initial growth and are slower in unrolling their central leaf rolls are more prone to attack and damage by *F. serrata* (Leslie, 2005). In South Africa sugarcane varieties that are affected the most are N27, N35 and N41 (Leslie, 2005).

1**.3. Host-plant resistance to insect pests**

1.3.1 Introduction

Pest and disease resistance is shown in the inherited ability of an organism to negate the effects, totally or partially, of a pathogen, insect, or other harmful factor (Sharma, 1997). Resistance is shown when symptoms are minimal to none which indicates that the pest cannot spread, or spreads with difficulty on the host (Ahman, 2006). The resistant characteristic of plants is usually a result of morphological and biochemical attributes of the plant which has an effect on the insect's behaviour and biology and leads to better survival and production of the plant (Sharma, 1997; Thayumanavan and Sadasivm, 2003; Gogi *et al*., 2010). By comparing resistant plants to more susceptible plants under similar conditions, one can deduce the degree of resistance of that plant (Sharma, 1997).

Naturally occurring pest resistant traits in plants can be of either induced or constitutive resistance (Broekgaarden *et al*., 2011). Induced resistance requires the plant to recognize that there is an invader, which in turn results in the plant producing proteins or metabolites that are harmful to the invader (Keen, 1999; Underwood and Rausher, 2002; War *et al*., 2012) (Figure 1.8). With induced resistance the plant detects the pest via at least one molecule produced by the pest. The molecule(s) may be a protein, fatty acid derivative (fatty acid-amino acid conjugates), or other chemical compound secreted by the pest (Alborn *et al*., 1997).

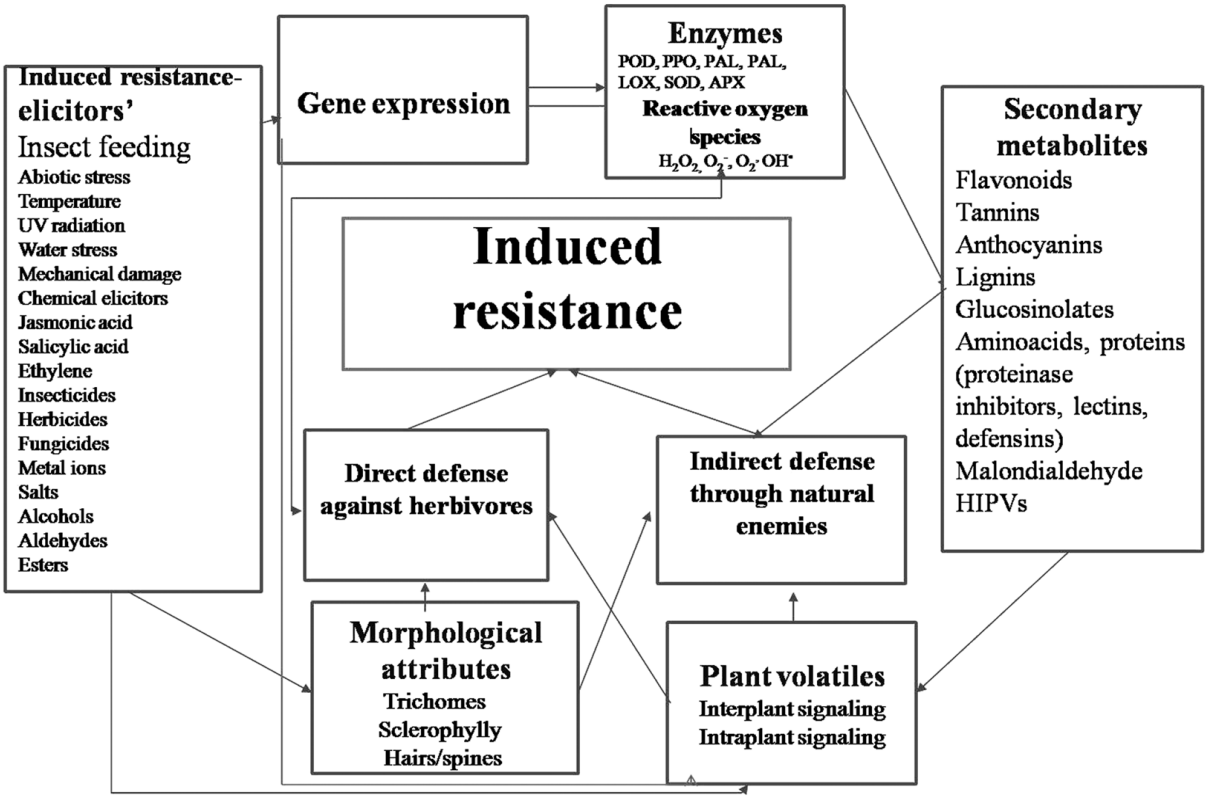

Figure 1.8 Mechanism of induced resistance in plants. POD, peroxidase; PPO, polyphenol oxidase; PAL, phenylalanine ammonia lyase; TAL, tyrosine alanine ammonia lyase; LOX, lipoxygenase; SOD, superoxide dismutase; APX, ascorbate peroxidase; HIPVs, herbivore induced plant volatiles. (War *et al*., 2012)

Constitutive resistance is the level of resistance already present in the plant, and is not dependent on the attack of a pest (Do Vale *et al*., 2001). It can be morphological, structural or chemical in nature (Keen, 1999; Underwood and Rausher, 2002).

Arimura *et al*. (2005) classified and described resistance in terms of direct and indirect defences. Direct defences are those which instantly impact on herbivores attacking the plant, such as physical barriers which include thorns, trichomes, waxes, spines, and chemical means using secondary plant metabolites or special defense proteins. Direct defences can be both constitutive and inducible. Indirect defences are mediated through other species such as natural enemies of the insect pest (Dudareva *et al*., 2006) (Figure 1.8).

There are three components of plant resistance, namely, antixenosis, antibiosis, and tolerance (Thayumanavan and Sadasivm, 2003). One or more of these mechanisms may be present in a resistant plant; however it is favourable for all three mechanisms to be present in a resistant variety (Ahman, 2006). Mathes and Charpentier (1969) describe four types of resistance to moth borers occurring in sugarcane. These are (i) the host-plant may be unsuitable for the moths to lay their eggs (ii) the host-plant may have negative effects on the borer due to physical and nutritional attributes of the plant (iii) the host-plant may be unsuitable for the entry of the borers and (iv) tolerance of the host-plant to borers. Number (i) can be defined as antixeniosis and (ii) and (iii) as antibiosis. These mechanisms will be discussed in more detail later on in the chapter.

1.3.2 Components of host-plant resistance

The three components of resistance, namely, antixenosis, antibiosis, and tolerance are they can be either physical or chemical in nature, or a combination of both.

Antixenosis is a resistant mechanism in plants that can be morphological, physical, or structural in nature and interferes with the behavioural aspect of insects such as mating, laying of eggs and the insects feeding (Thayumanavan and Sadasivm, 2003; Eickhoff *et al*., 2008; Gogi *et al*., 2010). It therefore results in the insect avoiding the host plant. It could be due to the colour, texture of the leaf surface, certain allelochemicals, or an interaction between all of these factors in the plant which deters the insect pest and prevents ovipositing from occurring on the plant (Gogi *et al*., 2010).

Antixenosis has been used in some cases to develop resistant varieties of crops (Kumarasinghe and Jepson, 2003). Examples are shown in rice that are resistant to *Cnaphalocrocis medinalis* Guenee (Lepidoptera: Crambidae) (rice fodder) as a result of crossing a susceptible cultivar with a wild rice cultivar having antixenotic characteristics; *Chilo infuscatellus* Snellen has a preference for ovipositing on sugarcane plants of 45 days old, whereas older plants will not be used for this purpose (Kumarasinghe and Jepson, 2003). In a paper published by Nibouche and Tibére (2010), mechanisms of resistance of two varieties (R570 and R579) to *C. sacchariphagus*, and its location in the plant were identified. In Cultivar R570, plants were artificially infested with *C. sacchariphagus* in the glasshouse. Within 48 hours after infestation there was a reduction in larvae numbers that had established on the plants. Bioassays carried out in the laboratory indicated that the reduction in larvae numbers was due to antixenosis on the lower surface of the leaf sheath. Susceptibility to the antixenosis was seen in the first, second and third instar stages. However, antixenosis was not seen on the leaf spindle or on the stalk. It was also concluded that antibiosis could not have been involved in the resistance shown as there was a low number of dead larvae on the plants and thus it was concluded that antixenosis was the main mechanism of resistance to *C. sacchariphagus*.

Antibiosis adversely affects the biology of an insect which tries to use the plant as a host and can be physical or chemical in nature (Thayumanavan and Sadasivm, 2003). Damage to the insect can be severe and often affects the larvae and eggs (Sharma, 1997). Insects that survive the effects of antibiosis may be permanently damaged having stunted growth, slower development processes, and a reduction in fecundity (Sharma, 1997; Padmaja *et al*., 2012). Antibiosis can be attributed to allelochemicals, growth inhibitors, and morphological factors preventing the attack of the insect (Eickhoff *et al*., 2008). Allelochemicals such as glycoalkaloids in potato, γ-tomatine in tomato, gossypol in cotton, and rutin and chlorogenic acid in tomato are harmful to insects that attack such plants (Sharma, 1997). Examples of some growth inhibitors imparting antibiosis are maysin in maize, coumestrol in soybean, and terpenoids in cotton which prevent the growth of insects that feed on them (Sharma, 1997). In a study conducted by Kumar *et al*. (2006) on antibiosis mechanisms of resistance to *C. partellus* in sorghum, it was found that antibiosis resulted in reduced pupal weight and less pupation. It was suggested that this could be due to secondary

plant substances present in the leaves. The levels of amino acids, tannins, phenolics, lignins, acid detergent fiber (ADF), and neutral detergent fiber (NDF) were linked to resistance of sorghum to *C. partellus* (Kumar *et al*., 2006).

Tolerance allows for normal growth and an increase in plant biomass, irrespective of the level of insect infestations (Sharma, 1997). Painter (1951) defined tolerance as, “a basis of resistance in which the plant shows an ability to grow and reproduce itself or repair injury to a marked degree in spite of supporting a population approximately equal to that damaging a susceptible host”. According to Reese *et al.* (1993), tolerance is the preferred mechanism of resistance because it does not negatively impact on the natural enemy populations, whereas antixenosis and antibiosis increase selection pressure on insect populations, which can lead to the development of more virulent biotypes and can also, have an adverse effect on control methods.

1.3.3 Physical defense mechanisms in plants

The surface of a plant is where organisms first come into contact with the plant in order to establish themselves with the plant, and therefore physical and chemical structures on the plant surface are important in contributing to pest resistance (Howe and Schaller, 2008). Trichomes and/or hairs on the surface of plants have been used to give rise to insect-resistant varieties (Peter *et al*., 1995). Trichomes can either be non-glandular, tiny hairs which physically deter insects, or they may be specialized glandular trichomes, morphological and chemical in nature, whereby they secrete substances which are stored or volatilized on the surface of the plant and are used to repel pests and prevent them from feeding (Johnson, 1975; Fernandes, 1994; Larkin *et al*., 2003, Martin and Glover, 2007; Howe and Schaller, 2008). Recent research also shows that trichomes may be involved in the early detection of pests whereby the trichomes are disturbed by the presence of moths or larvae and this leads to the plant gaining awareness of the pest which allows it to respond to the insect attack more quickly. The nature of response could be to increase the trichome density on new leaves (van Schie *et al*., 2007; Kobayashi *et al*., 2010).

Epicuticular wax on the surface of leaves may also play a role in protecting plants against insects (Eigenbrode and Espelie, 1995). In addition to preventing the plant from desiccation, they also result in a more slippery surface which prevents non-specialized insects from inhabiting the plant (Jenks *et al*., 1994; Riederer and Schreiber, 2001). The chemical and physical components of the wax layer play an important role in determining resistance (Howe and Schaller, 2008). In a study conducted on *C. partellus* it has been found that edge spines and leaf surface waxes play an important role in the reorientation of newly hatched *C. partellus* larvae which have drifted out onto leaves, and whose aim is to reach the whorl. The larvae are reorientated to the stalk in order for them to continue their climb. Therefore plant characteristics such as leaf surface waxes can be partially responsibly for different levels of resistance between varieties (Bernays *et al*., 1985).

Silicon in plants can also confer resistance to insects, and it has been shown that constitutive resistance using silicon as a physical defense mechanism is important against chewing insects. This has been shown for sugarcane that the application of silicon fertilizer resulted in increased resistance to penetration by *E. saccharina* (Keeping and Meyer, 2002; Kvedaras and Keeping, 2007). Another important physical attribute of plants involved in pest resistance is leaf toughness. This characteristic has an effect on insect penetration, preventing their piercing and sucking mouthparts from damaging plant tissues (Schaller, 2008). Leaf toughness is generally regarded as a physical factor. However the chemical composition of the cell wall contributes to leaf toughness (Schaller, 2008). Other physical attributes of plants contributing towards insect resistance are shape and colour. In a study was conducted by Kumarasinghe and Jepson (2003) on the antixenotic effect of sugarcane leaves on feeding and ovipositing by *Pyrilla perpusilla* Walker (Lophopidae: Homoptera), leaf colour was found to play an important role in choice of host for feeding.

1.3.4 Chemical defense mechanisms in plants

Chemical factors involved in plant resistance can be used in one of two ways. Firstly, chemicals can decrease the nutritional value of the plant as a food source, and secondly, they can deter insects by producing toxins. Plant primary metabolism gives

rise to carbohydrates, amino acids, and lipids that are vital nutrients for insects; and the availability of these nutrients has an effect on the life span, size, productiveness, and mortality of insects (Howe and Schaller, 2008).

Secondary metabolites play a vital role in defense mechanisms of plants (i.e., antixenosis, antibiosis and tolerance) (Wink, 1988). Phenolic compounds are secondary metabolites that are major compounds in plants (Mazid *et al*., 2011). Phenolic compounds include coumarin, lignin, flavonoids, and tannins (Mazid *et al*., 2011). Coumarins occur extensively in plants and are known to act as natural defense compounds against insects, fungi, and bacteria (Mazid *et al*., 2011). There are a number of cases where flavonoids are used in resistance against insects in plants. The C-glycosyl flavone, maysin, in maize silk tissues has insecticidal activity against *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) (Byrne *et al*., 1996; Rector *et al*., 2002). Tannins can affect the growth and development of insects, and can also behave as feeding repellents to a number of animals (Mazid *et al*., 2011). Terpenes are the largest group of secondary products and have a number of functions in plants, which include the formation of oils and resins involved in the defense against other organisms (Mazid *et al*., 2011). It has been shown that individual terpenes behave as insect antifeedants (Meisner *et al*., 1982; Van Beek and De Groot, 1986; Wickham and West, 1992). They have also been found to play a role in antibiosis in sugarcane against the woolly aphid *Ceratavacuna lanigera* Zehntner (Hemiptera: Aphididae) (Hunsigi *et al*., 2006).

Non-protein, nitrogen containing secondary metabolites includes alkaloids, cyanogenic glycosides, polyamines, polyamine phenylpropanoid conjugates (PPCs) and benzoxazinoids (Mazid *et al*., 2011). Dhurrin, a cyanogenic glycoside has been found in sugarcane leaves using HPLC, and it plays a role in constitutive and inducible resistance whereby the activity of IDP-glucose:p-hydroxymandelonitrile-O-glucosyltransferase and dhurrinase enzymes increases when sugarcane is attacked by the stalk borer *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) (De Rosa-Junior *et al*., 2007). It has been found that high concentrations of cyanide correlate with a reduction in feeding by first instar larvae of *C. partellus* in sorghum (Woodhead *et al*., 1980). Arginine is an important amino acid in plants and is involved in defense mechanisms against insects and pathogens. Arginine can be broken down by Jasmonic acid (JA)-induced arginase which in turn has an effect on

the insect's nutrition (Chen *et al*., 2005). Benzoxazinoids occur predominantly in the family Poaceae which includes maize, wheat and sugarcane (Singh *et al*., 2003). Benzoxazinoids concentrations are higher in younger plants and young, exposed tissues of older plants (Thackray *et al*., 1990). Aphids and stalk borers are deterred by benzoxazinoid compounds, which could be due to an anti-feeding effect of the compound and may also result in the inability of insects to detoxify other defense compounds in plants (Klun and Robinson, 1969; Argandona *et al*., 1980; Houseman *et al*., 1992; Barry *et al*., 1994; Ortego *et al*., 1998). Benzoxazinoids are known to be constitutive compounds but can also be synthesized due to an induced response from insect attack (Gutierrez *et al*., 1988; Huang *et al*., 2006; Wang *et al*., 2007).

1.3.5 Protein based defense mechanisms in plants

There are four classes of proteinases in insects, namely, serine, cysteine, aspartic acid proteinases and the metalloproteinases (Falco *et al*., 2001). Serine proteinase is found in Lepidoptera and cysteine proteinase activity in Coleopteran insects (Houseman *et al*., 1992; Gatehouse *et al*., 1985; Murdock *et al*., 1987). Plant proteinase inhibitors (PIs) are found in a number of plants and form part of their natural defence mechanisms against herbivores (Ryan, 1990). The inhibitors are more commonly found in plant parts more prone to attack, such as bulbs, leaves and seeds and may be of constitutive or wound-induced in nature (Falco *et al*., 2001). A number of papers have shown that these proteinase inhibitors have an effect on larval development but do not cause their death (Wolfson and Murdock, 1995). It is thought that the inhibitors have an effect on the digestive system of insects by inhibiting the activity of midgut enzymes therefore resulting in the reduced availability of amino acids required for protein synthesis, which in turn negatively impacts on growth, development, and reproduction of the insect (Falco *et al*., 2001). Alternatively, an indirect effect on insects can be caused whereby there is an increase in production of digestive proteinases to make up for the low levels of available amino acids, and this results in a reduction in amino acids needed for essential proteins (Broadway and Duffey, 1986). It has been shown in artificial diets incorporating soybean proteinases fed to sugarcane borers that a reduction in growth and development occurs in the borers (Falco *et al*., 2001).

Chitinase may also play a protective role against insects in plants by interrupting chitin-containing glycoproteins of the peritrophic matrix, which is a system that guards the gut cells from being damaged by digestive enzymes and microorganisms (Barbehenn, 2001). Lectins are sugar-binding proteins that occur in plants and other organisms, predominantly in legumes, that play a role in the defense mechanisms of the plant (Falco *et al*., 2001). The expression of lectin-like genes in sugarcane has been shown to be specific to certain tissues where expression is lower in the stalk and higher in the leaf roll, apical meristem and lateral buds (Falco *et al*., 2001).

Polyphenol oxidase (PPO) enzymes result in the browning of plant extracts and tissues that have been damaged by herbivory, wounding and JA (Constabel *et al*., 2000; Falco *et al*., 2001). It has been suggested that there is a role for PPOs in defending plants against insects. During feeding of the insect on the plant, o-quinones are produced due to mixing of PPO and phenolics and this leads to the modification of free amino acids and sulfhydryl groups in dietary proteins in the insect's mouth and gut (Falco *et al*., 2001). The formed phenolic decreases the nutritive value of the proteins (Constabel *et al*., 2000). The combination of PPOs with a phenolic substrate in glandular trichomes results in a glue-like substance which traps tiny insects (Falco *et al*., 2001). When PPOs are present in mesophyll tissue they result in proteins being modified and this leads to reduced digestibility of the protein in the insects gut during feeding (Falco *et al*., 2001).

Maize insect resistance cysteine proteinase (Mir1-CP) is a papain-like cysteine proteinase with an amino acid sequence similar to that of the cysteine proteinases from a number of baculoviruses that infect lepidopteran larvae through consumption (Rawlings *et al*., 1992). After ingestion by the insect, Mir1-CP proteolytic activity harms the peritrophic matrix (PM) (Pechan *et al*., 2002). Maize lines showing resistance to fall-armyworm (FAW) and other lepidopteran larvae were conventionally bred from wild germplasm from Antigua (Williams *et al*., 1990; Davis *et al*., 1998). After one hour of attack the maize varieties show a build-up of Mir1-CP in the whorl due to feeding of lepidopteran larvae (Pechan *et al*., 2000). Sugarcane shows cDNAs alike to that of the maize mir1 gene (Falco *et al*., 2001). The gene has been found to be expressed in the sugarcane callus, seeds, the root transition zone, and in the stalk of the plant (Jiang *et al*., 1995). Reports show that FAW resistant maize inbred Mp708 shows three times higher levels of JA before the attack by herbivores

compared to the susceptible maize inbred. It therefore seems that a section of Mp708's defence pathway is primed by JA with small amounts of protein always being present (Shivaji *et al*., 2010). It was shown by Mohan *et al*. (2008) that Mir1-CP had LC_{50} values close to that of *Bacillus thuringiensis* CryIIA toxin, in the range of 0.6 to 8 µg g^{-1}.

1.3.6 Indirect defense mechanisms in plants

Within the last few years another type of defense has been identified, which was first found in maize, and is now referred to as indirect defense. It has now been identified in a number of plant species in which the attack of insect results in the plant giving off complex amounts of volatiles into the atmosphere from their vegetative plant parts and these volatiles are known as herbivore induced plant volatiles (HIPVs). Enemies of these herbivores can be attracted to these HIPVs, and this is known as constitutive indirect defense (Turlings *et al*., 1990; Dicke and Sabelis, 1998; Baldwin *et al*., 2002).

There is a range of HIPVs known to exist in plants, including alkenes, alkanes, and two jasmonates (cis-jasmone and methyl jasmonate), but the main compounds seems to be C6 green leaf volatiles (GLVs), terpenes and products derived from the shikimic acid pathway (Preston *et al*., 2001; Farmer and Ryan, 1990; Holopainen, 2004; Van den Boom *et al*., 2004; Arimura *et al*., 2004; Turlings *et al*., 1998; Ferry *et al*., 2004). The volatiles can work in a number of different ways against herbivores; either attracting predators and parasites of the target herbivores, directly deterring the herbivore, or by priming the healthy plant parts of the plant under attack, or of the neighbouring vulnerable plants so that more efficient defense can take place in future attacks (De Moraes *et al*., 2001; Heil and Silva-Bueno, 2007; Ton *et al*., 2007).

Although indirect defense mechanisms are seen in natural hosts of *E. saccharina*, there have been no known records of sugarcane displaying indirect defense to this stalk borer (Conlong and Hastings, 1984). This could be due to loss of this trait during sugarcane plant breeding where only direct defence is actively selected for (Gouinguene *et al*., 2001; Degen *et al*., 2004). This has been seen in maize whereby a number of North American maize lines do not release (E)-β-caryophyllene in response to attack by *Diabrotica virgifera virgifera* LeConte (Coleoptera:

Chrysomelidae) (western corn rootworm), whilst European lines which do show indirect defense to the rootworm (Kollner *et al*., 2008).

1.3.7 Screening for host-plant resistance

1.3.7.1 Techniques for screening and assessing plants for resistance to stalk borers

Initial host-plant resistance screening studies should take place under controlled conditions in a glasshouse, or in the laboratory to increase precision (Ahman. 2006). Characteristics in the field such as soil, moisture, climate, and variable pest numbers reduce precision of field trials. Field assessments are generally performed at the last two stages of sugarcane selection programmes (Keeping, 2006). Designing experiments where the conditions are optimized for determining differences between sugarcane varieties in terms of their resistances takes into account the background of resistance mechanisms. For assessing both induced and constitutive resistance, bioassays using insects on plants can be used to compare insect numbers, plant symptoms, antibiosis, and antixenosis resistance components (Ahman, 2006).

Numerous methods have been used and explored to distinguish sugarcane varieties for resistance against stalk borers. These include measurement of internode rind hardness, forced penetration of larvae into stalks, ovipositing tests using moths, trials conducted under a controlled environment for artificial infestation of plants, and the incorporation of leaf powders into an artificial diet (Nibouche and Tibere,2010; Goebel and Way, 2009; Vercambre *et al*., 2001).

Black head stage egg masses and neonate larvae of *C. partellus* have been used to artificially infest maize varieties to determine their different resistances or susceptibilities (Kumar, 1997a). In sorghum and maize, the use of a “Bazooka” applicator for inoculating plants with *C. partellus* has been successful (Sharma *et al*., 1992). First instar larvae, together with a carrier, such as poppy seeds or corn cob grits, are transferred into a plastic bottle of the Bazooka and the leaf whorl of plants are infested with a single stroke that releases five to seven larvae (Sharma *et al*., 2008). A camel hair paint brush can also be used to manually deposit larvae onto the plant (Nibouche and Tibere, 2010). Generally, five to seven larvae are enough to

cause a fair amount of damage to the leaves and growing point (i.e. >90% damage) (Padmaja *et al*., 2012). The use of larvae for infestation of plants has been reported to be more effective than egg masses in host-plant resistance screening studies (Kumar, 1995).

A number of different damage parameters have been used in assessing sugarcane varieties for resistance to *Chilo* spp. These include foliar damage (leaf feeding damage), length of the stalk tunnelled, number of entry and exit holes in the stalk, stalk breakage, larval and pupal numbers, and deadhearts (Kumar, 1997b; Sharma *et al*., 2008). Due to the majority of injuries that cause yield loss occurring within the stalk of the plant, most studies on resistance use stalk damage parameters to assess resistance (Nibouche and Tibere, 2009). These damage parameters can be easily used when a small number of sugarcane varieties are being assessed, however, in large breeding programmes, where rapid progress is required, a single parameter such as leaf feeding damage can be used (Kumar, 1997a). Furthermore, *C. sacchariphagus* and *C. partellus* larvae initially feed on the leaves of their host before entering the stalk, resulting in leaf lesions. These leaf lesions give indications of young borer populations on the crop (Nibouche and Tibere, 2009). The use of leaf feeding injuries to assess resistance of sugarcane to *C. sacchariphagus* has been done by Conlong *et al*. (2004). Leaf damage on the whorl stage of maize has also been used to distinguish between susceptible and resistant varieties of maize (Kumar, 1997b).

The stage of the plant to be used in host-plant resistance screening studies must also be considered. Ampofo *et al*. (1986) used plants that were four weeks old for studies on maize resistance to *C. partellus*. However, it has also been found that plants of 2 weeks of age can distinguish between resistant and susceptible sugarcane varieties (Kumar, 1997a). *C. partellus* has been known to attack sorghum plants from two weeks after germination (Kumar *et al*., 2006).

1.3.7.2 The use of artificial diet bioassays in insect resistance screening

Artificial diets are vital in arthropod research (Blanco *et al*., 2009). Incorporating leaf material into an artificial diet can be useful in determining any constitutive resistance mechanisms in different plant varieties (Blanco *et al*., 2009). The use of artificial diets

in resistance screening studies is also useful in comparing sugarcane varieties under uniform conditions where variations from the environment are excluded (Padmaja *et al*., 2012). In order for successful resistance screening studies to take place, a large supply of insects in sufficient numbers is required, as well as a suitable artificial diet for rearing and maintaining insects to be used in resistance screening studies (Songa *et al*., 2001).

There are numerous commercial diets that have been developed to maximize insect growth and development by meeting all the nutritional requirements of the target insect (Blanco *et al*., 2009). If an artificial diet does not meet the nutritional requirements of the insect it can result in a negative effect on the insects' development and reproduction. Artificial diets should contain the correct proportions of nitrogen, lipids, carbohydrates, vitamins and minerals (Cohen, 2004). Carbohydrates have an effect on egg production and survival making them one of the most important components in the diet (Hari *et al*., 2008). Reduced fitness and reproduction has been observed in a number of economically important insects which have been soley raised on artificial diets (Kega *et al*., 2010). This is often referred to as bottleneck stress and can occur when an insect is taken out of its natural environment and has stress imposed to it that would not occur in nature, such as crowding, nutrition, temperature, humidity and lack of feeding choices (Cohen, 2004).

The incorporation of freeze-dried leaf powder into the artificial diet of *C. partellus* was done in an experiment to study the antibiosis resistance mechanism of 20 sorghum varieties (Kumar *et al*., 2006). Williams and Buckley (2008), also conducted a study whereby lyophilized leaf tissue of 20 maize varieties, varying in resistance, were incorporated into an artificial diet to determine their effects on the growth of fall armyworm (*Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)) and the southwestern corn borer (*Diatraea grandiosella* Dyar (Lepidoptera: Crambidae)). Differences in growth of these insects were observed between resistant and susceptible maize varieties incorporated into diets.

1.3.7.3 Surrogate insect resistance screening

Due to quarantine procedures, limitations to conduct host-plant resistance screening on sugarcane varieties to *C. sacchariphagus* occur in South Africa. The concept of 'surrogate insect resistance screening' was explored in this project, whereby *F. serrata* and *C. partellus* were used as surrogates for *C. sacchariphagus* in host-plant resistance studies. The concept of surrogate insect resistance screening is based on the theory that all three of these pests feed on the whorl of the plant, and therefore similar resistance mechanisms within the plant may act against them. *C. partellus* and *C. sacchariphagus* have an additional factor in common because they are both top borers. Among the few South African varieties with known resistance or susceptibility to *C. sacchariphagus*, there may be a correlation between *F. serrata* and *C. sacchariphagus* resistance rankings (Figure 1.9) suggesting that there could be some commonality in resistance mechanisms at least within the leaf whorl. Surrogate insect resistance screening has proven successful within the borer genus *Ostrinia* on maize (Overman, 1994).

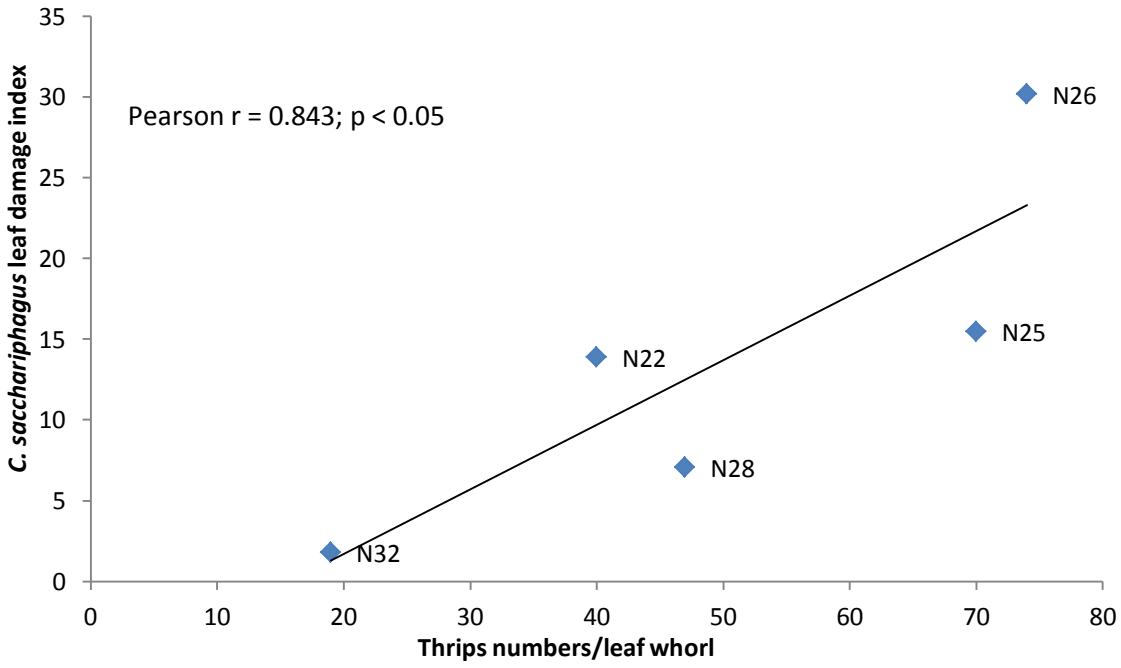

Figure 1.9 Relationship between *Fulmekiola serrata* numbers per leaf whorl (Anon, SASRI, South Africa) and *Chilo sacchariphagus* leaf damage index for five genotypes (for which data is available) (Conlong *et al*., 2004)

1.3.8 Near-infrared reflectance spectroscopy (NIRS) as a rapid screening tool in pest resistance

1.3.8.1 Background information

The analysis of plant constituents (e.g. proteins, carbohydrates, and lipids) is an integral part of numerous agricultural studies. However, chemical analyses are time consuming and expensive (Purcell *et al*., 2009). In plant breeding trials, large numbers may be required for analysis. This can lead to the analyst being forced to bulk samples in order for a sufficient amount to be obtained, and this in turn results in the accuracy of the experimental design being compromised (Foley *et al*., 1998). The use of NIRS has proven to be useful to overcome such issues. NIRS makes use of optical data, and is based on the reflectance from a sample in relation to the amount of radiation hitting it (Newgard, 2004). NIRS is associated with the absorption of electromagnetic radiation in the wavelength region from 750 to 2500 nm, next to the mid-infrared region and up to the visible region (Osborne, 1983; Workman and Shenk, 2004). The types of bonds that occur between atoms in plant tissues reflect the composition of the tissue, and spectroscopy can be used to determine information about the bonds between the atoms or groups of atoms (functional groups) (André and Lawler, 2003). The exposure of a sample to irradiation results in vibrations between the bonds which in turns results in stretching and bending. This results in a wave motion occurring in the bond at a frequency specific to the functional group. Absorption occurs of the incident light whose frequency matches that of the vibrations of the waves, and reflection or transmittance occurs of those frequencies that do not match. Vibrations of C-H, -O-H, S-H, and N-H bonds predominantly absorb NIR (Reich, 2005). These bonds are major constituents of organic compounds in plant tissues (André and Lawler, 2003). The type and number of bonds in the tissues are determined by the chemical make-up of the tissue and hence it is the chemical constituents in the tissues that determine the wavelengths and the amount of light that is absorbed (Foley *et al*., 1998). It is therefore the light that is reflected from a sample that gives information on the chemical composition of that specific sample.

NIRS has a number of advantages in that it provides a rapid and accurate analysis of materials, is non-destructive to samples being analysed, does not require expensive

and hazardous chemicals, is able to analyse a large number of nonhomogeneous samples, and a number of components in each sample can be seen in its spectrum from a single measurement, which in turn also reduces costs (Reich, 2005; Roggo *et al*., 2007; Mark and Campbell, 2008). However, there are also accompanying disadvantages with using NIRS. The initial cost of the instrumentation is large, and it is also a necessity to calibrate the instrument for each sample component to be analysed (Osborne, 1983; Workman and Shenk, 2004).

NIRS has been used by a number of industries including the oil, pharmaceutical, and agricultural industries. The use of NIRS is becoming increasingly popular and has become a routine method for analyses in many research fields (Foley *et al*., 1998). In agriculture, NIRS has a number of uses, which includes that of measuring grain quality, moisture content, and oil and protein contents (Throne *et al*., 2003). Additionally, NIRS has also been used to measure the composition of plants that are resistant to insects and pathogens (Andre and Lawler, 2003). The many advantages of NIRS, along with incremental advancements in the technology, have led to an increased use of NIRS in agriculture over other traditional methods. The majority of NIRS instruments that are being used are laboratory instruments which are kept in a controlled environment. However, a number of handheld NIRS instruments are also being used, mainly for measuring nitrogen contents of leaves to assist in optimizing fertilizer applications to crops. Portable NIRS instruments are also available that can scan the entire NIR spectrum. These specific instruments make use of fibre optic probes (Foley *et al*., 1998).

Overtones and amalgamations occur in the NIR spectrum due to scattering of light and the occurrence of absorptions in the mid-infrared region (Barton, 2002). This makes the direct interpretation of the NIR spectrum difficult because there are only a few areas of absorbance that are due to only one functional group (Reich, 2005). Thus statistical models are needed to confirm the intensity of the relationship between a specific absorbance and a laboratory assay of a specific constituent in a number of different tissues for the sample of interest. Near infrared spectroscopy (NIRS) is therefore a secondary method used, whereby chemical composition is determined by comparisons of the spectra with samples whose composition has been determined using laboratory methods (Figure 1.10) (Foley *et al*., 1998).

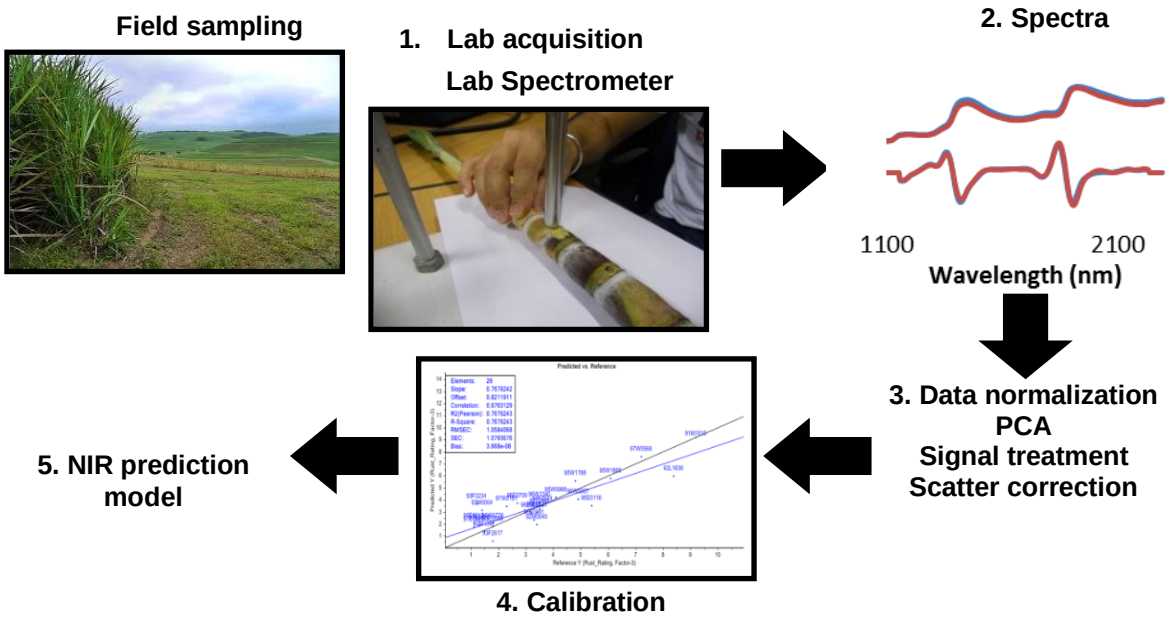

Figure 1.10 Steps involved in building near infrared models for predicting for components of interest such as *Eldana saccharina* resistance in sugarcane

1.3.8.2 Calibration and validation

Calibration is a process of developing models that relate spectral readings of a sample set to reference values which have been determined by wet chemistry or conventional means (Chen *et al*., 2002). Reference values may be that of measured nitrogen or protein concentrations, carbohydrates, grain yield, digestibility and intake of food by herbivores, susceptibility or resistance to insect attack, and others (Foley *et al*., 1998). It involves selecting representative samples and acquiring spectra using a spectrometer (Chen *et al*., 2002). Once the calibration has been built, it is important to test its quality, and this is done by a process called validation. Validation tests the ability of the calibration equation to predict a different sample set or an entire population of samples (Naes *et al*., 2002). Different types of validation methods can be used. An independent sample set, separate to the calibration data set can be used, or the same calibration data set can be used as is done in cross-validation (Foley *et al*., 1998). If the calibration model is accurate in predicting reference values

then it can ultimately be used to estimate the composition of samples for which they are not known (André and Lawler, 2003).

1.3.8.3 Chemometrics

Chemometrics is the name given to mathematical and statistical methods which are used to remove the complexity from spectral data and to obtain useful information from the data (Jørgensen, 2000). Chemometric methods used can be one of a number of different multivariate regression procedures, such as multiple linear regression (MLR), principal components regression (PCR), and partial least squares (PLS) regression (Foley *et al*., 1998). The use of such procedures allows one to see an overall view of the data, whereby major differences between groups and correlations can be evaluated (Wold, 1991). Pre-treatments and transformation of spectral data is used to reduce the effect of particle size, scattering and other factors on the NIR spectra. Transformation techniques include calculating first and second derivatives, de-trending, and standard normal variate (SNV) transformation (Jørgensen, 2000).

1.3.8.4 Evaluation of statistics from near infrared calibrations and validations

Evaluation of statistics from the calibrations and validations developed using NIRS is an important step to determine the accuracy and efficiency of a calibration model. Common statistics used are the standard deviation (SD), standard error of calibration (SEC), standard error of prediction (SEP), the coefficient of determination (R^2), slope of the regression equation, root mean square error of calibration (RMSEC), standard error of cross validation (SECV), root mean square error of cross validation (RMSECV), residual predictive deviation (RPD), standard error of prediction (SEP), and the root mean square error of prediction (RMSEP) (Dardenne, 2010; Fassio *et al*.,2007). The equations and interpretations of these statistics can be read in a number of textbooks (Naes *et al*., 2002).

1.3.8.5 Use of NIRS to predict for pest and disease resistance in plants

Plant constituents in different plant parts are often analysed using spectroscopic techniques. Investigation of new techniques and applications of these techniques is constantly underway. For example, plant breeders are developing NIRS calibrations that can assist in developing improved plant varieties in a shorter time frame (Purcell *et al*., 2009). Improved varieties of sugarcane are vital in protecting the plant against disease and insects, and are also important in producing higher yields of biomass and sucrose per hectare. The conventional development of new varieties can take up to 10 years, and is a resource and labour intensive procedure (Purcell *et al*., 2010).

Fiji leaf gall (FLG) is caused by infection by the Fiji disease virus (FDV) and is a serious disease in sugarcane in Australia that is transmitted by the sugarcane planthopper *Perkinsiella saccharicida* Kirkaldy (Hemiptera: Delphacidae) (Purcell *et al*., 2009). The main means of control is the use of resistant varieties and by using clean, disease free planting material. There is difficulty in rating sugarcane clones for resistance to the disease because infection rates cannot be controlled in field trials. Using glasshouses to perform ratings have also been shown to be unreliable because there are no correlations between the glasshouse results and field trial results. Therefore, as with many other diseases, the development and use of a rapid and in-field technique to detect resistance would be highly beneficial in sugarcane plant breeding programmes. In a paper published by Purcell *et al.* (2005), a number of benefits are listed with the use of NIRS as an early resistance screening method. These include earlier screening of varieties for the virus which will have a positive effect on the number of clones being brought forward to later stages in the selection programme, earlier detection of susceptible clones, a reduction in the need for field trials, which will in turn allow for better resource management, and generation of reliable data that can be used in future research projects.

As with the case of FDV in Australia, *E. saccharina* poses as a serious threat to the sugarcane industry in South Africa (Rutherford and Van Staden, 1996). The use and effectiveness of field trials is also limited because there are low numbers of the borer when the rainfall is high in some years. Thus a quick, accurate method is desired in

the early selection stages of a plant breeding programme. Larvae that have just hatched usually take approximately one week before physically boring into the stalk. Thus, the differences in survival and behaviour of larvae on the stalks of sugarcane varieties could be explained based on the biochemical effects on the surface of the stalk (Rutherford and Van Staden, 1996). Chromatography is usually used to differentiate between components involved in resistance. However chromatographic methods are not practical when it comes to screening a large number of samples, which is the case in early stages of a selection programme for sugarcane. Rutherford and Van Staden (1996) were able to come up with a NIR method to predict *E. saccharina* resistance by using a stepwise linear multiple regression model based on wax analysis. Using this information, Purcell *et al*. (2003) looked at the surface wax of sugarcane leaves using gas chromatography (GC) and spectroscopic methods, and thereafter, they were able to distinguish between sugarcane plant components using chemometric data treatment. Meyer (1997) reviewed a number of papers that suggest that flavonoids in the stalk bud scales and surface wax of the stalk could account for 55% of the variation in resistance among 30 NIRS analysed clones. Rutherford and Van Staden (1996) also deduced that wavelengths selected in multiple regression models indicate that alcohols and carbonyls contributed significantly to the wax component of insect resistance.

Near infrared spectroscopy (NIRS) has also been applied to smut (*Sporisorium scitamineum*) resistance in sugarcane, whereby on-site screening based on NIRS was investigated by Purcell *et al*. (2010). Smut is a disease which results in severe stunting of plants, which in turn leads to significant decreases in yields. In an experiment conducted by Purcell *et al*. (2010), 31 sugarcane samples were used for a validation trial. NIRS was used to obtain the spectra from stalk bud tissue which were then pre-treated and analysed using chemometrics. The smut ratings based on NIR were compared to ratings from field trials. The results obtained were promising and showed good potential for using NIRS as an early screening method for resistance in sugarcane to smut (Purcell *et al*., 2010).

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CHAPTER TWO

CHILO PARTELLUS (SWINHOE) (LEPIDOPTERA: CRAMBIDAE) COLONY ESTABLISHMENT, MAINTENANCE, AND OPTIMIZATION OF AN ARTIFICIAL DIET FOR USE IN CONSTITUTIVE RESISTANCE SCREENING BIOASSAYS

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Abstract

Chilo partellus and *Chilo sacchariphagus* are two stem borers that threaten the South African sugar industry at present. Sugarcane agro-ecosystems in KwaZulu-Natal were surveyed for *C. partellus*, and species confirmation took place using cytochrome oxidase I subunit (CO1) barcoding. A reliable supply of good quality insects is essential for host-plant resistant studies. The techniques used at the South African Sugar Research Institute (SASRI) for establishing and maintaining *C. partellus* colonies were developed. Artificial diets are developed to optimize insect growth and reproduction, and have to meet or exceed the nutritional requirements of target insects. Experiments were conducted to test different diets, with the incorporation of various ingredients and using different inoculation and rearing methods. Vials that were inoculated with two neonate larvae each gave higher mean larval weights and larval survival percentages than multicell trays and plastic jars. An improved artificial diet for rearing *C. partellus* was established; containing an

increased proportion of cane leaf powder (6.5% m/v), and incorporating non-fat milk powder (2.35% m/v) and whole egg powder (1.75% m/v). This diet gave higher mean larval survival percentages and mean larval weights than other diets tested. Because of an increased content of cane leaf powder (from 2.5% to 6.5% m/v), better discrimination between leaf powders from different sugarcane genotypes should become possible.

Keywords: *Chilo partellus*; *Chilo sacchariphagus*; host-plant resistance; cytochrome oxidase I subunit barcoding; sugarcane agro-ecosystem; artificial diet; constitutive resistance

2.1 Introduction

Chilo partellus (Swinhoe) (Lepidoptera: Crambidae) is a serious pest in Asia and southern African countries (Arabjafari and Jalali, 2007). The main host plants of *C. partellus* include crops such as maize (*Zea mays* L.), sorghum (*Sorghum bicolor L. Moench*), rice (*Oryza sativa* L.) and pearl millet (*Pennisetum glaucum*) (Sallam and Allsop, 2002). *C. partellus* can infest the crop from seedling stage up until maturity and affects the entire plant except for the roots. *C. partellus* has adapted to sugarcane in North Africa (Assefa and Conlong, 2009), and is present in the South African sugarcane agro-ecosystem (Hutchison *et al*., 2008). It may represent a threat similar to the one once posed by *Eldana saccharina* Walker (Lepidoptera: Pyralidae) before it adapted to feeding on sugarcane after which it became a major pest (Rutherford, personal communication).The fast spread of *C. partellus* from Malawi, and the associated crop losses and damage due to this borer prove how serious this pest can be if left uncontrolled (Conlong and Goebel, 2002). *Chilo sacchariphagus* Bojer originated in south East Asia and has been a serious insect pest of sugarcane since the 19th century in Reunion, Mauritius and Madagascar (Rochat *et al*., 2001; Conlong and Goebel, 2002). The potential for an invasion by *C. sacchariphagus* from Mozambique into South Africa poses a great risk to the South African sugarcane industry (Goebel, 2006; Bezuidenhout *et al*., 2008).

The use of host-plant resistance is one of the main methods of control for harmful borers such as *C. partellus* and *C. sacchariphagus* (Songa *et al*., 2001). The behavior of the borers is unpredictable and their numbers vary with changing seasons. As a result, field trials depending upon natural infestations of the borers are

unreliable for resistance screening studies. Artificial rearing of the target pests is therefore required. Incorporating leaf material of different sugarcane varieties into the artificial diet of insects can be used to test for the presence of constitutive antibiosis resistance mechanisms in different sugarcane varieties, which can be useful in arthropod research (Blanco *et al*., 2009). In order for successful resistance screening studies to take place, a large supply of insects in sufficient numbers is required, which requires a suitable artificial diet for rearing and maintaining insects to be used in resistance screening studies (Songa *et al*., 2001). Thus, colonies need to be established in a controlled environment. The success of the research depends on the supply of the insect in sufficient numbers and at the correct stages of its life cycle (Songa *et al*., 2001). Mass rearing can be defined as “the production of insects in numbers per generation exceeding 10 thousand to 1 million times the mean productivity of the native female population” (Taneja and Nwanze, 1990). The maintenance of the colony involves periodically incorporating wild populations of the species into the laboratory colony. This is done in order to preserve and maintain the heterozygosity in the pest population and to avoid deviation from the natural pest behaviour (Onyango *et al*., 1994). A suitable rearing facility is required in order to establish colonies successfully. Requirements in the facility are sufficient laboratory space, equipment, diet components, trained staff and different rooms are required to perform specific functions in rearing insect colonies. The rooms where larvae and moths are kept should have conditions (temperature, humidity and light) suitable to those of the insect and representative to conditions in the field (Tende *et al*., 2010).

There have been various artificial diets used for rearing *C. partellus*, and that have been adapted and improved over time (Figure 2.1) (Taneja and Nwanze, 1990). Generally, artificial diets of insects should contain portions of nitrogen, lipids, carbohydrates, vitamins and minerals (Cohen, 2004). A list of the most important diet components and their functions in insects is shown in Table 2.1 (Cohen, 2004). It is important that the components in an insect’s diet be available in sufficient amounts, or else the insect will feed at a slower rate and less efficiently. In a number of cases, where economically important pests have been reared solely on artificial diet, reduction in fitness and reproduction has occurred, which in turn leads to slower developmental times and reduced fecundity (Kega *et al*., 2010). This is often referred to as bottleneck stress, which occurs when insects are taken out of their natural

environment and have factors imposed on them which did not occur in nature. Stresses that can impose themselves to insects in a rearing environment include crowding, nutrition, temperature, humidity, antimicrobial agents and lack of feeding choices (Cohen, 2004).

C. sacchariphagus is not yet found in South Africa and therefore cannot be used to carry out studies in this country, unless under quarantine. The concept of 'surrogate insect resistance screening' will be explored, whereby *C. partellus* could be used as a potential surrogate for *C. sacchariphagus* in host-plant resistance studies. The concept is based on the fact that both these pests feed on the whorl of the plant, and both are top borers, and therefore similar resistance mechanisms within the plant may act against them equally. The concept of 'surrogate insect resistance screening' has proven successful within the borer genus *Ostrinia* on maize (Overman, 1994). Cytochrome oxidase I subunit (CO1) barcoding allows for the identification of organisms by looking at the similarity of their DNA sequence to a set of reference taxa (Habeeb and Sanjayan, 2011). It allows for the discrimination of closely related species of lepidopterans and can therefore be used to select which target insects can be best used as surrogate insects in resistance studies (Herbert *et al*., 2003).

The objectives of this chapter were to (a) survey the sugarcane agro-ecosystem in KwaZulu-Natal for the presence of *C. partellus* and to identify its various hosts; (b) discriminate between borers using cytochrome oxidase I subunit barcoding for the selection of target insects for use as surrogate insects in resistance studies (c) describe rearing methods used for establishing and maintaining *C. partellus* at the South African Sugar Research Institute (SASRI); and (d) establish a new artificial diet, to carry out constitutive resistance studies on sugarcane varieties, with an increased proportion of cane leaf powder, without having a detrimental effect on the nutrient composition within the diet. Inoculation and rearing methods were also compared in order to establish which method would have the least negative impact on larval survival and growth.

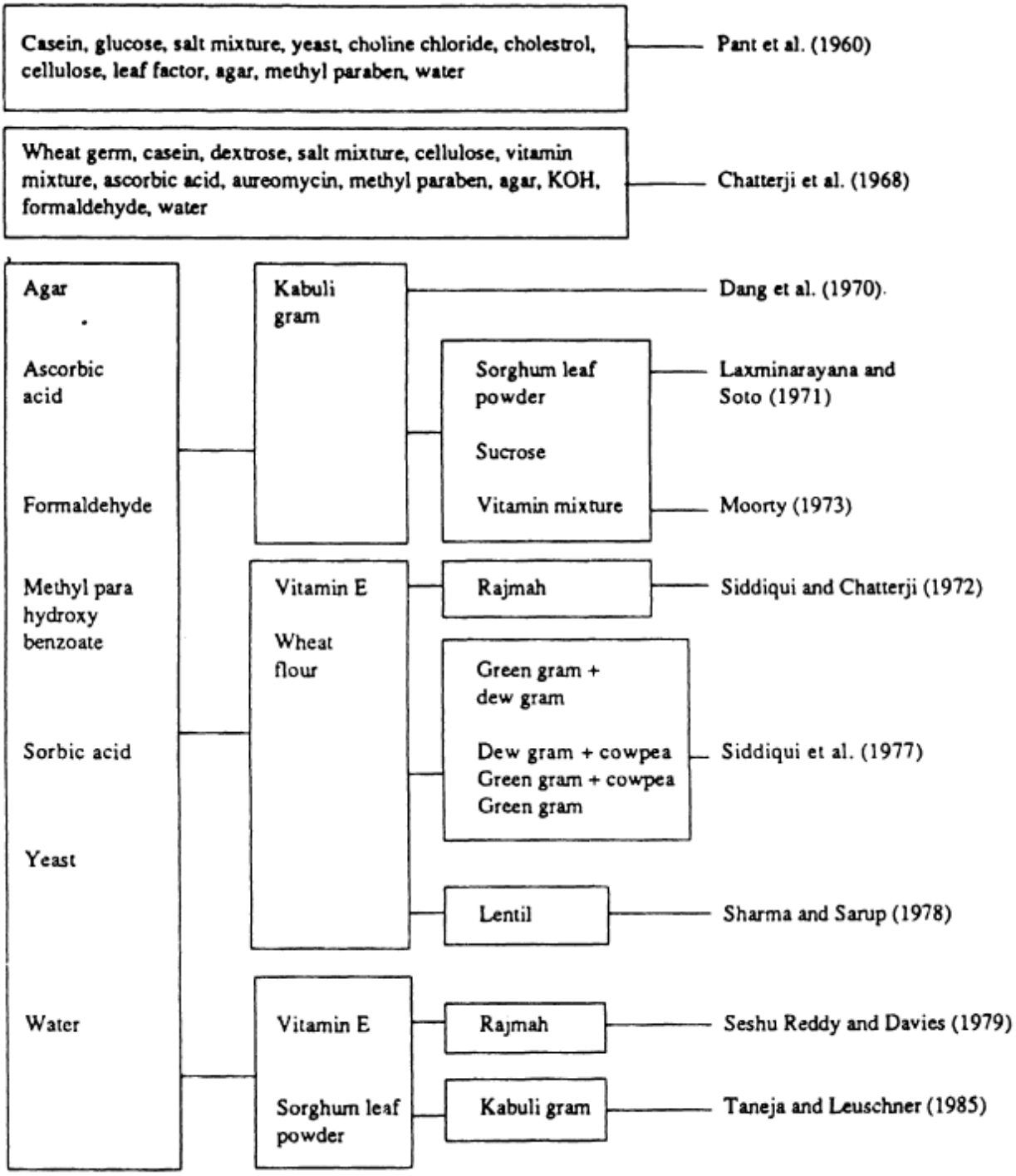


Figure 2.1 Diets used for mass rearing *Chilo partellus* (Taneja and Nwanze, 1990)

Table 2.1 Important diet components and their functions in insects (Cohen, 2004)

Diet Component	Function
Proteins	Source of Nitrogen. Broken down into amino acids which are then used to build proteins for use in the insects body (Muscles, cell membranes, enzymes, etc.)
Lipids (sterols, oils, fats, phospholipids)	Building cell membranes, hormones, transport of nutrients, energy source, structural material for making other molecules.
Carbohydrates	Building material and energy source.
Water soluble vitamins (Vitamin B and C)	Vitamin B is used as a co-factor in metabolism, or as a growth factor. Vitamin C is a phagostimulant, antioxidant, cuticle sclerotization, and possibly for defense.
Lipid-soluble vitamins	Vitamin A is important for eye pigment formation and other pigments needed for growth. Antioxidants and form part of membranes and vacoules. Vitamin E has an effect of fertility/fecundity, is an antioxidant, and may also have other functions.
Minerals	Various minerals have different functions. E.g. Potassium is involved in some chemical reactions, and is found in a number of structures. Important minerals include potassium, phosphorous, magnesium, iron, and selenium.
Feeding stimulants	Stimualte the feeding process. Includes gamma amino butyric acid, sinigrin, waxes, and plant secondary compounds.
Protective agents	Prevents contamination of microbials, oxidation, and nutrient distruction.
Nutritionally inert ingredients	Used to provide texture by use of fillers. E.g. cellulose used as a powder, girt, or flakes. May also be included for bulking the diet or to carry other substances. May also include wheat germ, soy flour, bean meals, or other material from plants.
Water	Required for all life processes.
Emulsifiers	Stabilizers which allows lipid- and aqueous-phase substances to mix and interact for long times.
Gelling agents and stabilizers	Have a number of functions such as making a high water content diet solid,keeps the diet components mixed, and prevents reactions between ingredients from occuring.

2.2 Materials and methods

2.2.1 Surveying the sugarcane agro-ecosystem for *Chilo partellus*

Field collections of putative *C. partellus* were carried out from infested host plants at three different sites. The sites visited were Sezela, Mount Edgecombe and Tinley Manor (all based in KwaZulu-Natal, South Africa). The stalks and leaves of the host plants were examined for borer damage symptoms and the presence of larvae. Stalks were dissected longitudinally using a cane knife (SASRI Technical Services) to recover the larvae. Recovered larvae were immediately placed in 25 ml sized plastic vials (Lasec, Durban, South Africa) containing 8 ml of the standard artificial

diet adapted from Onyango and Ochieng'-Odero (1994) (Table 2.2). Host plants found to be infested were observed and recorded. Two larvae from each site were stored in empty plastic vials containing ethanol to be used for species identification purposes. The rest of the larvae collected were left to feed on the diet and were to be used in colony establishment.

Table 2.2 Artificial diet prepared in vials for mass rearing of *Chilo partellus* at SASRI, Mount Edgecombe (adapted from Onyango and Ochieng'-Odero (1994), by replacing maize whorl powder with cane leaf whorl powder)

Fraction	Ingredients	Quantity for 1L
A	Distilled water (ml)	400
	Brewer's yeast (g)	23
	Sorbic acid (g)	0.6
	Nipagin (g)	1.5
	Ascorbic acid (g)	2.5
	Vitamin E (cold water soluble) (g)	2
	Cane leaf whorl powder (g)	25
	Chickpea flour (g)	90
	Sucrose (g)	35
B	Agar powder (g)	12.5
	Distilled water (ml)	400
C	Formaldehyde (40%) (ml)	3

2.2.2 *Chilo partellus* identification

The objectives were to show that the larva collected from the field were larvae of *C. partellus.*

2.2.2.1 DNA extraction and quantification

DNA from two larvae collected from each site (a total of six larvae) was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. DNA quantification was performed using a NanoDrop® spectrophotometer (ND-1000) (Thermo Fisher Scientific, Wilmington, USA).

2.2.2.2 PCR amplification

PCR amplification was carried out using the Kapa Robust 2G Kit (Kapa Biosystems, Massachusetts, USA) components as per manufacturer's instructions. The positive control used included *E. saccharina* DNA (SASRI insect rearing unit, Mount Edgecombe, South Africa), and the negative control had no DNA. Three replications were performed for the larva from each site. Primers used for PCR amplification of the mitochondrial cytochrome oxidase I subunit (COI) were COI – 5' F GGTCAACAAATCATAAAGATATTGG and COI – 5' R TAAACTTCAGGGTGACCAAAAAATCA (Former *et al*., 1994). The components were made up into a 30 µl reaction mixture with a final concentration of 0.5 µM for each primer, 0.2 mM of dNTP mix, 0.75 U of KAPA2G Robust HotStart DNA polymerase, 1 x KAPAEnhancer 1, 30 ng of extracted DNA and 1 x KAPA2G Buffer A. PCR amplification was performed in a Bio-Rad MyCycler^{TM} thermal cycler (Bio-Rad Laboratories Inc., USA) and the PCR parameters were as follows: 1 cycle of initial denaturation at 94^{o}C for 2 min, 38 cycles of 94^{o}C for 30 sec, 55^{o}C for 50 sec, 72^{o}C for 1 min 30 sec, and a final extension step of 72^{o}C for 10 min.

2.2.2.3 Gel electrophoresis

Gel electrophoresis was performed in order to ensure the presence of DNA and to estimate the DNA fragment size. A 1.2% agarose gel was prepared by dissolving 0.6 g of agarose gel in 50 ml running buffer (45 mM Tris, 45mM boric acid, 1mM EDTA; pH 8.0) which was made up of 200 ml 5 x TBE and 1800 ml distilled water. The solution was then poured into a 50 ml casting tray inserted with a comb, forming 15 wells, which was allowed to set. Once the gel was set, it was placed into an electrophoresis chamber filled with 1 x TBE running buffer. The molecular solutions were made up using 1.5 µl loading dye and GelRed mix (10:1 ratio) and 5 µl of the amplified DNA sample. The solute ions were then carefully dispensed into individual wells in the gel material and a potential difference of 70 V cm^{-1} was applied to create an electric field. DNA fragments were observed using short wavelength UV light (320 nm) beside a molecular weight marker (GeneRuler^{TM} 100bp DNA Ladder Plus (Fermentas Life Sciences)).

2.2.2.4 PCR purification

The remaining 25 µl of DNA sample was used for PCR purification which was carried out using the Promega Wizard SV Gel and PCR Clean-up System (Promega, Madison, USA) as per manufacturer's instructions. DNA quantification was performed using a NanoDrop® spectrophotometer (ND-1000).

2.2.2.5 Sequencing

Purified PCR product was sequenced according to the method used by Platt *et al*. (2007), using the BigDye Terminator v3.1 Cycle Sequencing Kit components (Applied Biosystems, California, USA) and analysed using an ABI PRISM 310 genetic analyser (Applied Biosystems, USA).

2.2.2.6 Sequence analysis and phylogeny

Resulting nucleotide sequences were submitted to the BOLD (Barcode of Life Data) program (Ratnasingham and Hebert, 2007) for initial identification. Sequences were analysed and aligned using Geneious^{TM} 5.5.6 software (Kearse *et al*., 2012). Sequences were then moved into BioEdit sequence alignment editor (Hall, 1999). Alignments were constructed using MAFFT (a multiple sequence alignment program) (Katoh and Standley, 2013). The evolutionary history of the taxa analysed was represented using the bootstrap consensus tree using 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentages of replicate trees in which the related taxa are clustered together in the bootstrap test were displayed next to the branches (Felsenstein, 1985). The tree was constructed to scale, where the lengths of branches were in the same units as those of evolutionary distances used to build the phylogeny tree. The Jukes-Cantor method (Jukes and Cantor, 1969) was used to compute evolutionary distances, and units were in the number of base substitutions per site. Pairwise deletion was used, where all positions having alignment gaps and missing data were eliminated in pairwise sequence comparisons. A total of 537

positions were present in the final dataset. Phylogenies were built using Mega4 (Molecular Evolutionary Genetic Analysis) software (Tamura *et al*., 2007).

2.2.3 *Chilo partellus* colony establishment and rearing

2.2.3.1 Rearing facilities and equipment

C. partellus larvae were reared according to the protocol used by SASRI, and are described below. The rearing facility for *C. partellus* at SASRI consisted of six rooms which were used for various rearing activities. These were the preparation room, the larval growth room, the parasite assessment room, the pupal development and adult emergence room, and lastly the gestation room.

Larval and pupal development took place in rearing rooms maintained at 27 +/- 2^{o}C, 65 +/- 5% and an 8 hour light: 16 hour dark photophase provided by 65 watt colour 19 Triphos fluorescent tubes. Lighting was supplemented by natural lighting through the windows. Emerged adults, mating, completion of their gestation period and egg-laying took place in rearing rooms maintained at 27 +/- 2^{o}C, 70 +/- 5% and an 8 hour light: 16 hour dark photophase provided by 65 watt colour Triphos fluorescent tubes. Light was also supplemented by natural lighting from windows. Inoculation of neonate larvae was done in rooms maintained at human comfort conditions (22 +/- 2^{o}C, ambient humidity). Light was provided by fluorescent tubes (colour 21 or cool white; nm spectrum; 400-500 lux).

2.2.3.2 Diet preparation and pouring

Collected larvae were reared and maintained on the diet shown in Table 2.2. The method for diet preparation was as follows: The Brewer's yeast (NCP, Modderfontein, Johannesburg, South Africa), sorbic acid (Sigma Life Sciences, United States), ascorbic acid (Warren Chem Specialities, Cape Town, South Africa), vitamin E (Polychem Supplies cc, Congella), ground chickpea (S.E. Mart, Durban, South Africa), sucrose (Huletts, Durban, South Africa), and crushed cane tops and leaves (SASRI, KwaZulu-Natal, South Africa) from Fraction A were weighed and mixed together thoroughly. The sample of nipagin (Sharon Laboratories, Israel) was

dissolved in the ethanol from Fraction A. The distilled water from Fraction A was autoclaved and then allowed to cool to 60-70^{o}C. It was then added to the dry ingredients from Fraction A, together with the dissolved nipagin, and mixed together for 3 minutes in the blender (6.7 litre bowl capacity) (Kenwood, United Kingdom). The agar powder (Polychem supplies cc, Congella) and distilled water in Fraction B were mixed together, autoclaved, and allowed to cool to 60-70^{o}C. Fraction A and Fraction B ingredients were mixed together, after which the 40% formaldehyde volume (Merck (PTY) Ltd., Johannesburg, South Africa) from Fraction C was added, and mixing in the blender took place for a further 3 minutes. Eight millilitres of diet was dispensed into 25 ml sized vials (Lasec, Durban, South Africa) and placed open on the laminar bench where they were exposed to UV light for 1 hour.

2.2.3.3 Diet inoculations

The surface of the diet in each vial was punctured once with the end of a sterilized plastic rod to facilitate penetration of larva. Two active neonate larvae were transferred into a single 25 ml vial using a clean, sterilized paintbrush (Winsor and Newton, London, UK). Each vial was sealed with a ventilated lid. The vials and ventilated lids were sterilized in a 4.3% solution of Jik (Protea Chemicals, Johannesburg, South Africa) and water to prevent contamination.

2.2.3.4 Larval growth

The inoculated vials were transferred into the larval growth rooms, and at 25-27 days after inoculation, they were screened for the presence of pupae. The pupae were placed into multicell trays (Interpac, Woodland, California) and sealed with clingwrap (Multiwrap (PTY) Ltd., Wadeville, South Africa) using a wrapping machine. Each cell was aerated by checking that the machine punched a hole through the plastic wrap.

2.2.3.5 Adult emergence and ovipositing

The pupae in the multicell trays were transferred to the adult emergence and ovipositing room for adult emergence to take place. Adults were collected daily, and a maximum of 20 males and females were placed into a 30 cm x 30 cm x 30 cm Perspex box with sleeves (Perspex from Maizey Plastics, Johannesburg, South Africa and assembled at SASRI), with a stocking rate of 1 female: 1 male always being maintained. Wax paper (Masscash (PTY) Ltd., Johannesburg, South Africa) ovipositing substrates were placed in each box together with a 250 ml container with fresh water and four 0.375" x 1-1/2" dental wicks (Shanghai Ristea Industries Co. Ltd., Shanghai, China) for the adults to drink from. The wax paper ovipositing substrates were folded into a number of pleats to form an appropriate substrate for ovipositing to take place. Eggs were collected on a daily basis and placed into plastic tubing which was sealed with a heat sealer. The sealed bags containing the ovipositing substrates were labelled with the date. Eggs were then placed in an incubator (TriLab, Pinetown, South Africa) maintained at 24^{o}C. Perspex boxes were discarded after five days.

2.2.3.6 Egg surface sterilization

Surface sterilization took place of eggs that were five days or older (not hatching). This was done by removing the eggs from the substrate and placing them onto a Petri dish (Concorde Plastic (PTY) Ltd., Johannesburg, South Africa) with autoclaved Whatman No. 1 filter paper (Schleicher & Schuell, Whatman International Ltd, Maidstone, England). Under the fume cupboard, a solution of 15 ml formaldehyde and 85 ml distilled water was made up. This 5.25% formaldehyde solution was then added to the eggs in the Petri dish and left for 15 minutes, after which the formaldehyde solution was carefully poured off. The eggs were then rinsed twice with distilled water for 30 seconds each time. Eggs were finally transferred onto a clean piece of autoclaved damp Whatman No. 1 filter paper in a Petri dish and placed back into the incubator until hatching took place for inoculations.

2.2.4 Colony maintenance

In order to maintain the established colonies, *C. partellus* borers were occasionally collected from their hosts in the agro-ecosystems surrounding sugarcane fields. The hosts were dissected and the larva or pupa extracted from the plant material. The collected insects were reared in isolation for their first generation to prevent any microbial contamination of the existing colony.

2.2.5 Optimizing the artificial diet of *Chilo partellus*

Eight separate experiments were conducted to compare different diets (Tables 2.3-2.5), where all of the diets were prepared and poured using the same method established at SASRI, which are described above in Section 2.3.3.2, except where specified. Ingredients within the diets were adjusted accordingly to maintain a constant level of nitrogen, potassium and phosphorous in all the diets for comparative purposes and to prevent a negative impact on the insect's growth and survival. The incorporation of egg powder (Sunspray Food Ingredients (PTY) Ltd., Johannesburg, South Africa) and non-fat milk powder (Lactalis Ingredients, Bourgbarre, France) into the diets played an important role in maintaining the correct proportion of nutrients within the diet. The control (represented as Diet One) used in each experiment (with the exception of Experiment Two) was the standard diet described by Onyango and Ochieng'-Odero (1994), where the variety NCo376 was sourced for cane leaf whorl powder

Diets made in 25 ml sized plastic vials are shown in Table 2.3. Approximately 8 ml of diet was dispensed into the plastic vials. The diet surface was punctured using the back end of a sterilized rod to facilitate larval penetration. Each diet was made up of three replications of 20 vials each, arranged in a completely randomized design. Two neonate *C. partellus* larvae were introduced into each vial using a fine tipped, sterilized paintbrush (Winsor and Newton, London, UK), after which each vial was closed using a gauzed ventilated lid to prevent larvae from escaping and to prevent contamination. Vials were placed into open plastic containers (Lasec, Durban, South Africa) for each diet, and left in the rearing facility for 27 days (i.e. just before pupation) (Figure 2.2a). In Experiments Two and Three, vials were placed upside

down in the plastic containers to further reduce the death of larvae as a result of their movement away from the diet. *C. partellus* larvae are known to be positively phototropic and negatively geotropic (Scheltes, 1978).

Plastic jars with gauzed lids (Lasec, Durban, South Africa) of 500 ml capacity were used for Experiments Four and Five(Table 2.4). Approximately 150 ml of diet was dispensed into each console jar, and the surface of the diet was punctured ten times using the back end of a sterilized rod. Five replications were used for Experiments Fourand 10 replications were used for Experiment Five, where each replication consisted of one jar. Inoculations were performed differently to that of the vials and multicell trays, where neonate larvae were used. In Experiment Four a, 50 sterilized *C. partellus* eggs in the black-head stage were placed onto the surface of Whatman No. 1 filter paper (Schleicher & Schuell, Whatman International Ltd, Maidstone, England) (Figure 2.2b). The filter paper was carefully placed onto the inside of the console jar lid, and jars were then closed and kept upside down to ensure that emerging larvae would move upwards toward the diet (Figure 2.2c). The same was repeated for Experiment Four b, except 100 sterilized eggs were used. For Experiment Five, 100 sterilized and non -sterilized eggs each were used for Diet One and Two, respectively. Jars were kept in the rearing facility for 27 days. Jars were observed on a daily basis, and once larvae had been seen to emerge, jars were turned upright to prevent the diet from eventual collapse.

Table 2.5 shows diets used for experiments conducted using multicell trays (Interpac, Woodland, California). Each tray consisted of 32, 10 ml sized cells, into which 8 ml of diet was dispensed per cell. The diet was punctured using the back end of a sterilized rod. Three replications were used for each diet, with each replication consisting of one multicell tray. Inoculations were performed the same way as that for the vials, except with one neonate larva inoculated per cell. Cells were generously covered with sago to prevent the escape of larvae (Figure 2.2d). Trays were then placed into the rearing facility for 27 days.

After 27 days, larvae were removed from their respective diets, and larval weight and larval survival was measured and recorded for each diet.

Table 2.3 Diets used in the *Chilo partellus* diet suitability experiments using vials, where the control diet is the standard diet used at SASRI for rearing *C. partellus*. Amounts shown are to make up 1 L of diet.

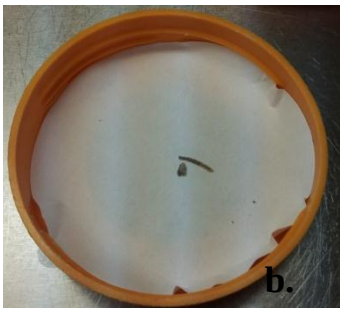
	1 Litre diet															
	Experiment 1					Experiment 2		Experiment 3				Experiment 4				
	Control							Control				Control				
	1	2	3	4	5	1	2	1	2	3	4	1	2	3	4	
FRACTION A																
Distilled water (ml)	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	
Brewer's yeast (g)	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	
Sorbic acid (g)	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	
Nipagin (g)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	
Ethanol (ml)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
Ascorbic acid (g)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	
Vitamin E (Cold water soluble) (g)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
Cane leaf whorl powder (g)	25	35	45	55	65	65	65	25	65	65	45	25	65	65	45	
Chickpea (g)	90	70	52.5	35.5	18	18	18	90	18	18	52.5	90	18	18	52.5	
Sucrose (g)	35	35	35	35	35	35	25.5	35	35	35	35	35	35	35	35	
Non-fat milk powder (g)	0	0	0	0	0	0	23.5	0	0	23.5	0	0	0	23.5	0	
Whole egg powder (g)	0	10	17.5	24.5	32	32	32	0	32	17.5	17.5	0	32	17.5	17.5	
FRACTION B																
Agar (g)	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	
Distilled water (ml)	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	
FRACTION C																
Formaldehyde (ml)	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	

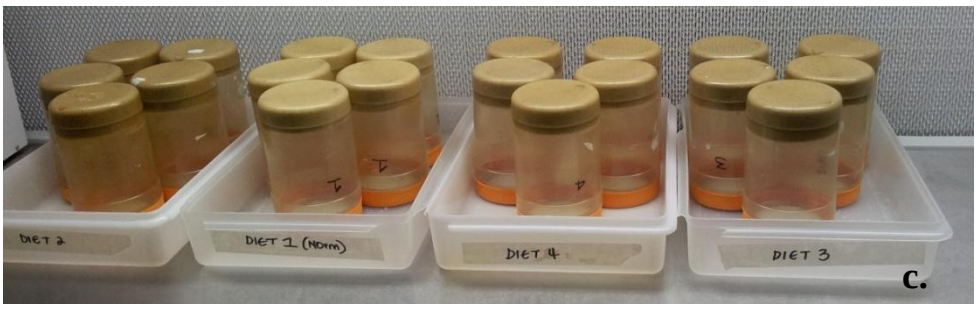
Table 2.4 Diets used in the *Chilo partellus* diet suitability experiments using 500 ml plastic jars, where the control diet is the standard diet used at SASRI for rearing *C. partellus.* Amounts shown are to make up 1 L of diet.

	1 Litre diet					
	Experiment 4a and 4b				Experiment 5	
	Control					
	1	2	3	4	1	2
FRACTION A					**Sterilized eggs**	**Non-sterilized eggs**
Distilled water (ml)	400	400	400	400	400	400
Brewer's yeast (g)	23	23	23	23	23	23
Sorbic acid (g)	0.6	0.6	0.6	0.6	0.6	0.6
Nipagin (g)	1.5	1.5	1.5	1.5	1.5	1.5
Ethanol (ml)	5	5	5	5	5	5
Ascorbic acid (g)	2.5	2.5	2.5	2.5	2.5	2.5
Vitamin E (Cold water soluble) (g)	2	2	2	2	2	2
Cane leaf whorl powder (g)	25	65	65	45	65	65
Chickpea (g)	90	18	18	52.5	18	18
Sucrose (g)	35	35	35	35	35	35
Non-fat milk powder (g)	0	0	23.5	0	23.5	23.5
Whole egg powder (g)	0	32	17.5	17.5	17.5	17.5
FRACTION B						
Agar (g)	12.5	12.5	12.5	12.5	12.5	12.5
Distilled water (ml)	400	400	400	400	400	400
FRACTION C						
Formaldehyde (ml)	3	3	3	3	3	3

Table 2.5 Diets used in the *Chilo partellus* diet suitability experiments using multicell trays. Amounts shown are to make up 1 L of diet.

1 Litre diet Experiment 6			
	1	**2**	**3**
FRACTION A			
Distilled water (ml)	400	400	400
Brewer's yeast (g)	23	23	23
Sorbic acid (g)	0.6	0.6	0.6
Nipagin (g)	1.5	1.5	1.5
Ethanol (ml)	5	5	5
Ascorbic acid (g)	2.5	2.5	2.5
Vitamin E (Cold water soluble) (g)	2	2	2
Cane leaf whorl powder (g)	25	65	65
Chickpea (g)	90	18	18
Sucrose (g)	35	35	25.5
Non-fat milk powder (g)	0	0	23.5
Whole egg powder (g)	0	32	17.5
FRACTION B			
Agar (g)	12.5	12.5	12.5
Distilled water (ml)	400	400	400
FRACTION C			
Formaldehyde (ml)	3	3	3





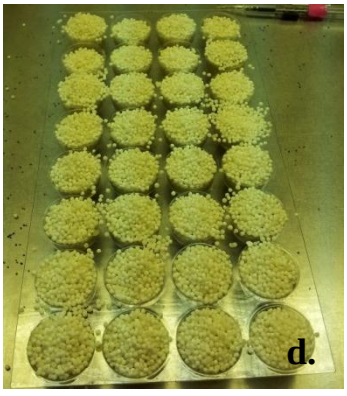


Figure 2.2 Different techniques used for rearing *Chilo partellus* (a) 25 ml vials with guazed lids; (b) Whatman No. 1 filter paper on lid of plastic jar with 50 *C. partellus* sterilized black-head stage eggs; (c) 500 ml plastic jars; (d) Multicell trays.

2.2.6 Statistical analysis

Software used to analyse data was GenStat release 14th edition (VSN International, Hemel Hempstead, UK) (Payne *et al*., 2011). Mean larval weights and larval survival for each diet was used for statistical analysis. Prior to analysis, the W-test for normality (Shapiro and Wilk, 1965) was used on the data for each parameter. These were subjected to $\log_{10}$ or square root transformations where necessary. The data for mean larval weight was subjected to Residual Maximum Likelihood (REML) variance component analysis (Harville, 1977) and means separated by the Sidak test (Abdi, 2007). Mean larval survival number was analysed using General Linear Mixed Model (GLMM) analysis (Breslow, 1993) and the Sidak test used to separate means. The standard error of the mean (SEM) was calculated for each parameter and presented in tables along with the means.

2.3 Results

2.3.1 *Chilo partellus* host identification

The hosts identified at the three sites surveyed for *C. partellus* included wild sorghum (*Sorghum halapense* (L.) Pers.) (Figure 2.3a) and Job's tears (*Coix lacryma-jobi* L.*)* (Figure 2.3b).

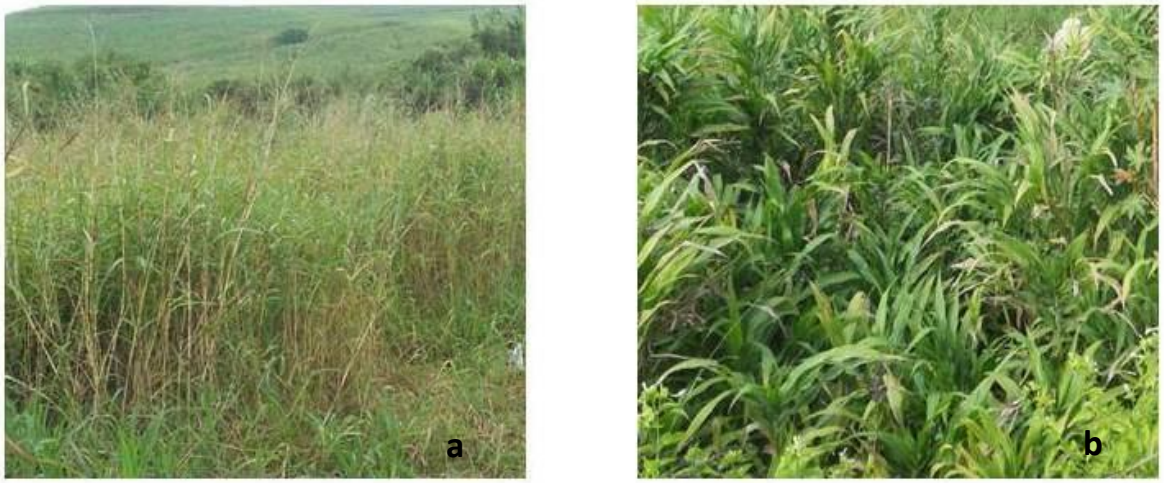


Figure 2.3 Observed hosts of *Chilo partellus* (a) wild sorghum (*Sorghum halepense* (L.) Pers.); (b) Job's tears (*Coix lacryma-jobi* L.*)*

2.3.2 *Chilo partellus* identification using molecular phylogeny

The samples collected from the three different sites were PCR amplified using standard COI primers (LCO forward and HCO reverse), and the sequences generated from this study were submitted to BOLD / BLAST (Ratnasingham and Hebert, 2007) for comparative analysis against the BOLD / BLAST database. The sequences obtained from the specimens from the three different sites were all identical and the BLAST / BOLD search confirmed the species collected to be that of *C. partellus. C. partellus* isolates grouped together with the *C. partellus* reference strain (KINS091-10.COI-5P, BOLD) in the neighbour-joining tree, and showed 100 percent bootstrap support (Figure 2.4). Furthermore, COI gene sequences were able to differentiate between *Chilo* species, separating *C. partellus* and *C. sacchariphagus* into two different groupings (Figure 2.4). The phylogeny shows a closer relationship between the Lepidoptera species sharing the same genus (viz. *C. partellus* and *C. sacchariphagus*), versus *E. saccharina* which falls out of this group.

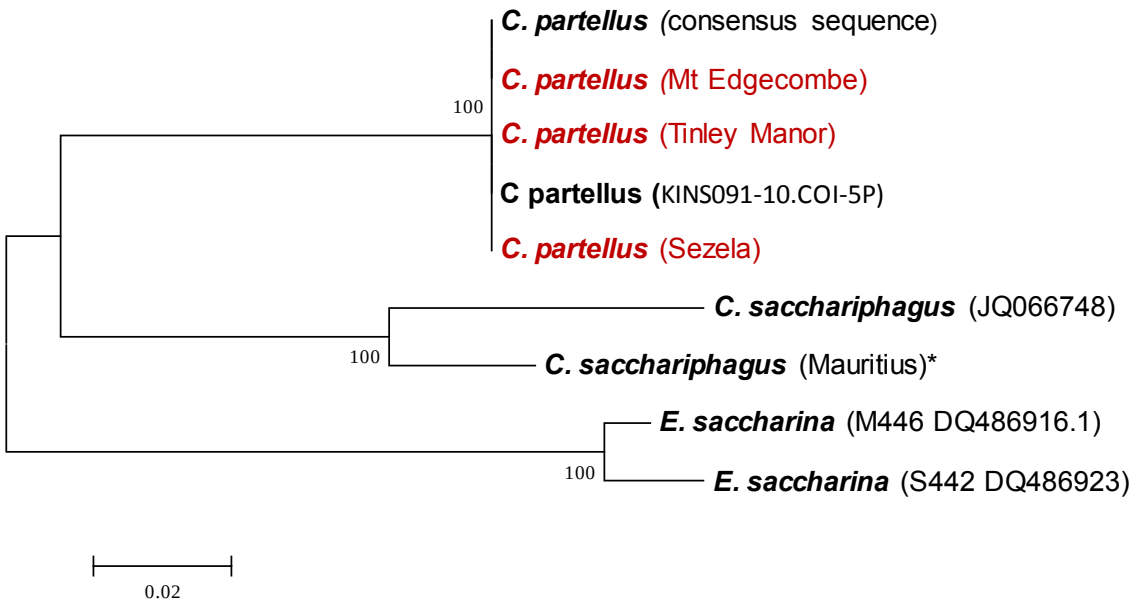


Figure 2.4 A neighbour-joining tree showing *Chilo* phylogeny based on the COI gene sequence for *Chilo partellus* obtained in KwaZulu-Natal (red) and *Chilo* reference sequences from BOLD (black). The scale bar represents 2 estimated changes per 100 nucleotides. *Eldana saccharina* was used as out-group.

* *C. sacchariphagus* from Kediri, East Java 28-1-2010, Adult moths, collected by Mike Way, *Chilo sacchariphagus* (96.98 BOLD Status = early release)

2.3.3 Optimization of the *Chilo partellus* artificial diet

In Experiment One; there was a significant difference between diets with respect to mean larval weight (Appendix 2.1). Diets One and Two differed significantly from Diets Three and Four in terms of mean larval weight (Table 2.6). Mean larval weight was significantly higher for Diets Three and Four, which had cane leaf powder of 45g and 55g per 1L, respectively. There was no significant difference between diets with respect to larval survival in Experiment One (Appendix 2.2). However, Diet Three gave the highest survival percentage (50.83%) (Table 2.6).

There was a significant difference between diets for mean larval weight in Experiment Two (Appendix 2.3). Diet Two, which incorporated non-fat milk powder, resulted in a significantly higher larval weight than Diet One, which had a zero level of non-fat milk powder (Table 2.6). However, there was no significant difference between the diets in terms of larval survival (Appendix 2.4), although Diet Two gave a higher survival percentage (45%) than Diet One (30.17%) (Table 2.6).

In Experiment Three a, there was a significant difference between diets with respect to mean larval weight (Appendix 2.5). Diet Two had a significantly lower mean larval weight, whilst Diet Four had a significantly higher mean larval weight compared to the rest of the diets (Table 2.6). There was no significant difference in mean larval weight between Diets One and Three, although Diet Three gave a higher larval weight than Diet One (Table 2.6). There was also a significant difference between diets with respect to survival percentage for this experiment (Appendix 2.6). Diets Three and Four resulted in significantly higher survival percentages than did Diets One and Two, with Diet Three (65g cane leaf powder + 23.5g non-fat milk powder + 17.5g whole egg powder in 1L) resulting in the highest survival percentage of larvae (73.3%) (Table 2.6).

Again, there was a significant difference between diets with respect to mean larval weight for Experiment Three b (repeat of Experiment Three a) (Appendix 2.7), where Diet Three gave a significantly higher mean larval weight compared to the rest of the diets, and Diet Two gave a significantly lower mean larval weight than Diets Three and Four (Table 2.6). These results concurred with those of Experiment Three a. There was also a significant difference between diets for larval survival (Appendix 2.8), where Diet Four resulted in a significantly lower larval survival percentage than

Diet Three, which showed the highest larval survival percentage (45.83%) (Table 2.6).

In Experiment Four a, using the 500 ml plastic jars, there was a significant difference between diets for mean larval weight (Appendix 2.9). Diet three (65g cane leaf powder + 23.5g non-fat milk powder + 17.5g whole egg powder in1L) gave a significantly higher mean larval weight than the other diets (Table 2.7). Diets One and Two gave the lowest mean larval weights, although they did not differ significantly from larvae living on Diet Four. Again, in Experiment Four b there was a significant difference between diets for mean larval weight (Appendix 2.10). As with Experiment Four a, diet three (65g cane leaf powder + 23.5g non-fat milk powder + 17.5g whole egg powder in 1L) gave a significantly higher mean larval weight compared to the rest of the diets. Experiment Five, which was used to compare the use of sterilized versus non-sterilized eggs, there were no significant differences between the two methods, with mean larval weight being very similar for both methods (Table 2.7).

There was a significant difference between diets with respect to larval weight for Experiment Six when using multicell trays (Appendix 2.12). Diet Three (65g cane leaf powder of NCo376 + 23.5 g non-fat milk powder + 17.5g whole egg powder in 1L) gave a significantly higher mean larval weight compared to the other diets (Table 2.8). No significant difference between diets for larval survival was found for Experiment Six (Table 2.8).

Overall, vials gave higher larval survival percentages than the plastic jars and multicell trays, which gave very low larval survival percentages. The diets with increased proportions of cane leaf powder and with the incorporation of non-fat milk powder and egg powder gave higher mean larval weights than the standard diet in all inoculation techniques tested. The diet containing 65 g cane leaf powder + 17.5 g egg powder + 23.5 g milk powder in 1L total was the best diet overall because it consistently gave a high mean larval weight and larval survival percentages in Experiments Two, Three, Four, Five, Six and Eight. Larval survival percentage was also better for the diet containing 65 g cane leaf powder + 17.5 g egg powder + 23.5 g milk powder in 1L compared to the other diets when tested in all of the experiments, and gave the highest overall larval survival percentage (73.3%) in Experiment Three a when using vials.

Table 2.6 Effect of different artificial diets in vials on the survival and weight of *Chilo partellus* larvae. Larval weight data was subjected to REML variance component analysis and survival data was subjected to GLMM analysis, the Sidak test was used to separate means, and $\log_{10}$ or square root transformation was used where necessary. Untransformed data is presented here.

Experiment	Diet	Mean larval weight (g)	Survival No. (%)
1	1	0.03939 ± 0.0040a	40 ± 4.491a
	2	0.04838 ± 0.0061a	36.67 ± 4.418a
	3	0.09324 ± 0.0052b	50.83 ± 4.583a
	4	0.07446 ± 0.0261b	38.33 ± 4.457a
	5	0.04512 ± 0.0037a	41.67 ± 4.519a
2	1	0.00778 ± 0.0013a	39.17 ± 4.475a
	2	0.0492 ± 0.0035a	45 ± 4.561a
3	1	0.05434 ± 0.0108b	25 ± 3.969a
	2	0.00386 ± 0.0014a	12.5 ± 3.032a
	3	0.06686 ± 0.0038b	73.3 ± 4.054b
	4	0.1158 ± 0.0051c	62.5 ± 4.438b
4	1	0.03146 ± 0.0042ab	30 ± 4.201ab
	2	0.01937 ± 0.0025a	34.17 ± 4.348ab
	3	0.07046 ± 0.0109c	45.83 ± 4.568b
	4	0.03732 ± 0.0038b	28.33 ± 4.131a

Mean ± SE, values in a column followed by the same letter are not significantly different using the Sidak test (P = 0.05)

Table 2.7 Effect of different artificial diets and inoculation techniques using 500 ml plastic jars on the weight of *Chilo partellus* larvae. Larval weight and survival number were collected 27 days after inoculation. Larval weaight data was subjected to REML variance component analysis, Sidak test, and $\log_{10}$ transformation was used where necessary. Untransformed data is presented here.

Experiment	Diet	Mean larval weight (g)	Survival No. (%)
5	1	0.0283 ± 0.005a	2.8
	2	0.0141 ± 0.0044a	3.6
	3	0.0756 ± 0.0069b	14
	4	0.0429 ± 0.0045a	16.4
6	1	0.0184 ± 0.0030a	22
	2	0.0126 ± 0.0079a	3.6
	3	0.0852 ± 0.0103b	4.4
	4	0.0141 ± 0.0055a	5.2
7	1	0.0241 ± 0.0026a	7.4
	2	0.0227 ± 0.0018a	8.6

Mean ± SE, values in a column followed by the same letter are not significantly different using the Sidak test (P = 0.05)

Table 2.8 Effect of different artificial diets using multicell trays on the mean larval weight of *Chilo partellus.* Larval weight data was subjected to REML variance component analysis and survival data was subjected to GLMM analysis, the Sidak test was used to separate means, and $\log_{10}$ or square root transformation was used where necessary. Untransformed data is presented here.

Experiment	Diet	Survival No. (%)	Mean larval weight (g)
8	1	5.556 ± 2.214a	0.079 ± 0.0102b
	2	1.852 ± 1.303a	0.0319 ± 0.0173a
	3	16.667 ± 3.603a	0.1231 ± 0.0127d

Mean ± SE, values in a column followed by the same letter are not significantly different using the Sidak test (P = 0.05)

2.4 Discussion

2.4.1 *Chilo partellus* host identification and colony establishment

Borer larvae collected from the selected sugarcane agro-ecosystems in KwaZulu-Natal were confirmed to be *C. partellus*. This may indicate that *C. partellus* poses a similar threat to that shown by *E. saccharina* before it became a serious pest of sugarcane in South Africa. The two plant species that were found to host *C. partellus* surrounding sugarcane fields in KwaZulu-Natal concur with the literature (Arabjafari and Jalali, 2007; Hutchison *et al*., 2008). In a paper published by Birkett *et al*. (2006) it is stated that the larva of borers such as *C .partellus* and *B. fusca* are polyphagous, in that they attack a wide range of wild and cultivated grasses (Poaceae), (Ong'amo *et al*., 2006; Calatayud *et al*., 2014; Moolman *et al*., 2014). Similiarly, hosts of *E. saccharina* also include a number of wild and cultivated grasses and wetland sedges (Potgieter *et al*., 2012). The populations of the wild grasses surrounding sugarcane fields are therefore very important in managing stem borers in Africa. Although *C. partellus* is not yet a serious pest of sugarcane in South Africa, it has been found in sugarcane bordering maize fields in southern Africa, and also has been found to be a predominant pest of sugarcane in Ethiopia (Way and Kfir, 1997; Assefa and Conlong, 2009).

As expected, *C. partellus* and *C. sacchariphagus* show a closer genetic relationship compared to that with *E. saccharina* Both *C. sacchariphagus* and *C. partellus* feed on the whorl of the plant, after which they become top-borers (Sarup *et al*., 1985; Way and Turner, 1999; Hutchison *et al*., 2008). These similar feeding mechanisms and a close relationship support that *C. partellus* is better suited for resistance screening studies carried out in South Africa for determining *C. sacchariphagus* resistance in sugarcane

With an efficient insectary in place at SASRI, more efficient research can take place as good quality insects can be supplied in sufficient quantities, and at the necessary developmental stages, for specific research requirements such as screening large numbers of progeny for cultivar resistance (Tende *et al*., 2010).

2.4.2 Optimization of the *Chilo partellus* artificial diet

The results show that the vials gave a better larval survival percentage than the plastic jars or multicell trays. The low larval survival percentage in the multicell trays could be due to the larvae escaping from the small cells within the trays. The sago covering the diet was used to prevent this, but due to *C. partellus* larva being positively phototrophic, they were still able to escape from the cells towards the light (Scheltes, 1978).The reason for the lower larval survival in the plastic jars could be due to a possible requirement that the larvae encounter the side of the container before entering the diet. This would be more likely to occur in the much lower diameter vials.

The incorporation of cane leaf whorl powder into an artificial diet appears useful for testing sugarcane varieties in a controlled manner (Kumar *et al*., 2006). Increasing the cane leaf powder in the diet will allow for the quantification of the whorl based constitutive antibiosis component of resistance in sugarcane varieties against *C. partellus* larvae.

Whole egg powder was initially incorporated into the diets with increased proportions of cane leaf powder in order to maintain the nitrogen content of the diets for comparative purposes. However, it can be seen from the results of Experiment One, that Diet Five, which had the highest whole egg powder content (32 g) was toxic to the larvae and mean larval weight and survival decreased. Therefore, non-fat milk powder was incorporated into a diet to diversify sources of nitrogen, and the whole egg powder content was reduced. Approximately 65 g cane leaf powder together with whole egg powder and non-fat milk powder was found to be more optimal in 1L of the artificial diet of *C. partellus*, as opposed to the standard diet containing 25 g cane leaf powder and no whole egg powder and non-fat milk powder per 1 L used for routine rearing. From these results it is clear that the source of nutrients has an effect on insect growth and development. Carpenter and Bloem (2002) suggest that the type or source of protein and sugars from specific ingredients in the diet are more important in making an artificial diet better suited for an insect, than the actual amount of protein within the diet. The results also show that there is a general increase in mean larval weight with an increase in the cane leaf powder component within the diet. This could be due to an increase in the plant tissue within the diet,

which is more favourable for the larvae to feed on and more similar to the natural diet of the borer, reducing the 'bottleneck effect' which is commonly observed (Kega *et al*., 2010)

After analysing the results, it was decided that the diet containing the 65 g cane leaf powder + 23.5 g non-fat milk powder + 17.5 g whole egg powder per 1L food (Table 2.9), in vials that would be used to carry out experiments for varietal comparisons using the artificial diet of *C. partellus*. This diet gave consistently good mean larval weights and survival percentages across all experiments.

Table 2.9 The diet established for *Chilo partellus* for carrying out varietal constitutive resistance comparisons.

FRACTION A	**Amount per 1 Litre**
Distilled water (ml)	400
Brewer's yeast (g)	23
Sorbic acid (g)	0.6
Nipagin (g)	1.5
Ethanol (ml)	5
Ascorbic acid (g)	2.5
Vitamin E (cold water soluble) (g)	2
Cane leaf whorl powder (g)	65
Chickpea (g)	18
Sucrose (g)	35
Non-fat milk powder (g)	23.5
Whole egg powder (g)	17.5
FRACTION B	
Agar (g)	12.5
Distilled water (ml)	400
FRACTION C	
Formaldehyde (40%) (ml)	3

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2.6 Appendix

Appendix 2.1 Results of REML for mean larval weight (g) for Experiment One

Fixed term	Wald statistic	n.d.f.	F static	d.d.f.	F pr
Diet	63.26	4	15.81	244	<0.001

Appendix 2.2 Results of GLMM for larval survival percentage (%) for Experiment One

Fixed term	Wald statistic	n.d.f.	F static	d.d.f.	F pr
Diet	5.96	4	1.49	595	0.203

Appendix 2.3 Results of REML for mean larval weight (g) for Experiment Two

Fixed term	Wald statistic	n.d.f.	F static	d.d.f.	F pr
Diet	109.02	1	109.02	99	<0.001

Appendix 2.4 Results of GLMM for larval survival percentage (%) for Experiment Two

Fixed term	Wald statistic	n.d.f.	F static	d.d.f.	F pr
Diet	0.83	1	0.83	238	0.363

Appendix 2.5 Results of REML for mean larval weight (g) for Experiment Three

Fixed term	Wald statistic	n.d.f.	F static	d.d.f.	F pr
Diet	111.75	3	37.25	204	<0.001

Appendix 2.6 Results of GLMM for larval survival percentage (%) for Experiment Three

Fixed term	Wald statistic	n.d.f.	F static	d.d.f.	F pr
Diet	105.63	3	35.21	476	<0.001

Appendix 2.7 Results of REML for mean larval weight (g) for Experiment Four

Fixed term	Wald statistic	n.d.f.	F static	d.d.f.	F pr
Diet	53.28	3	17.76	162	<0.001

Appendix 2.8 Results of GLMM for larval survival percentage (%) for Experiment Four

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Diet	9.67	3	3.22	476	0.022

Appendix 2.9 Results of REML for mean larval weight (g) for Experiment Five

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Diet	44.11	3	14.7	99	<0.001

Appendix 2.10 Results of REML for mean larval weight (g) for Experiment Six

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Diet	66.33	3	22.11	77	<0.001

Appendix 2.11 Results of REML for mean larval weight (g) for Experiment Seven

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Diet	1.4	1	1.4	124.6	0.238

Appendix 2.12 Results of GLMM for mean larval weight (g) for Experiment Eight

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Diet	384.43	2	192.21	567.8	<0.001

CHAPTER THREE

EVALUATION OF CONSTITUTIVE RESISTANCE IN SUGARCANE TO *CHILO PARTELLUS* (SWINHOE) (LEPIDOPTERA: CRAMBIDAE) USING ARTIFICIAL DIET BIOASSAYS

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Abstract

Stalk borers feed on crops such as sugarcane, maize and sorghum in sub-Saharan Africa. *Chilo partellus* and *Chilo sacchariphagus* are serious pests on a number of hosts, and pose threats to the sugarcane industry in South Africa. The use of host-plant resistance may become important in controlling these pests, and new and improved varieties are important in maintaining good yields. Constitutive antibiosis resistance to *C. partellus* larvae was explored in a diverse collection of 20 sugarcane varieties, by incorporating crushed dried leaf powder into an artificial diet. There were significant differences in larval weight, total weight and larval survival in diets incorporating leaf powder from different sugarcane varieties. Feeding with diets containing M1135/64 and N24 leaf powders consistently gave lower larval weights and larval survival, whereas varieties M1025/70, R573 and N25 gave higher larval weights and larval survival.This suggests that M1025/70, R573 and N25 could be more susceptible, with little to no constitutive resistance against *C. partellus*. The concept of insect surrogacy was also explored, whereby known ratings of specific sugarcane varieties to *C. sacchariphagus* were compared to the results obtained for

constitutive resistance against *C. partellus* from this study using artificial diets. Some correlations were observed for specific sugarcane varieties, such as N25 and R570, with respect to *C. partellus* and *C. sacchariphagus* resistance. However, further investigations using different resistance screening methods will be required to determine different components of resistance in these sugarcane varieties.

Keywords: sugarcane; *Chilo partellus*; *Chilo sacchariphagus*; antibiosis; constitutive resistance; artificial diet; insect surrogacy.

3.1 Introduction

Stalk borers are one of the most serious insect pests of grass crops such as sugarcane (*Saccharum officinarum* L), maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) in sub-Saharan Africa (Kfir *et al*., 2002). *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) is found in India, Pakistan, Kenya, Somalia, Tanzania and South Africa (Kumar, 1997). *Chilo sacchariphagus* Bojer (Lepidoptera: Crambidae) is a major pest in Mozambique, Mauritius, Reunion and Madagascar (Goebel and Way, 2009). Borer damage can result in the total loss of a crop in a very short time, as well as reduce the quality of the crop (Fenton, 1952). *C. partellus* and *C. sacchariphagus* are serious pests on a number of hosts and pose threats to the sugarcane industry in South Africa. Chemical and biological control of these pests would be expensive, whereas host-plant resistance offers a safe and relatively cheap method of control (Epidi *et al*., 2004).

There are a number of different factors affecting the establishment of insects in plants. These include non-preference for ovipositing, factors affecting feeding, difficulty in metabolizing ingested food, and reduced growth and fecundity of the insect (Kumar *et al*., 2006; Panchal and Kachole, 2013). Constitutive resistance in plants can be structural, morphological, or chemical in nature and does not require the attack of the insect to be expressed, unlike induced resistance which requires the recognition of an invader in order for resistance to occur (Keen, 1999). Preformed chemicals synthesized via secondary metabolism are found in plants, and include phenolics, terpenoids and steroids, which may have a toxic effect on insect herbivores (Keen, 1999; War *et al*., 2012). A good knowledge of the different

mechanisms of resistance in plants is vital for the effective use of resistant sources in improving crop management. Due to large genotype x environment interactions, the use of field trials makes it difficult to quantify the different mechanisms of resistance in plants (Kumar *et al*., 2006). The incorporation of leaf powder of different sugarcane varieties into an insect's artificial diet is therefore very useful in quantifying any constitutive antibiosis resistance mechanisms present within sugarcane varieties. The level of resistance of varieties determined using this method may differ to the level of resistance of varieties determined in the field, due to the lack of strong genotype x environment interactions (Kumar, 1997). However, according to Padmaja *et al*. (2012), the analyses of resistance of certain sugarcane varieties by incorporating them into artificial diets, is a useful method in comparing varieties under uniform conditions, without any variations from the environment. The incorporation of freeze-dried leaf powder into the artificial diet of *C. partellus* has been done in an experiment to study the antibiosis resistance mechanism of 20 sorghum varieties (Kumar *et al*., 2006). A similar study was conducted by Epidi *et al*. (2004), also using sorghum varieties in the artificial diet of *C. partellus*. Williams and Buckley (2008) conducted a study whereby lyophilized leaf tissue of 20 maize varieties, varying in resistance, were incorporated into an artificial diet to determine their effects on the growth of fall armyworm (*Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)) and the southwestern corn borer (*Diatraea grandiosella* Dyar (Lepidoptera: Crambidae)). Differences in growth of these insects were observed between resistant and susceptible maize varieties incorporated into diets.

Most host-plant resistance studies on borers focus on the resistance mechanisms within the stalk because this is where majority of the damage is caused, which results in yield losses. However, young larvae of *C. sacchariphagus* and *C. partellus* also feed on the leaves before entering the stalk of the plant and this ultimately results in leaf lesions. Although these leaf lesions do not always significantly impact on the crop economically, they can generate an estimate of the borer populations and can confirm whether leaf resistance occurs, which ultimately contributes to a reduction in borer populations (Nibouche and Tibere, 2009). It has been found that leaves of sorghum contain resistance to *C. partellus* larvae, and in maize, leaf resistance occurs against borers such as *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), *C. partellus* and *Diatraea* spp. (Guthrie *et al*., 1970; Pathak 1990).

In this study, leaf powder from a selected range of resistant and susceptible sugarcane varieties was incorporated into the established artificial diet of *C. partellus*, and used to determine any constitutive antibiosis resistance mechanisms in the selected sugarcane varieties to *C. partellus*. Constitutive resistance effects of sugarcane varieties were expressed in terms of larval weight, larval survival and pupal numbers. Results from this study were compared to existing *C. sacchariphagus* ratings of specific sugarcane varieties based on previous studies, to determine if correlations between *C. partellus* and *C. sacchariphagus* resistance and susceptible sugarcane varieties occur. *C. sacchariphagus* has not yet been found in South Africa, and therefore cannot be used to carry out host-plant resistance studies in the country, unless under quarantine. If sugarcane varieties which appear to be resistant to *C. partellus* are also resistant to *C. sacchariphagus* then the concept of 'surrogate insect resistance screening' will prove effective. Among the few South African varieties with known resistance or susceptibility to *C. sacchariphagus*, there appears to be a correlation with *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae) rankings (See Chapter 1, Page 35). All of these pests feed on the whorl of the plant, and therefore similar resistance mechanisms within the plant may act against them. Additionally, *C. partellus* and *C. sacchariphagus* are both top borers belonging to the same genus. The concept of 'surrogate insect resistance screening' has proven successful within the borer genus *Ostrinia* on maize (Overman, 1994).

3.2 Methods and materials

3.2.1 Selection of sugarcane varieties

Twenty sugarcane varieties were selected for this study (Table 3.1). Sugarcane varieties N22, N28, N25, N26 and N32 were selected based on *C. sacchariphagus* leaf feeding index ratings (CLI) and from information obtained on *C. sacchariphagus* ratings from the paper published by Conlong *et al*. (2004). The 'leaf feeding index' is a non-destructive measurement of damage by borers used to assess their leaf feeding behaviour and to determine susceptibility or resistance of different maize and sorghum varieties to the borers *C. partellus* and *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae). The remaining sugarcane varieties were selected based on numbers of *F. serrata* obtained from field trials run by the South African Sugar Research Institute

(SASRI, KwaZulu-Natal, South Africa). R570 and R576 are resistant and susceptible sugarcane varieties to *C. sacchariphagus* respectively, according to a study done by Nibouche and Tibere (2010), and were therefore also used in this study. NCo376 was used as a control variety because it is the standard variety being used in the artificial diet of *C. partellus* at SASRI.

Table 3.1 Sugarcane varieties incorporated into the artificial diet of *Chilo partellus* for constitutive antibiosis resistance mechanism studies. R = Resistant, S = Susceptible.

Variety	**Av. No. Thrips/ leaf whorl**	***C. sacchariphagus* leaf Index) (Conlong *et al*., 2004)**	**Total *C. s* Rating (low value = R; high value = S) (Conlong *et al*., 2004)**
Co1287	21		
Co6505	67		
R 576	27		
R 573	45		
R 570	49		
R 572	127		
R 568	153		
N24	38		
N22	40	13.9	23(I)
N28	47	7.1	6 (R)
N25	70	15.5	36 (S)
N26	74	30.2	44(S)
N31	91		
M1135/64	19		
M861/60	21		
M1025/70	58		
N27	120		
N21	165		22(I)
N32	19	1.8	19(IR)
NCo376			

3.2.2 Incorporation of selected sugarcane varieties into an artificial diet of *Chilo partellus*

The selected sugarcane varieties were collected from field trials established at SASRI (KwaZulu-Natal, South Africa). Sugarcane was six months of age when collected because this was representative of the age that *C. sacchariphagus* usually attacks sugarcane, which is three to seven months old (Goebel, 2006; Bezuidenhout *et al*., 2008). Five stalks were collected per variety, and the leaves removed from the growing point for each stalk. Leaves were cut into small pieces using secateurs, and placed into brown paper bags (WOLFF Wholesalers, Edenvale, South Africa) for drying. Leaves were dried using a Scientific Series 9000 incubator (TriLab, Pinetown, South Africa) at 65^{o}C for 24 hours, after which they were sent to the mill at SASRI for

crushing into a powder. The cane leaf powder was then incorporated into the new diet which had previously been established and can be seen in Table 3.2. 20 artificial diets could not all be made in one day due to equipment and time constraints and therefore sugarcane varieties were divided into five batches, where cane leaf powder from each variety in a particular batch was incorporated into the artificial diet on the same day (Table 3.3). The variety NCo376, used as the standard variety in the artificial diet of *C. partellus* at SASRI, was used as a control for each batch for comparative purposes.

The method for diet preparation was as follows: The Brewer's yeast (NCP, Johannesburg, South Africa), sorbic acid (Sigma Life sciences, United States), ascorbic acid (Warren Chem Specialities, Cape Town, South Africa), vitamin E (Polychem Supplies cc, Congella), ground chickpea (S.E. Mart, Durban, South Africa), sucrose (Huletts, Durban, South Africa), non-fat milk powder (Lactalis Ingredients, Bourgbarre, France), whole egg powder (Sunspray Food Ingredients (PTY) Ltd., Johannesburg, South Africa) and crushed cane tops and leaves (SASRI, KwaZulu-Natal, South Africa) for a specific genotypic from fraction A were weighed and mixed together thoroughly. The nipagin (Sharon Laboratories, Israel) was dissolved in the ethanol from fraction A. The distilled water from fraction A was autoclaved and then allowed to cool to 60-70^{o}C. It was then added, together with the dissolved nipagin, to the dry ingredients from fraction A and mixed together for 3 minutes in the blender (6.7 Litre bowl capacity) (Kenwood, United Kingdom). The agar powder (Polychem supplies cc, Congella) and distilled water in fraction B were mixed together, autoclaved and allowed to cool to 60-70^{o}C. Fraction A and fraction B ingredients were mixed together, after which the formaldehyde 40% (Merck (PTY) Ltd., Johannesburg, South Africa) from fraction C was added and mixing in the blender took place for a further 3 minutes. 8 ml of diet was dispensed into 25 ml sized vials (Lasec, Durban, South Africa) and placed open on the laminar bench where they were exposed to UV light for 1 hour.

Three replications of 20 vials each were used for each variety in a completely randomized design (CRD). After each batch of diets were made, vials for each diet made up using a specific variety were placed into a zip lock plastic bag (Glenpak, Johannesburg, South Africa), which were labelled with the variety name, and sealed and stored in a refrigerator at 4^{o}C overnight. The following day, plastic bags were

removed from the fridge and warmed to room temperature before being inoculated with *C. partellus* larvae. Larvae for inoculations were obtained from the established colony at SASRI. The surface of the diet in each vial was punctured once with the end of a sterilized plastic rod to facilitate penetration of larvae. Two active neonate larvae were transferred into a single 25 ml vial using a clean, sterilized paintbrush (Winsor and Newton, London, UK). Each vial was sealed with a ventilated lid. The vials and ventilated lids were sterilized in a 4.3% solution of Jik (Protea Chemicals, Johannesburg, South Africa) and water to prevent contamination. Vials were then placed upside down in plastic containers and kept in a larval growth room maintained at 27 +/- 2^{o}C, 65 +/- 5% and an 8 hour light: 16 hour dark photophase provided by 65 watt colour 19 Triphos fluorescent tubes for 27 days.

After the larvae had been feeding on the diet for 27 days, they were removed from the diet in the vials, and larval number, pupal number, pupal weight and larval weight were recorded for all larvae recovered.

Table 3.2 The artificial diet used in constitutive resistance mechanism studies

FRACTION A	**Amount per 1 Litre**
Distilled water (ml)	400
Brewer's yeast (g)	23
Sorbic acid (g)	0.6
Nipagin (g)	1.5
Ethanol (ml)	5
Ascorbic acid (g)	2.5
Vitamin E (Cold water soluble) (g)	2
Cane leaf whorl powder (g)	65
Chickpea (g)	18
Sucrose (g)	35
Non-fat milk powder (g)	23.5
Whole egg powder (g)	17.5
FRACTION B	
Agar (g)	12.5
Distilled water (ml)	400
FRACTION C	
Formaldehyde (ml)	3

Table 3.3 Leaf whorl powders of different sugarcane varieties were incorporated into artificial diet, and inoculated with *Chilo partellus* larvae on the same day. Varieties were tested in batches due to equipment constraints (different batches are colour coded)

Variety	Batch
Control 1 (NCo376)	1
N31	1
N28	1
R572	1
M861/60	1
M1025/70	1
Control 2 (NCo376)	2
M1135/64	2
N24	2
N32	2
R568	2
N25	2
Control 3 (NCo376)	3
N26	3
R570	3
Co1287	3
R576	3
Control 4 (NCo376)	4
Co6505	4
N21	4
Control 5 (NCo376)	5
N27	5
N22	5
R573	5

3.2.3 Statistical analysis

Software used to analyse data was GenStat release 14th edition (VSN International, Hemel, Hempstead, UK) (Payne *et al*., 2011). Mean larval weight, mean total *C. partellus* (pupae plus larvae) weight and larval survival were used for statistical analysis in GenStat. Prior to analysis, the W-test for normality (Shapiro and Wilk, 1965) was used on the data for each parameter, and it was subjected to $\log_{10}$ transformation where necessary. The data for mean larval weight was subjected to Residual Maximum Likelihood (REML) variance component analysis (Harville, 1977) and means were separated using the Sidak test (Abdi, 2007). Mean larval survival number was analysed using General Linear Mixed Model (GLMM) analysis (Breslow, 1993) and the Sidak test used to separate means. The standard error of the mean

(SEM) was calculated for each parameter and presented in tables along with the means.

3.3 Results

Mean larval weight, total (larvae plus pupae) *C. partellus* weight and larval survival of *C. partellus* larvae reared on artificial diet incorporating cane leaf powder of 20 sugarcane varieties showed significant differences between the varieties tested (Appendix 3.1, 3.2 and 3.3). For the diets where pupae were found, mean pupal weight was calculated. However, there was no significant difference for mean pupal weight between diets incorporating cane tops from different sugarcane varieties (Appendix 3.4).

Varieties were ranked from lowest larval weight to highest larval weight in Table 3.4, with varieties highlighted in the same colour being from the same batch. There was no significant difference for mean larval weight between sugarcane varieties Co6505, the control diets (NCo376), M1135/64, N27 and N24. All these varieties had the lowest larval weights. Varieties N31, N25, R572, R568, M1025/70, N28 and M861/60 did not differ significantly from each other. M861/60 had a significantly higher mean larval weight compared to all sugarcane varieties, except to N31, N25, R572, R568 and N28. Mean total weight showed a similar trend to that of mean larval weight (Table 3.4).

Larval survival percentage was ranked lowest to highest (Table 3.5). The diet incorporating the variety R573 showed the highest larval survival percentage of 85%, which was significantly higher than the larval survival percentages of diets incorporating varieties NCo376 (controls from batch 3 and batch 1), N24, N26 and M1135/64, which had the lowest larval survival percentages. While N27 gave the second highest larval survival percentage of 82.5% (Table 3.5), it was ranked with one of the lower mean larval weights (Table 3.4). Similarly, R568 gave a fairly high mean larval weight, whilst its larval survival percentage was below 80%. The number of pupae found in each diet was shown to be higher in diets which gave a higher mean larval weight (Table 3.4). N28 gave the highest pupal number with 12 pupae recovered in total, followed by M1025/70 which had seven pupae recovered from the

diet in total. Zero pupa were found in diets incorporating sugarcane varieties Co6505, NCo376, Co1287, N21, N32, R573 and N31.

Table 3.4 Effect of leaf powders in artificial diet from 20 different sugarcane varieties on *Chilo partellus* larval and pupal weights (g). (NCo376 was used as a control variety for each batch – colour coded). The data was subjected to REML variance component analysis, Sidak test and log_{10} transformation was used where necessary. Untransformed data is presented here.

Variety	Mean larval weight (g)	Number of Pupae	Mean pupal weight (g)	Mean total Chilo weight (g)
NCo376*	**0.0609**	**0**		**0.0609**
Co6505	0.04847 ± 0.002573a	0	*	0.04847 ± 0.002573a
Control 1	0.05931 ± 0.003818ab	0	*	0.05931 ± 0.003818ab
Control 5	0.05997 ± 0.004478ab	0	0.0685	0.06006 ± 0.004432ab
Control 3	0.06027 ± 0.004007ab	0	*	0.06027 ± 0.004007abc
Control 2	0.06243 ± 0.005477abc	0	*	0.06243 ± 0.005477abc
Control 4	0.06251 ± 0.003781abcd	0	*	0.06251 ± 0.003781abc
M1135/64	0.06531 ± 0.005702abcde	3	0.0761	0.06574 ± 0.005486abc
N27	0.07227 ± 0.004283abcdef	1	0.0688	0.07224 ± 0.00424abcd
N24	0.07515 ± 0.005569abcdefg	1	0.0616	0.07495 ± 0.005493abcde
R 570	0.07661 ± 0.004117bcdefg	1	0.0564	0.07636 ± 0.004073bcde
Co1287	0.07825 ± 0.005154bcdefg	0	*	0.07825 ± 0.005154bcde
N21	0.07825 ± 0.003472bcdefg	0	*	0.07825 ± 0.003472bcde
N22	0.07941 ± 0.004835bcdefg	2	0.04695	0.07873 ± 0.004758bcde
N26	0.08124 ± 0.005051bcdefg	2	0.05305	0.08048 ± 0.004943bcdef
N32	0.08253 ± 0.004311bcdefg	0	*	0.08253 ± 0.004311bcdef
R 576	0.08897 ± 0.004359cefgh	2	0.05365	0.08811 ± 0.004295cdefg
R 573	0.09587 ± 0.004274fghi	0	*	0.09587 ± 0.004274defgh
N31	0.10245 ± 0.008625ghij	0	*	0.10245 ± 0.008625efgh
N25	0.11 ± 0.005462hij	7	0.0553	0.1065 ± 0.005278fgh
R 572	0.11234 ± 0.004562hij	2	0.05505	0.1111 ± 0.004549gh
R 568	0.11285 ± 0.005757hij	5	0.07626	0.1107 ± 0.005562gh
M1025/70	0.11503 ± 0.004555ij	5	0.05872	0.11213 ± 0.004507gh
N28	0.12142 ± 0.008054ij	12	0.06479	0.11361 ± 0.007257gh
M861/60	0.1233 ± 0.005226j	7	0.06911	0.11904 ± 0.005093h
Mean	0.09		0.061	
SD	0.022		0.009	
CV%	24.94		14.63	

Mean ± SE, values in a column followed by the same letter are not significantly different using the Sidak test (P = 0.05)

Table 3.5 Effect of leaf powders in artificial diet from 20 different sugarcane varieties on *Chilo partellus* survival percentage. (NCo376 was used as a control variety for each batch - colour coded). Mean larval survival number was analysed using GLMM analysis and the Sidak test (F pr. < 0.001, Appendix 3.3).

Variety	Survival %
NCo376*	**67.82**
Control 3	55.8 ± 4.552a
N24	59 ± 4.506ab
N26	61.67 ± 4.457abc
Control 1	61.7 ± 4.457abc
M1135/64	62.5 ± 4.438abc
N31	63.3 ± 4.418abcd
Control 2	65 ± 4.372abcd
R 570	65.8 ± 4.348abcd
R 576	68.3 ± 4.264abcd
Co1287	70 ± 4.201abcd
R 568	70.8 ± 4.167abcd
N32	71.7 ± 4.131abcd
N28	71.7 ± 4.131abcd
M861/60	74 ± 4.054abcd
Co6505	75.8 ± 3.877abcd
Control 4	77.5 ± 3.828abcd
R 572	77.5 ± 3.828abcd
Control 5	79.1 ± 3.723bcd
N22	79.1 ± 3.723bcd
N21	80 ± 3.416cd
N25	80.8 ± 3.608bcd
M1025/70	80.8 ± 3.608bcd
N27	82.5 ± 3.483cd
R 573	85 ± 3.273d
Mean	72.41
SD	8.3189
CV%	11.49

Mean ± SE, values in a column followed by the same letter are not significantly different using the Sidak test (P = 0.05)

3.4 Discussion

There were variations in larval weight, total *C. partellus* weight and larval survival in the diets incorporating cane leaf powder from different sugarcane varieties. In a

similar study conducted by Kumar *et al* (2006), differences in larval survival, larval weight, pupal weight and other larval parameters were also observed when incorporating leaf powders of different sorghum varieties into a diet for *C. partellus*. Sugarcane varieties M1135/64, R570, N26 and N24 gave consistently lower larval weights and larval survival when they were incorporated into the artificial diet of *C. partellus*. This suggests that these sugarcane varieties do not promote larval growth and therefore have a higher constitutive resistance to *C. partellus* larvae when compared to the other sugarcane varieties. Conversely, varieties M1025/70, R573 and N25 gave higher mean larval weights and larval survivals when incorporated into the diet, which suggests that they are more susceptible varieties, which have little to no constitutive resistance against *C. partellus*. This indicates that the more susceptible varieties meet the nutritional requirements required by the larvae to ensure the larval growth can be completed in a shorter time frame (Arabjafari and Jalali, 2007). The mechanisms in plants accounting for the negative effects on larval survival and growth are non-preference and antibiosis (Davis *et al*., 1999). The antibiosis resistance mechanisms present in resistant sugarcane varieties may be because of secondary plant substances in the leaves or also due to low nutritional quality of the food (Kumar *et al*., 2006). In a study conducted by Kumar (1997), to determine the effect of dry leaf powders and fresh leaf juices on *C. partellus* larval development, it was suggested that non-nutritional factors (allelochemicals) in plant tissues are lost during the preparation of the diet, due to the heat involved. Therefore, the survival and development of larvae was directly related to nutritional factors in the plant tissues. *C. partellus* resistance in sorghum is associated with low sugar content and an increased level of amino acids, tannins, total phenols, neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignins in the plant (Kumar *et al*., 2006). Plants may contain large amounts of preformed chemicals produced through secondary metabolism, such as various phenolics, terpenoids and steroids. In some tissues, these chemicals may be in high concentrations, which can be very toxic to insects (Keen, 1999). In artificial diet bioassays conducted by Kumar *et al*. (1993a, b), it was shown that *C. partellus* larvae displayed feeding non-preference, indicated by the low amounts of food ingested by resistant maize varieties compared to a susceptible variety.

Not all of the sugarcane varieties showed a correlation between larval weight and larval survival. Varieties N27 and N21 gave high survival percentages of 82.5% and 80% respectively, whereas their mean larval weights were relatively low compared to other varieties (0.07227 g and 0.07825 g respectively). This could be due to more competition between larvae in vials, resulting in less available food for individual larvae to feed on. Another explanation could be that antibiosis had an effect on the larvae, stunting their weight, however this is more unlikely as antibiosis also reduces survival of the insect (Sharma, 1997; Padmaja *et al*., 2012). On the other hand, N26 and N31 had low larval survival percentages (61.67% and 63.3% respectively), whereas their larval weights were high. The lower number of larvae could have reduced competition for food within the vials and therefore more food was available for individual larvae.

When comparing some of the results from this study to the ratings shown for *C. sacchariphagus* in a study conducted by Conlong *et al* (2004), some interesting observations could be made. N25 gave a fairly high Chilo leaf feeding index (CLI) and *C. sacchariphagus* rating (the higher the rating the more susceptible the variety) compared to the other varieties (Table 3.1), which correlates with the results from this study, where N25 gave high larval weight and larval survival for *C. partellus* compared to other sugarcane varieties, indicating its lower constitutive resistance. Moreover, *F. serrata* numbers obtained for N25 were fairly high (Table 3.1) as well. This backs the concept of insect surrogacy, where similar feeding mechanisms of *C. sacchariphagus, C. partellus* and *F. serrata* result in plants having similar resistance mechanisms acting against them. However, N26 was shown to have a very high CLI and *C. sacchariphagus* rating by Conlong *et al* (2004), as well as high *F. serrata* numbers, whereas in this study *C. partellus* had a fairly low larval survival (61.67%) and an intermediate larval weight (0.08124 g) when reared on diet incorporating cane tops from N26. This could indicate that N26 has a poor constitutive resistance but a strong induced resistance against *C. partellus*, if the concept of insect surrogacy for *C. partellus* and *C. sacchariphagus* is used. According to the Chilo leaf feeding index and ratings obtained for the variety N28 (Conlong *et al*., 2004), it was shown to be the most resistant variety to *C. sacchariphagus*. However, a high larval weight and larval survival for *C. partellus* was observed when larvae were reared on the diet incorporating N28 cane leaf powder. This could suggest that the mechanism of

resistance for N28 is not that of the constitutive type, but rather that of antixenosis present on the surface of the leaves, or that of induced resistance, which requires the insect to come into contact with the plant, which in turn results in compounds such as phytoalexins or pathogenesis related (PR) proteins being produced (Ahman, 2006). R570 is fairly resistant to *C. sacchariphagus* according to Nibouche and Tibere (2009); and in this study, *C. partellus* gave a relatively low larval survival and mean larval weight in the diet containing R570, indicating that it has strong constitutive resistance against *C. partellus*.

There seemed to be a correlation between pupal number and mean larval weight, where the diets resulting in higher larval weight also resulted in a higher number of pupae being found and vice versa. This suggests that the diets with high larval weights and pupal numbers result in faster growth of the borer, while those diets with low larval weights and pupal numbers reduce larval growth. Antibiosis is also shown by reduced pupal weight and reduced pupation and adult emergence (Kumar *et al*., 2006).

The incorporation of cane leaf powder of different sugarcane varieties into the artificial diet of *C. partellus* is useful in overcoming the variation of infestation that would be seen under field conditions and allows one to test different sugarcane varieties under uniform conditions (Kumar *et al*., 2006). The use of an artificial diet to establish mechanisms of resistance to borers can be used, but should not replace conventional field trials for screening germplasm. The resistance level of sugarcane varieties established using this technique may not conform to those in the field due to the absence of strong genotype x environment interactions (Kumar, 1997). The results from this study suggest that specific sugarcane varieties do contain some degree of constitutive antibiosis as a resistance mechanism against *C. partellus* larvae. However, it is a complex interaction of factors that determine the resistance or susceptibility of sugarcane varieties to stem borers, which needs to be explored further. Sugarcane varieties, such as Co1287, M1135/64, R570, N26 and N24, which show some form of constitutive antibiosis resistance to *C. partellus* in this study, should be tested further, and have the potential to be used in resistance breeding programmes to increase the diversity of resistance to *C. partellus*, and ultimately *C. sacchariphagus*.

3.5 References

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3.6 Appendix

Appendix 3.1 Results of REML analysis for mean larval weight (g) for comparisons of 20 sugarcane varieties in *Chilo partellus* artificial diet

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	461.9	23	20.08	1995	<0.001

Appendix 3.2 Results of REML analysis for mean total weight (g) for comparisons of 20 sugarcane varieties in *Chilo partellus* artificial diet

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	421.62	23	18.33	2046	<0.001

Appendix 3.3 Results of GLMM analysis for larval survival percentage (%) for comparisons of 20 sugarcane varieties in *Chilo partellus* artificial diet

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	94.38	23	4.1	2837	<0.001

Appendix 3.4 Results of REML analysis for mean pupal weight (g) for comparisons of 20 sugarcane varieties in *Chilo partellus* artificial diet

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	11.12	13	0.86	37	0.603

CHAPTER FOUR

EVALUATION OF OVIPOSITIONAL ANTIXENOSIS IN SUGARCANE VARIETIES TO *CHILO PARTELLUS* (SWINHOE) (LEPIDOPTERA: CRAMBIDAE)

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Abstract

Chilo partellus and *Chilo sacchariphagus* are both top borers that pose a threat to the sugarcane industry in South Africa. *C. partellus* was used as a surrogate insect for *C. sacchariphagus* in ovipositionalstudies on sugarcane because *C. sacchariphagus* is not yet present in South Africa. Both pests belong to the same family and have the same feeding mechanisms; therefore similar defense mechanisms in plants may operate against them. The concept of ovipositional antixenosis behaviour of insects is based on the theory that female insects will choose their hosts in a hierarchal manner, laying most of their eggs on the preferred plant. This could be due to characteristics of the plant which either fail to allow for ovipositing, including essential stimuli (attractants), or containing ovipositional-inhibiting stimuli (repellents). In this study, differences in 20 selected sugarcane varieties with respect to ovipositing of *C. partellus* moths were investigated. Two experiments were conducted, whereby the 20 sugarcane varieties were planted into 98 well trays in a completely randomized design and replicated five and 10 times for Experiment One and Two, respectively.

Individual trays were placed into BugDorm® rearing tents when plants were still in their seedling stage and moths placed into the cages for ovipositing to take place. No statistically significant differences were found between sugarcane varieties for both egg number and batch number for both experiments (F pr > 0.05). However, sugarcane varieties N28, Co1287 and M1025/70 gave the highest egg and batch numbers consistently for both experiments, whereas N32 and R573 gave consistently low egg and batch numbers. **Keywords:** *Chilo partellus*, *Chilo sacchariphagus*, sugarcane, surrogate, oviposition, antixenosis.

4.1 Introduction

Chilo partellus (Swinhoe) (Lepidoptera: Crambidae) is a serious pest of sorghum and maize in Eastern and Southern Africa. *Chilo sacchariphagus* Bojer (Lepidoptera: Crambidae) originated in south East Asia and has been a serious insect pest of sugarcane since the 19th century in Reunion, Mauritius and Madagascar (Rochat *et al*., 2001; Conlong and Goebel, 2002). Larvae of these borers cause damage to plants by initially feeding on the leaves, and later by boring into the stalk, which ultimately weakens the plant (Midega *et al*., 2010). “Deadhearts” are also formed due to damage caused to the growing points by the feeding larvae, and this leads to eventual death of the plant (Midega *et al*., 2010). Although *C. sacchariphagus* has not spread outside Mozambique into adjacent countries, the potential for an invasion by *C. sacchariphagus* into South Africa poses a great risk to the South African sugarcane industry (Goebel, 2006; Bezuidenhout *et al*., 2008). The control of this borer is very important and would save large amounts of money worldwide if it is controlled in the early stages (Bezuidenhout *et al*., 2008). *C. partellus* has adapted to sugarcane in Ethiopia and is present in the South African sugarcane agro-ecosystem (Way and Kfir, 1997; Assefa and Conlong, 2009). *C. partellus* may represent a threat similar to the one once posed by *Eldana saccharina* Walker (Lepidoptera: Pyralidae) before it added sugarcane to its list of host plants. Chemical and biological control of these pests can be expensive and harmful to the environment; whereas host-plant resistance offers a safe and relatively cheap method of control (Epidi *et al*., 2004).

The concept of ovipositional antixenosis behaviour is based on the idea that female insects will choose their hosts in a hierarchal manner, laying most of their eggs on

the preferred plant (Thompson and Pellmyr, 1991). This in turn will have an effect on the process of establishment of the insect on the plant (Varshney *et al*., 2007). It has not yet been confirmed whether host-preference directly effects larval performance, but in some cases there has been a strong correlation between host preference and larval performance. This could be as a result of females basing their choice of host on plant properties (morphological and chemical), that could influence larval behaviour (Midega *et al*., 2010). Resistance of plants to ovipositing is caused by characteristics of the plant which either fail to support ovipositing, including stimuli (attractants), or by plants containing ovipositional-inhibiting stimuli (repellents) (Kumarasinghe and Jepson, 2003). The surface of a plant is important in deterring or attracting other organisms, and it is where organisms first come into contact with the plant in order to establish themselves on the plant, and therefore physical and chemical structures on the plant surface are important in contributing to pest resistance (Howe and Schaller, 2008). Trichomes and/or hairs on the surface of plants have been used to give rise to insect-resistant cultivars (Peter *et al*., 1995). Trichomes can either be non-glandular, tiny hairs which physically deter insects, or they may be specialized glandular trichomes, morphological and chemical in nature, whereby they secrete substances which are stored or volatilized on the surface of the plant and are used to repel pests and prevent them from feeding (Johnson, 1975; Fernandes, 1994; Larkin *et al*., 2003, Martin and Glover, 2007; Howe and Schaller, 2008).

Chemical compounds may influence the choice of plant for ovipositing and the response of the female to the plant (Thompson and Pellmyr, 1991). *Papilio machaon* Linnaeus (Lepidoptera: Papilionoidae) (Swallowtail butterflies) show ovipositional responses to plants containing differing proportions of compounds, where only one compound present shows a weak ovipositional response, but when all compounds are present strong responses are observed (Thompson and Pellmyr, 1991). Odour, reflectance (i.e. colour of the host to the insect) and shape of the host plant also influence the behaviour of an insect to a plant for ovipositing (Kumarasinghe and Jepson, 2003). In some cases, ovipositional antixenosis effects have been used to develop resistant cultivars. An example can be seen in rice, where a cultivar resistant to rice folder *Cnaphalocrocis medinalis* Guenee (Lepidoptera: Crambidae) was developed after a susceptible cultivar was crossed with an antixenotic wild rice *Oryza*

brachyantha Roehr. Ovipositional responses to plants can also be seen in *Chilo infuscatellus* Snell which prefer to lay their eggs on 45 day old plants and in *C. sacchariphagus* which prefer to lay their eggs on the upper surface of leaves (Kumarasinghe and Jepson, 2003). In *C. partellus*, it has been shown that the female moths prefer to oviposit on Napier grass (*Pennisetum purpureum* Schumach) rather than on maize (*Zea mays* L.), even though subsequent larval development on Napier grass is poor (Midega *et al*., 2010). This observation has been used to develop a push-pull management strategy for controlling the pest in maize fields.

C. sacchariphagus has not yet been found in South Africa and therefore cannot be used to carry out host-plant resistance studies in the country, unless under quarantine. If sugarcane varieties which appear to be resistant to *C. partellus* are also resistant to *C. sacchariphagus* then the concept of 'surrogate insect resistance screening' will prove effective. Among the few South African varieties with known resistance or susceptibility to *C. sacchariphagus*, there appears to be a correlation with *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae) (thrips) rankings (Chapter One, Page 35). All of these pests feed on the whorl of the plant and therefore similar resistance mechanisms within the plant may act against them. Additionally, *C. partellus* and *C. sacchariphagus* are both top borers. The concept of 'surrogate insect resistance screening' has proven successful within the borer genus *Ostrinia* on maize (Overman, 1994).

Most host-preference studies using ovipositing Lepidoptera have been based on the use of simultaneous choice trials (Thompson and Pellmyr, 1991). In this study, *C. partellus* moths are used to test 20 selected sugarcane varieties at their seedling stage, in a series of simultaneous choice trials, for any ovipositional antixenosis resistance mechanisms. *C. partellus* eggs are laid on both the upper and under sides of the leaves, as well as on the stem. They are usually laid in clusters of approximately 100 eggs per female (Hutchison *et al*., 2008). Sugarcane varieties were discriminated based on the number of eggs and number of egg batches laid on them by female moths of *C. partellus.*

4.2 Materials and methods

4.2.1 Selection of sugarcane varieties

Twenty sugarcane varieties were selected for this study (Table 4.1). Varieties N22, N28, N25, N26 and N32 were selected based on *C. sacchariphagus* leaf feeding index ratings and from information obtained on *C. sacchariphagus* ratings from the paper published by Conlong *et al*., (2004). The 'leaf feeding index' is a non-destructive measurement of damage by borers used to assess their leaf feeding behaviour and to determine susceptibility or resistance of different maize and sorghum varieties to *C. partellus* and *Busseola fusca* Fuller (Lepidoptera: Noctuidae) borers. The remaining varieties were selected based on *F. serrata* numbers obtained from a field trial run by the South African Sugar Research Institute (SASRI, KwaZulu-Natal, South Africa). Thrips has a similar feeding mechanism to *Chilo* spp. (both feed on the whorl of the plant), and therefore similar resistance mechanisms in plants could act against all of these pests. R570 and R576 are resistant and susceptible varieties to *C. sacchariphagus,* respectively, according to a study done by Nibouche and Tibere (2010) and were therefore also used in this study.

Table 4.1 Sugarcane varieties selected to be incorporated into the artificial diet of *Chilo partellus* for ovipositional (antixenosis) resistance mechanism studies. R = Resistant, S = Susceptible.

Variety	**Av. No. Thrips/ leaf whorl**	***C. sacchariphagus* leaf feeding index) (Conlong *et al*., 2004)**	**Total *C. s* Rating (low value = R; high value = S) (Conlong *et al*., 2004)**
Co1287	21		
Co6505	67		
R 576	27		
R 573	45		
R 570	49		
R 572	127		
R 568	153		
N24	38		
N22	40	13.9	23 (I)
N28	47	7.1	6 (R)
N25	70	15.5	36 (S)
N26	74	30.2	44 (S)
N31	91		
M1135/64	19		
M861/60	21		
M1025/70	58		
N27	120		
N21	165		22 (I)
N32	19	1.8	19 (I)

4.2.2 Oviposition experiments using *Chilo partellus* moths in a controlled environment

Antixenosis for ovipositing was studied under multi-choice conditions in a controlled environment ideal for that of *C. partellus* moths. The room used to conduct experiments was maintained at 27 +/- 2^{o}C, 70 +/- 5% and an 8 hour light: 16 hour dark photophase provided by 65 watt colour Triphos fluorescent tubes. Light was also supplemented by natural lighting from windows. Insects were obtained from the colony established at the South African Sugar Research Institute (SASRI, Kwazulu-Natal, South Africa). Stalks from the selected sugarcane varieties were collected from established field trials at SASRI and used for planting. Stalks were cut into single budded setts using secateurs, and then hot water treated at 50^{o}C for 30 minutes in a water bath (Lasec, Durban, South Africa). The setts were planted into 98 well trays (Hygrotech, Pretoria, South Africa) for germination to take place. The medium in which they were planted was a 1:1 ratio of potting mix (Grovida, Durban, South Africa) and vermiculite (Grovida, Durban, South Africa). Trays were kept in a glasshouse and manually watered on a daily basis. Twenty-day-old seedlings were used for experiments because this is the stage when female stem borer moths lay eggs on plants under natural conditions (Kumar *et al*., 2007). The method used for experiments can be seen in Figure 4.1.

Two different ovipositional experiments were conducted, with the number of moth pairs used differing for each one. Twenty seedlings of the 20 selected varieties were planted per 98 well tray in a completely randomized design, with one row and one column of wells left empty between varieties (Appendix 4.1). There were five replications in the first experiment, with each replication consisting of one tray. Research randomizer (Urbaniak, 1997) was used to obtain the random design. Trays were placed into BugDorm® insect rearing tents (BugDorm-2400, Megaview Science Co. Ltd., Taiwan), with each BugDorm® housing one tray. Five pairs of newly emerged *C. partellus* moths were released into each BugDorm® rearing tent for the first experiment. The moths were provided with water in a cotton swab (Shanghai Ristea Industries Co. Ltd., China). Moths were allowed five days for mating and ovipositing, after which trays were removed from the BugDorms, and egg number

and egg batch number counted per variety for each replication. A Niko SMZ 1500 stereoscopic zoom microscope was used to count the eggs on the leaves more precisely (Nikon, New York, USA). A Nikon DS-Fi1 camera (Nikon, New York, USA) fitted to the microscope was used to take stereo images of *C. partellus* eggs and egg batches on leaves. Experiment Two was conducted in exactly the same way as Experiment One, except 10 pairs of *C. partellus* moths were used in each BugDorm® tent, with 10 replications.

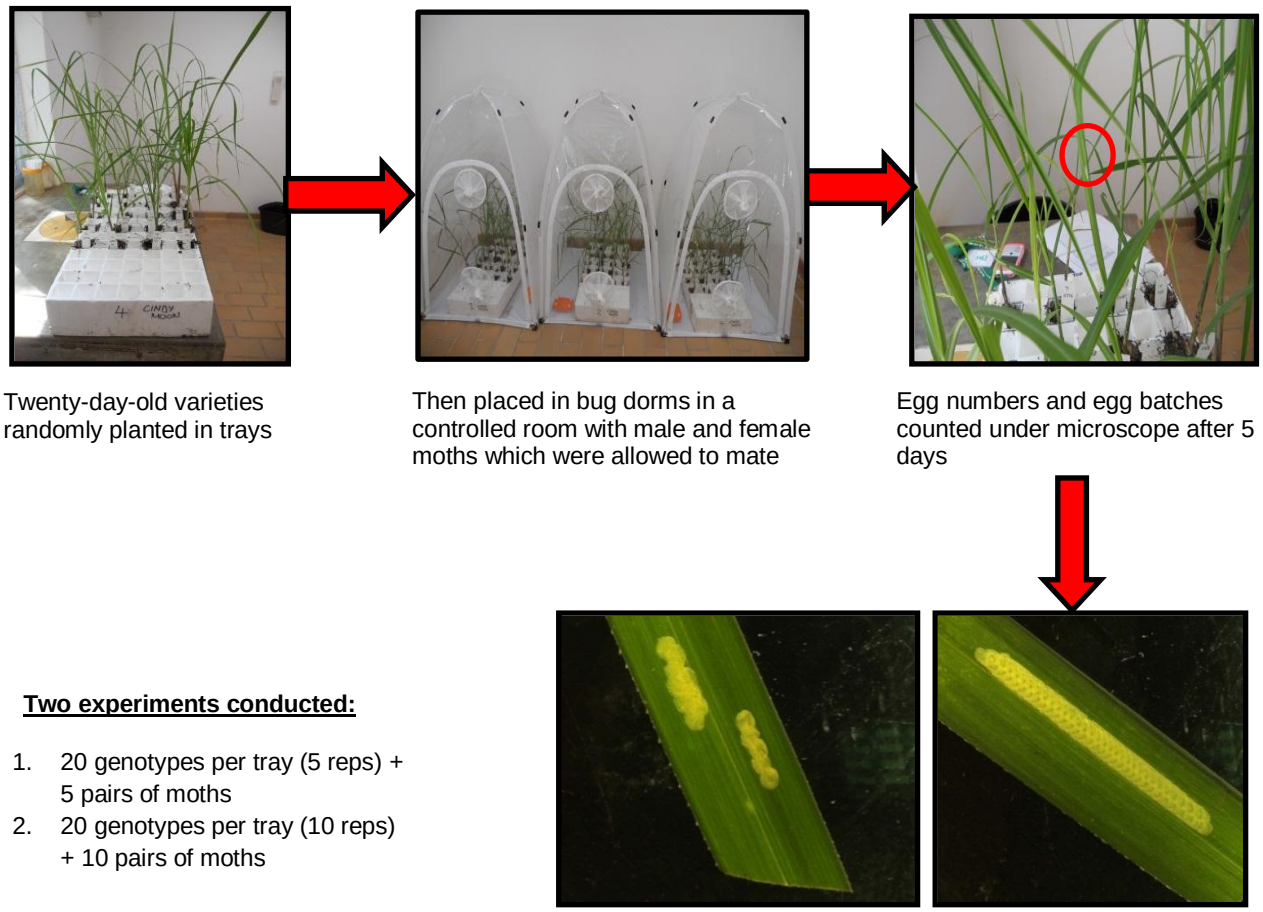

Figure 4.1 Method used for ovipositional experiments using *Chilo partellus* moths

4.2.3 Statistical analysis

Software used to analyse data was GenStat release 14th edition (VSN International, Hemel Hempstead, UK) (Payne *et al*., 2011). Number of eggs and number of egg batches were used for statistical analysis for all experiments.. Prior to analysis, the W-test for normality (Shapiro and Wilk, 1965) was used on the data and it was

subjected to $\log_{10}$ transformations where necessary. The data was subjected to general analysis of variance (ANOVA) (Shapiro and Wilk, 1965) and means were separated using the Sidak test (Abdi, 2007). The standard error of the mean (SEM) was calculated for each parameter and presented in tables.

4.3 Results

There were no statistically significant differences between sugarcane varieties for the mean number of eggs laid and for the mean number of egg batches for both Experiments One and Two, where F pr. values were all greater than 0.05 (Appendices 4.2, 4.3, 4.4 and 4.5). However, tendencies in the mean number of eggs and mean batch numbers between varieties were still observed. In Experiment One, where five pairs of moths were used, the variety R568 gave the highest mean number of eggs across replications (115.6), closely followed by N24 (113.8), N27 (103.8) and Co1287 (99.8) (Table 4.2). The least number of mean eggs in Experiment One was shown by varieties N32 (23.6), followed by R574 (26.6), M1135/64 (29.4) and R576 (36.6) (Table 4.2). Similar results were shown for mean batch numbers per variety for Experiment One. In Experiment Two, where 10 pairs of moths were used, N28 gave the highest mean number of eggs of 143, followed by Co1287 (134.6) and M1025/70 (114.6) (Table 4.2). R573 gave a very low mean batch number in Experiment Two of 11.4, followed by N32 (34.6) and R570 (35.4) (Table 4.2).

Table 4.2 Mean *Chilo partellus* egg batch and egg numbers per plant for Experiments One and Two. General ANOVA was used to analyse the data, means are followed by their standard error (SE) values (Exp 1 egg number F pr = 0.833, Appendix 4.2, Exp 1 batch number F pr. = 0.886, Appendix 4.3, Exp. 2 egg number F pr. = 0.439, Appendix 4.4, Exp 2 batch number F pr. = 0.195, Appendix 4.5).

	Mean batch number		**Mean egg number**	
	Experiment		**Experiment**	
Variety	**1**	**2**	**1**	**2**
CO1287	3.6±1.887	6.4±2.638	99.8±39.65	134.6±50.24
CO6505	2±1.304	3.4±1.208	52±30.4	93.6±45.92
M1025/70	3±1.14	4.4±2.315	83±29.63	114.6±64.55
M1135/64	1.4±0.678	3.4±1.536	29.4±18.53	83.8±39.35
M861/60	2.8±1.158	4.4±0.748	69±28.2	110±25.18
N21	2.8±1.715	3.4±1.536	72.8±38.67	68±23.83
N22	3.8±1.158	2±0.447	73.8±27.7	35±7.04
N24	3.4±0.98	3.2±1.855	113.8±36.12	96.2±51.09
N25	4.6±2.293	3.2±1.02	75±37.59	79.4±28.96
N26	2.4±0.872	2.6±1.166	70±30.19	73.8±35.16
N27	5±3.114	2.4±0.51	103.8±40.57	69.2±17.81
N28	3.4±1.778	6±1.871	73.4±28.06	143±46.22
N31	3.4±2.676	4±1.949	62.8±52.08	96.4±40.18
N32	1.2±0.374	0.8±0.374	23.6±9.35	34.6±16.41
R568	5±3.146	2.2±0.917	115.6±81.03	48.4±22.91
R570	2.6±0.927	1.2±0.735	74.6±26.51	35.4±20.48
R572	2±0.548	4.6±1.778	42.4±14.52	91±31.73
R573	1.4±0.4	0.6±0.245	67±27.88	11.4±6.46
R574	1.4±0.678	3.8±1.828	26.6±14.04	94.6±45.05
R576	1.2±0.735	4.8±1.393	36.6±16.43	91.8±30.08
Mean	2.82	3.34	68.25	80.24
SD[a]	1.22	1.56	27.30	34.28

[a] SD =standard deviation

4.4 Discussion

No statistically significant differences occurred between varieties with respect to the number of batches and the number of eggs laid by *C. partellus* moths, and therefore no substantial conclusions can be made. . The experimental plan used in this study, whereby groups of female and male insects were placed into a cage with test plants, and the final number of eggs laid counted, is a common method for determining oviposition behaviour. However, this method may not be the ideal method because the final distribution of eggs could be represented by a combination of females that have different degrees of preference and ranking of host plants for oviposition. Furthermore, there may be competition among females for sites of oviposition which would ultimately result in a more uniform distribution of eggs (Thompson and Pellmyr, 1991).

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4.6 Appendix

Appendix 4.1 Layout and randomization of sugarcane varieties in trays for ovipositional experiments using *C. partellus* moths

short side of tray

long side of tray

1	6	11	16			
2	7	12	17			
3	8	13	18			
4	9	14	19			
5	10	15	20			

8	11	19	15			
7	1	2	14			
13	10	6	12			
4	9	18	16			
20	17	5	3			

20	9	5	11			
15	1	4	7			
19	16	10	14			
17	2	12	13			
3	18	6	8			

11	7	1	14			
20	19	2	8			
9	4	6	12			
13	5	15	17			
3	16	18	10			

14	16	3	4			
18	19	9	10			
13	20	8	15			
17	6	5	11			
7	12	2	1			

Genotype code	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Genotype name	Co1287	Co6505	R576	R573	R570	R572	R574	R568	N24	N22
Genotype code	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Genotype name	N28	N25	N26	N31	M1135/64	M861/60	M1025/70	N27	N21	N32

Appendix 4.2 Experiment One (five pairs of moths): Results of ANOVA for egg number

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	CV%
Rep stratum	4	66660	16665	3.01		42.3
Rep.*Units* stratum						109
Variety	19	70820	3727	0.67	0.833	
Residual	76	420566	5534			
Total	99	558047				

Appendix 4.3 Experiment One (five pairs of moths): Results of ANOVA for batch number

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	CV%
Rep stratum	4	119.76	29.94	2.47		43.4
Rep.*Units* stratum						123.4
Variety	19	140.76	7.41	0.61	0.886	
Residual	76	920.24	12.11			
Total	99	1180.76				

Appendix 4.4 Experiment Two (10 pairs of moths): Results of ANOVA for egg number

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	CV%
Rep stratum	4	77051	19263	3.38		38.7
Rep.*Units* stratum						
Variety	19	111648	5876	1.03	0.439	94.1
Residual	76	433603	5705			
Total	99	622302				

Appendix 4.5 Experiment Two (10 pairs of moths): Results of ANOVA for batch number

Source of	d.f.	s.s.	m.s.	v.r.	F pr.	CV%
Rep stratum	4	155.14	38.785	4.22		41.7
Rep.*Units*						
Variety	19	230.84	12.149	1.32	0.195	90.8
Residual	76	698.46	9.19			
Total	99	1084.44				

Appendix 4.6 Experiment One and Two combined: Results of ANOVA for batch number

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	224.42	56.11	5.24	
Rep.*Units* stratum					
Exp	1	13.52	13.52	1.26	0.263
Variety	19	200.32	10.54	0.99	0.481
Exp.Variety	19	171.28	9.01	0.84	0.654
Residual	156	1669.18	10.70		
Total	199	2278.72			

Appendix 4.7 Experiment One and Two combined: Results of ANOVA for egg number

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	126882.	31721.	5.68	
Rep.*Units* stratum					
Exp	1	7188.	7188.	1.29	0.258
Variety	19	97355.	5124.	0.92	0.562
Exp.Variety	19	85114.	4480.	0.80	0.702
Residual	156	870998.	5583.		
Total	199	1187537.			

CHAPTER FIVE

EVALUATION OF RESISTANCE OF SUGARCANE VARIETIES TO *CHILO PARTELLUS* (SWINHOE) (LEPIDOPTERA: CRAMBIDAE) UNDER GLASSHOUSE CONDITIONS

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Abstract

Chilo sacchariphagus and *Chilo partellus* are two borers which are a threat to the South African sugarcane industry. *C. partellus* and *C. sacchariphagus* both initially feed on the whorl of their hosts before boring into the stalk, resulting in destruction to the growing point and extensive stalk damage. Host-plant resistance plays a pivotal role in controlling such pests and therefore it is important to identify sugarcane varieties that could have resistance against them. Glasshouse trials conducted in pots were used to compare 21 sugarcane varieties for their resistance or susceptibility to *C. partellus*. Leaf whorls of 21 day old plants were inoculated with 10 neonate larvae, and after 30 days varieties were assessed for various damage parameters. Results from these trials give preliminary indications as to whether *Fulmekiola serrata* and *Chilo* resistances are correlated; whether *C. partellus* or *F. serrata* could serve as surrogates in assessing resistance to *C. sacchariphagus*; and whether *C. partellus* itself poses a threat to sugarcane in South Africa. There was a significant difference between sugarcane varieties for the mean number of

shotholes/lesions, mean number of borings and mean number of larvae recovered. Results from this study indicate that N32, N28 and R570 show high levels of resistance against *C. partellus,* which concur with *C. sacchariphagus* ratings obtained from previous studies. Similarly, the variety R576 gave a susceptible rating to *C. sacchariphagus*, and an intermediate-suscpetible rating to *C. partellus*. Correlations of shotholes versus thrips numbers gave poor Pearson r values, however specific varieties such as N21, R568, and R572 which had high thrips numbers were also shown to be susceptible or to have intermediate ratings to *C. partellus* in the pot trials.

Keywords: *Chilo partellus*, *Chilo sacchariphagus*, sugarcane, host-plant resistance, sugarcane varieties, *Fulmekiola serrata*, surrogates.

5.1 Introduction

Chilo partellus (Swinhoe) (Lepidoptera: Crambidae) is one of the most important pests of sorghum (*Sorghum bicolor* L. Moench) and maize (*Zea mays* L.) worldwide and can cause serious damage to these crops (Kumar *et al*., 2007). *Chilo sacchariphagus* Bojer (Lepidoptera: Crambidae) is a serious pest of sugarcane (*Saccharum* L.) in Asia, on islands of the Indian Ocean and in Mozambique (Nibouche and Tibere, 2010). Damage caused by *C. sacchariphagus* to sugarcane in Reunion can result in losses of up to 25% in cane yield (Nibouche and Tibere, 2010). *C. partellus* and *C. sacchariphagus* are top borers that both pose a threat to the South African sugar industry. Early detection and control of these borers is therefore a biosecurity imperative (Conlong and Goebel, 2002; Bezuidenhout *et al*., 2008).

C. partellus and *C. sacchariphagus* have similar feeding mechanisms, whereby they feed directly from the vegetative tissue where sucrose is stored, which in turn has an effect on yield and quality (Vercambre *et al*., 2001). Larvae of these borers initially feed on the leaf whorl of their host, resulting in shotholes in the emerging leaves, which eventually become elongated lesions on fully elongated leaves (Padmaja *et al*., 2012). Older larvae then bore into the stem and damage the growing point of the plant resulting in ‘deadhearts’ (Kumar *et al*., 2007). Larvae continue to feed within the stem resulting in extensive tunnelling (Kumar *et al*., 2007; Padmaja *et al*., 2012). The

consequence is reduced foliage, early leaf senescence, interruption of translocation of nutrients and metabolites, decreased plant vigour, lodging and eventual death (Padmaja *et al*., 2012).

The use of resistant varieties is the most essential part of an integrated pest management (IPM) system. IPM has been used successfully to control a number of pests in sugarcane, including *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) (Vercambre *et al*., 2001) and more recently *Eldana saccharina* Walker (Lepidoptera: Pyralidae) (Rutherford, 2014). The use of resistant varieties reduces the amount of insecticides required in controlling these pests, which in turn allows for natural enemy populations to be sustained (Vercambre *et al*., 2001). Naturally occurring pest resistant traits in plants can be of either induced or constitutive resistance (Broekgaarden *et al*., 2011). Induced resistance requires the plant to recognize that there is an invader, which in turn results in the plant producing proteins or metabolites that restrict the invader (Keen, 1999; Underwood and Rausher, 2002). Constitutive resistance is the level of resistance already present in the plant and is not dependent on the attack of a pest (Do Vale *et al*., 2001). It can be morphological, structural or chemical in nature (Keen, 1999; Underwood and Rausher, 2002). There are three components of plant resistance to insect herbivores, namely, antixenosis, antibiosis and tolerance (Thayumanavan and Sadasivm, 2003). One or more of these mechanisms may be present in a resistant plant; however it is favourable for all three mechanisms to be present in a resistant variety (Ahman, 2006). A summary of these resistance mechanisms can be seen in Table 5.1. In sugarcane, three main types of resistance mechanisms have been thought to act against borer populations. These include ovipositional antixenosis, difficulty in larvae entering a plant either due to resistance in the stalk or due to resistance on the leaves where larvae feed initially and antibiosis acting against already established larvae (Mathes and Charpentier, 1969).

Table 5.1 Type of insect resistance mechanisms in plants and their related responses (adapted from Kumar, 1997)

Type of resistance mechanism	Response of insect or plant
Antixenosis	Repulsion of insects: avoidance or departure from plants Feeding: inhibition Oviposition: inhibition
Antibiosis	Metabolism of food ingested by insects: nutrition, metabolic disturbance Negative impact on larval development Reduced survival of larvae and adults
Tolerance	Repair, regeneration of damaged tissue of plants

In this study, 21 selected and diverse sugarcane varieties were whorl inoculated with *C. partellus* larvae under glasshouse conditions. A leaf feeding injury assessment was made and larval survival and the extent of subsequent borings were also assessed. This study also aimed to give a better indication as to whether *C. partellus* may pose a threat to the sugarcane industry, and to give preliminary indications as to whether it could be used as a surrogate insect in assessing resistance to *C. sacchariphagus*, by comparing results to previous ratings of sugarcane varieties to *C. sacchariphagus.* There is also a possibility that *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae) (thrips) can be used as a surrogate because it also feeds on the whorl of the plant, and because there may be a correlation between resistance rankings for *C. sacchariphagus* and thrips (see Chapter 1, Page 35).

5.2 Materials and methods

5.2.1 Selection of sugarcane varieties

Twenty one sugarcane varieties were selected for this study (Table 5.2). Sugarcane varieties N22, N28, N25, N26 and N32 were selected based on *C. sacchariphagus* leaf feeding index ratings and from information obtained on *C. sacchariphagus* ratings from a paper published by Conlong *et al*., (2004). The 'leaf feeding index' is a non-destructive measurement of damage by borers used to assess their leaf feeding behaviour, and to determine the susceptibility or resistance of different maize and sorghum varieties to *C. partellus* and *Busseola fusca* Fuller (Lepidoptera: Noctuidae) borers (Van den Berg and Van der Westhuizen, 1998). The remaining sugarcane

varieties were selected based on *F. serrata* numbers obtained from a field trial run by the South African Sugar Research Institute (SASRI, KwaZulu-Natal, South Africa). R570 and R576 are resistant and susceptible sugarcane varieties to *C. sacchariphagus,* respectively, according to a study done by Nibouche and Tibere (2010) and were therefore also used in this study. The glasshouse trial was performed twice. R574 was not available from the field for planting in the second trial, therefore it was decided that NCo376 would be used because it is the standard variety used in the artificial diet of *C. partellus* at SASRI.

Table 5.2 Sugarcane varieties used in glasshouse screening studies for resistance to *Chilo partellus*. R = Resistant, S = Susceptible.

Variety	**Av. No. Thrips/ leaf whorl**	***C. sacchariphagus* leaf feeding index) (Conlong *et al*., 2004)**	**Total *C. s* Rating (low value = R; high value = S) (Conlong *et al*., 2004)**
Co1287	21		
Co6505	67		
R 576	27		
R 573	45		
R 570	49		
R 572	127		
R 568	153		
N24	38		
N22	40	13.9	23 (I)
N28	47	7.1	6 (R)
N25	70	15.5	36 (S)
N26	74	30.2	44 (S)
N31	91		
M1135/64	19		
M861/60	21		
M1025/70	58		
N27	120		
N21	165		22 (I)
N32	19	1.8	19 (I)

5.2.2 Pot trials used to evaluate damage on sugarcane by *Chilo partellus* under glasshouse conditions

This experiment took place in a glasshouse at the South African Sugar Research Institute (SASRI, KwaZulu-Natal, South Africa). Stalks from the selected sugarcane varieties were collected from established field trials at SASRI and used for planting. Stalks were cut into single budded setts using secateurs, and then hot water treated in a water bath (Lasec, Durban, South Africa) at 50^{o}C for 30 minutes. Setts were

planted into 25 Litre PVC pots (30 cm diameter and 45 cm deep) (Grovida, Durban, South Africa) containing cleaned, coarse Umgeni river sand (Chain sands, Durban, South Africa). Pots were placed into troughs (Wardkiss, Durban, South Africa) filled with water to ensure that they would not dry out and that ants could not predate inoculated larvae. The experiment was laid out in a completely randomized design, with a total of five replications (Appendix 5.1). Each replication consisted of 20 pots representing all 20 sugarcane varieties (Figure 5.1b). Pots were arranged in double rows of 10 pots each for each replication, and spaced so that they did not come into contact with each other, to avoid contact between neighbouring plants. Planting was done over three weeks, with one sett being planted in each pot per week, until a total of three setts of the same variety were planted in each pot (Figure 5.1a). Planting was done in this manner for inoculation purposes. Pots were labelled with the variety name using pot labels (Wardkiss, Durban, South Africa). They were watered manually on a daily basis until inoculation of larvae took place, after which troughs were maintained to always be filled with water. Plants were fertilized monthly with 4:1:1 (44) N:P:K fertilizer (16 g/pot) (Grovida, Durban, South Africa).

When each plant reached 21 days of age, inoculations were performed. This is the stage when female stem borer moths lay eggs on plants under natural conditions (Kumar *et al*., 2007). Neonate larvae of *C. partellus* were provided by the established colony at SASRI. Inoculations took place over a period of three weeks, with the first-planted plant per pot being inoculated in the first week, the second in the second week, and the third plant in the third week until all the plants in the pot trial had been inoculated (Figure 5.1a). Each plant was inoculated with 10 neonate *C. partellus* (Nibouche and Tibere, 2010) larvae, and they were released directly into the central whorl of each plant with a camel hair brush (Winsor and Newton, London, UK). According to Sharma (1997), five to seven larvae usually causes a sufficient amount of damage to susceptible sugarcane varieties. Inoculated plants were labelled with the date using white labels (Wardkiss, Durban, South Africa). Inoculations took place in the early morning between 07h00 and 11h00 to avoid larval death due to high temperatures.

Thirty days after inoculating, damage assessments were made. Plants were cut at their base and used for analysis. The number of entry/exit holes (borings) due to larvae was counted (Figure 5.2a). Stalks were split open throughout their entire

length and the total length of tunnels in each stem was recorded (Figure 5.2b). Upon dissection of the plants, the number of larvae and pupae found were counted. Live larvae that were found on the outside of the plant were considered established. Larval weight of recovered larva was also recorded. The number of damaged leaves and the number of shotholes or lesions per leaf were counted and recorded (Figure 5.2c). Two different leaf feeding damage rating scales were used. One of the rating scales was on a scale of 0 to 4 (Figure 5.3), where 0 = no visible leaf injury, 1 = few shotholes or lesions observed on leaves, 2 = medium damage, 3 = heavy damage, and 4 = extensive and severe damage to leaves. The second rating scale was based on a method used by Conlong *et al*. (2004), which uses the number of damaged leaves and the number of feeding holes as part of a rating system on a scale of 1 to 9. A stalk damage rating system was also used, based on the same scale as that of the leaf feeding damage rating on a scale of 0 to 4 (Figure 5.4). An overall rating was given to each variety, based on the total number of borings, total number of larvae recovered and mean number of shotholes/lesions over the two pot trials that were carried outThis was a subjective rating whereby R = resistant, IR = intermediate-resistant, I = intermediate, IS = intermediate-susceptible, and S = susceptible

After the first pot trial was complete, a second pot trial was conducted in the exact same way. However, the Variety R574 was replaced with Variety NCo376 as there was no R574 planting material available from the field. The glasshouse was fogged using Doom Fogger (Checkers Hyper, Durban, South Africa) to kill off any predators or moths that may have an effect on the trial.

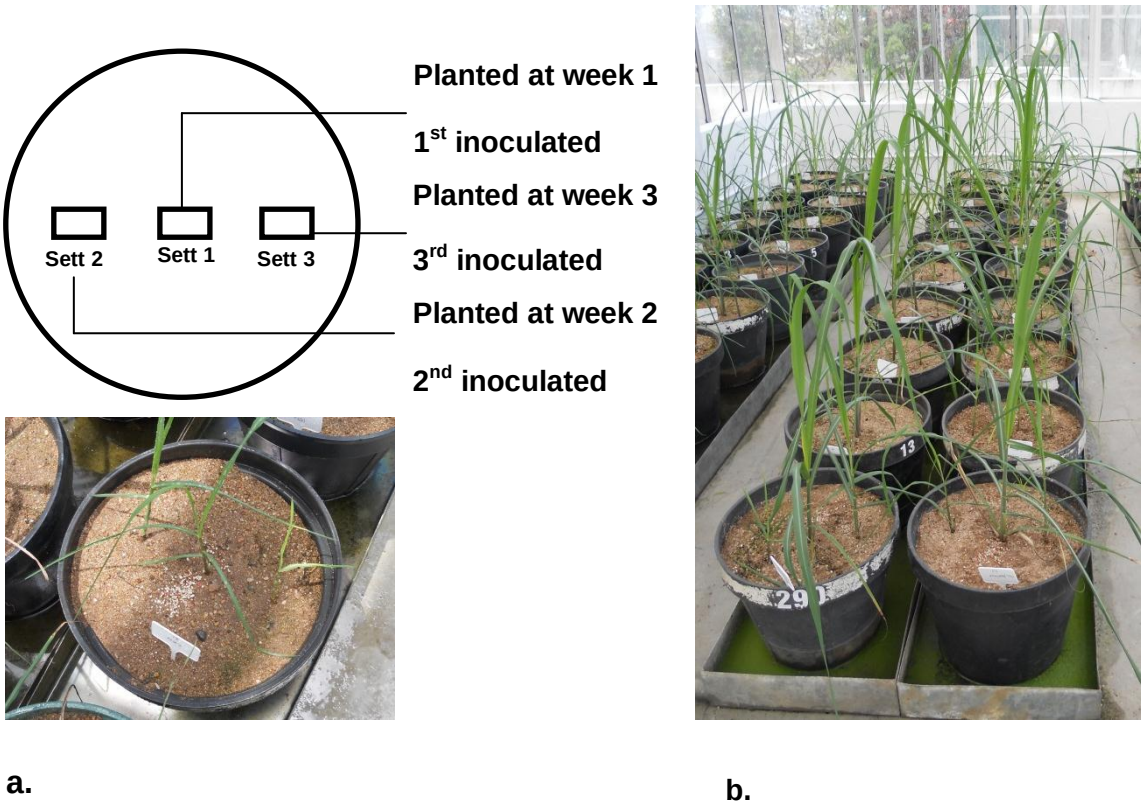

Figure 5.1 Layout of (a) individual pots and (b) a replication from the pot trial used for *Chilo partellus* resistance studies

Figure 5.2 Damage parameters used to assess *Chilo partellus* damage to sugarcane varieties (a) entry/exit holes (borings) (b) tunnel length and (c) Shotholes or lesions

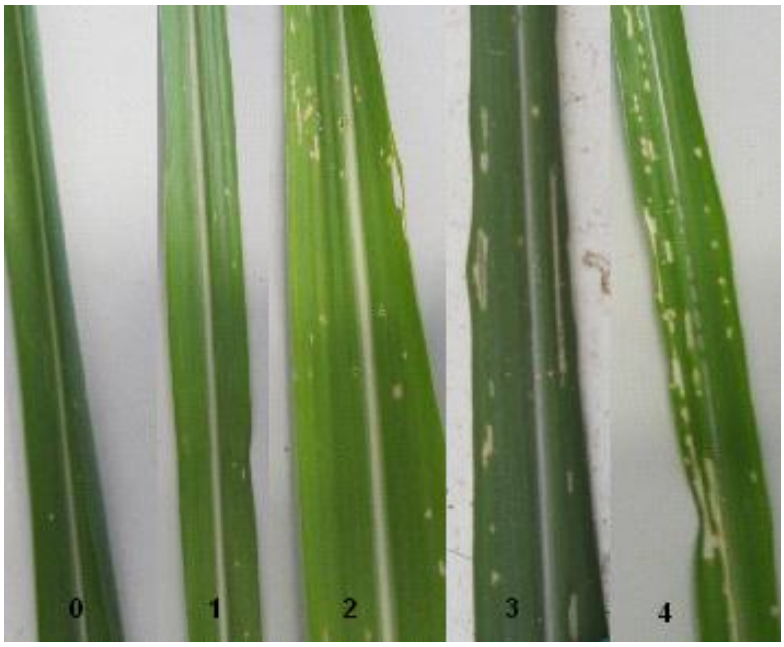

Figure 5.3 Examples of damage caused to sugarcane leaves by *Chilo partellus* used as a visual leaf rating system on a scale of 0 to 4

Figure 5.4 Examples of damage caused to sugarcane stalks by *Chilo partellus* used as a visual stalk rating system on a scale of 0 to 4

5.2.3 Statistical analysis

Software used to analyse data was GenStat release 14th edition (VSN International, Hemel Hempstead, UK) (Payne *et al*., 2011). The two pot trials conducted were analysed separately. Prior to analysis, the W-test for normality (Shapiro and Wilk, 1965) was used on the data for each parameter, and it was subjected to $\log_{10}$ or square root transformations where necessary. The data was subjected to Residual Maximum Likelihood (REML) variance component analysis (Harville, 1977) and means were separated using the Sidak test (Abdi, 2007). The standard error of the mean (SEM) was calculated and presented in tables alongside the means for each parameter.

5.3 Results

5.3.1 Damage assessment of sugarcane varieties for the first pot trial

There was a significant difference between sugarcane varieties for the mean number of shotholes/lesions, mean number of borings, and mean number of larvae counted in the first pot trial (Appendices 5.2, 5.3, 5.4). A summary of the damage parameters for all the sugarcane varieties for the first pot trial can be seen in Table 5.3. Variety M1025/70 had a higher mean number of shotholes/lesions than the other sugarcane varieties, significantly higher than N21, N24 and N32, which had the lowest mean number of shotholes/lesions. R576 also had a significantly higher mean shothole/lesion number compared to N32, which had the lowest mean number of shotholes/lesions. R576 had a significantly higher mean number of borings compared to all the sugarcane varieties, with the exception of sugarcane varietyM1025/70. M1025/70 had a significantly higher mean number of borings compared to R570, R574, Co1287, M1135/64 and N32, which all had the least mean number of borings. R570 and N32 were the only two sugarcane varieties found to have no borings. A total of 52 borings were counted for R576 across all replications, followed by N31 and M1025/70 of which 27 and 24 borings were counted, respectively. R576 had a significantly higher mean number of larvae compared to N32, where no larvae were found. The remaining sugarcane varieties did not differ

significantly from each other with respect to the mean number of larvae. The highest number of larvae found was for R576, with a total of 14 larvae found across all replications, followed by R568 with a total of 13 larvae counted across all replications. No larvae were found for R570, R574, M1134/64 and N32. No significant differences occurred between sugarcane varieties for mean larval weight and mean tunnel length (Appendices 5.5, 5.6). However, tunnel length was highest in N31 (14.125 cm), R572 (13.5 cm) and N27 (9.167 cm).

Pupae were recovered from stalks of varieties R572, R574, R576 and N25, with R574 and R576 having the highest number of pupae found (Table 5.3).

The mean leaf feeding scores can also be seen in Table 5.3. Varieties having a mean leaf feeding score of above 3 (heavy damage), when using the scale of 0-4, included R576 and M1025/70. This seems to concur with results from the other damage parameters which show the same varieties to have higher numbers of borings and larvae. Varieties showing a leaf feeding score of below 2 (few shotholes/lesions on leaves) included R570, R573, M1135/64, N24 and N32. When using the second leaf feeding rating system, score of of 1-9, similar results were observed. Again sugarcane varieties R576, Co6505, N31 and N22, had the highest scores, whilst R570, R573, M1135/64, N21, N24 and N32 had the lowest leaf feeding scores. Overall, the mean stalk feeding scores were a lot lower than the mean leaf feeding scores, using a scale of 0-4. The highest scores were observed for M1025/70, N21, N22, N27 and R568, while a stalk damage score of 0 was observed for R570 and N32 (Table 5.3).

In a study conducted by Conlong *et al*. (2004), an objective was to determine whether the assessment of leaf damage (i.e shotholes) could be used to replace the laborious task of destructive stalk splitting and the counting of borings. Similarly, in this study we wanted to determine if there was a correlation between the number of stalk borings and the number of shotholes on the leaves which could indicate whether the counting of shotholes could replace the laborious task of assessing stalk damage (Figure 5.5). A significant correlation between the two parameters was found with a Pearson r of 0.682 ($p < 0.05$) for Pot Trial One.

Table 5.3 Comparison of damage parameters caused by *Chilo partellus* larvae on 20 selected sugarcane varieties of sugarcane grown in Pot Trial One. The data was subjected to REML variance component analysis, Sidak test, and data $\log_{10}$ or square root transformation used where necessary. Untransformed data is presented here.

Variety	Mean no. shotholes per variety[a]	Total no. borings[b]	Mean borings per variety[a]	Total no. larvae[b]	Mean no. larvae per variety[a]	Mean larval weight (g)[a]	Mean tunnel length (cm)[a]	Total no. pupae[b]	Mean leaf feeding score (0-4)[a]	Leaf feeding score (1-9)	Mean stalk feeding score (0-4)[a]
R568	25.46±3.482abc	7	0.4667±0.2153ab	13	0.8667±0.3634ab	0.01009±0.00449	1.875±0.718	0	2.7	3	0.8
R570	15.71±2.708abc	0	0a	0	0ab	0	0	0	1.8	2	0
R572	28.46±4.75abc	5	0.3333±0.2323ab	3	0.2±0.1447ab	0.03575±0.02075	13.5±5.5	1	2.5	3	0.6
R573	17.2±3.303abc	6	0.4±0.2726ab	2	0.1333±0.0909ab	0.0115±0.0114	0	0	1.8	2	0.4
R574	23.67±4.929abc	1	0.0667±0.0667a	0	0ab	0.06667±0.00667	20±*	3	2.1	3	0.5
R576	43.92±4.438bc	52	3.4667±1.2105c	14	0.875±0.2562b	0.04662±0.01669	5.411±1.513	3	3.3	5	2
Co1287	27±5.302abc	5	0.3333±0.3333a	2	0.1333±0.1333ab	0.017±0.0067	0.75±0.25	0	2.0	3	0.5
Co6505	31.92±8.324abc	14	0.9333±0.4194ab	1	0.0667±0.0667ab	0.0167±*	2.5±*	0	2.5	4	0.7
M1025/70	49.72±6.311c	24	1.6±0.3879bc	5	0.3333±0.126ab	0.06696±0.02044	6.667±2.25	0	3.2	5	2
M1135/64	14.48±4.031abc	1	0.0667±0.0667a	0	0ab	0	5±*	0	1.6	2	0.1
M861/60	21.5±2.619abc	18	1.2±0.4598ab	1	0.0667±0.0667ab	0.0234±*	30±*	0	2.6	3	0.4
N21	14.64±1.847ab	12	0.8±0.4047ab	5	0.333±0.2108ab	0.04726±0.02698	2±0.5	0	2.2	2	0.8
N22	31.85±4.92abc	10	0.6667±0.1869ab	7	0.4667±0.2153ab	0.02899±0.01586	2.167±0.441	0	2.8	4	0.8
N24	11.82±11.82ab	11	0.7333±0.3305ab	2	0.1333±0.0909ab	0.00525±0.00515	1.75±0.25	0	1.8	2	0.3
N25	24.06±4.974abc	12	0.8±0.4276ab	5	0.3125±0.176ab	0.01482±0.00726	2±*	1	2.1	3	0.4
N26	23.85±2.713abc	12	0.8±0.3546ab	4	0.2667±0.2063ab	0.01155±0.00804	0	0	2.1	3	0.3
N27	27.36±6.641abc	7	0.4667±0.2906ab	2	0.1333±0.0909ab	0.165±0.0408	9.167±5.419	0	2.0	3	0.9
N28	23.73±6.633abc	7	0.4667±0.2153ab	1	0.0667±0.0667ab	0.0001±*	0	0	2.2	3	0.3
N31	31.28±4.302abc	27	1.8±0.579bc	13	0.8667±0.3065ab	0.03303±0.00791	14.125±4.185	0	2.8	4	1.8
N32	11.42±1.953a	0	0a	0	0a	0	0	0	1.3	2	0
mean	24.95	11.55	0.77	4.00	0.26	0.03	5.85	0.40	2.24	3.05	0.68
SD	9.91	12.03	0.80	4.48	0.29	0.04	7.97	0.94	0.54	0.94	0.60

Mean ± SE, values in a column followed by the same letter are not significantly different using the Sidak test (P = 0.05); *Standard errors could not be calculated due to only one recording; [a] mean over all plants per pot (inoculations) for all replications per variety;[b] Total over all plants per pot (inoculations) for all replications per variety

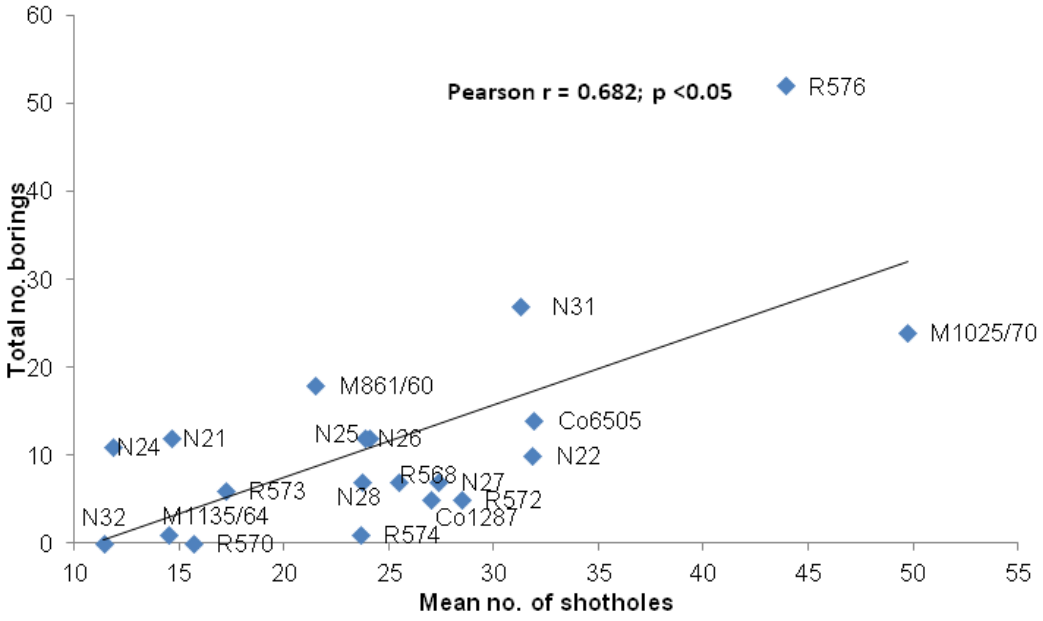

Figure 5.5 A significant correlation between the total number of *Chilo partellus* borings and the mean number of shotholes for 20 sugarcane varieties grown in Pot Trial One (PT1 Pearson correlation r = 0.682; p < 0.05).

5.3.2 Damage assessment of sugarcane varieties for the second pot trial

There was a significant difference between sugarcane varieties with respect to the mean number of shotholes/lesions, mean number of borings and mean number of larvae (Appendices 5.7, 5.8, 5.9). A summary of the damage parameters for all the sugarcane varieties for the second pot trial can be seen in Table 5.4. N22 and N25 had the highest mean number of shotholes/lesions, being significantly higher than N24 and R573 which had the least mean number of shotholes/lesions. Co1287 also had significantly higher mean number of shotholes than N24 and R573. The remaining sugarcane varieties did not differ significantly from each other with respect to the mean number of shotholes/lesions. M1025/70 had the highest mean number of borings and was significantly higher than the majority of the sugarcane varieties. M861/60 had the least mean number of borings and was significantly lower to that of M1025/70, N22 and N31. M1025/70 gave the highest number of total borings (47),

followed by N22 and N31 which had 28 and 26 borings in total respectively. The highest mean number of larvae was found in M1025/70, R572, Co6505, N21 and N31, while the lowest was found in M861/60, R570, Co1287, M1135/64, N24 and N25. There was no significant difference found between sugarcane varieties for mean larval weight and mean tunnel length (Appendices 5.10, 5.11).

Pupae were recovered from stalks of sugarcane varieties R568, Co1287, M1025/70 and N25. However, the maximum number of pupae recovered was one per variety, which was not significant enough to draw any relevant conclusions.

The mean leaf feeding scores can also be seen in Table 5.4. Sugarcane varieties having a mean leaf feeding score of above 3 (heavy damage), when using the scale of 0-4, included M1025/70 and N22. This seems to correlate with results from the other damage parameters which show the same varieties to have higher numbers of borings and larvae. Varieties showing a leaf feeding score of below 2 (few shotholes/lesions on leaves) included R573, R570, M1135/64, M861/60, N24 and N32. When using the second leaf feeding score rating system, of 1-9, similar results were observed. Sugarcane varieties R568, Co1287, M1025/70, N21, N25 and N22, had the highest scores, whilst R570, R573, R576, M1135/64, M861/60, N26, N28 and N32 gave the lowest leaf feeding scores. With respect to the mean stalk feeding score, varieties R570, M1135/64, M861/60, N24, N28 and N32 gave scores below 1, while M1025/70 gave the highest score of 2.6 (Table 5.4).

A correlation occurred between for the mean number of shotholes/lesions and the total number of borings found and is shown in Figure 5.6. A high number of larvae were associated with an increase in the number of borings and mean number of shotholes/lesions.

Table 5.4 Comparison of damage parameters caused by *Chilo partellus* larvae on 20 selected sugarcane varieties of sugarcane grown in Pot Trial Two. The data was subjected to REML variance component analysis, Sidak test, and data $\log_{10}$ or square root transformation used where necessary. Untransformed data is presented here.

Variety	Mean no. shotholes per variety[a]	Total no. borings[b]	Avg borings per variety[a]	Total no. larvae[b]	Mean no. larvae per variety[a]	Mean larva weight (g)[a]	Mean tunnel length (cm)[a]	Total no. pupae found[b]	Mean leaf feeding score (0-4)[c]	Leaf feeding score (1-9)	Mean stalk feeding score (0-4)[a]
R568	35.12±5.608b	17	1.1333±0.2906abc	6	0.4286±0.1373ab	0.0465±0.00798	10.545±2.155	1	2.6	4	1.8
R570	18.43±4.578ab	20	1.3333±0.8819ab	2	0.1333±0.0909a	0.1285±0.00855	9.8±2.871	0	1.6	2	0.8
R572	29.68±5.476ab	15	1.6±0.636abc	12	1.2±0.2906bc	0.03475±0.00694	8.444±2.28	0	2.4	3	2
R573	10±3.928ab	8	0.5333±0.2557ab	4	0.2667±0.1182a	0.0255±0.00911	3.667±1.382	0	1.3	2	1
R576	17.11±4.988ab	9	0.6±0.235ab	7	0.4667±0.1919ab	0.03165±0.01353	6.167±1.833	0	2	2	1.4
Co1287	40.21±9.156ab	12	0.8±0.3677ab	2	0.1333±0.0909a	0.09343±0.05148	12.5±5.994	1	2.4	4	1.7
Co6505	18.57±4.458ab	17	1.1333±0.3887abc	12	0.8±0.3117abc	0.05331±0.01327	9.357±1.543	0	2.3	2	1.6
M1025/70	33.14±4.672b	47	3.1333±0.5595c	21	1.3125±0.2366c	0.05658±0.01013	11.5±2.014	1	3.2	4	2.6
M1135/64	13.45±6.131a	4	0.2667±0.1817ab	2	0.1333±0.0909a	0.06055±0.00055	10.333±5.044	0	1.0	2	0.8
M861/60	10.35±5.021ab	3	0.2±0.2a	0	0a	0	3±*	0	0.9	2	0.2
N21	32.31±6.667ab	22	1.6±0.3491abc	12	0.8±0.1447abc	0.02573±0.00655	4.646±0.905	0	2.3	4	1
N22	46.33±6.285ab	28	1.8667±0.4667abc	7	0.4667±0.1652ab	0.02476±0.00634	7.842±1.298	0	3.1	5	2
N24	10.4±3.305ab	3	0.2±0.1069ab	2	0.1333±0.0909a	0.0296±0.0295	2.25±0.25	0	1.6	2	0.1
N25	46.03±7.307ab	8	0.5333±0.3362ab	1	0.0667±0.0667a	0.0661±0.0066	16.667±3.073	1	2.7	5	2
N26	20.61±4.561ab	11	0.7333±0.3003ab	7	0.4667±0.1919ab	0.03331±0.01131	5.75±1.493	0	2.0	2	0.9
N27	28.8±5.447ab	8	0.4667±0.1652ab	7	0.4667±0.1919ab	0.02164±0.00649	8.571±2.581	0	2.5	3	1
N28	17.23±4.413ab	6	0.4±0.2138ab	4	0.2667±0.1533a	0.00907±0.00897	8.75±3.614	0	2	2	0.6
N31	28.62±4.461ab	26	1.7333±0.3838bc	12	0.8±0.1447abc	0.04104±0.00807	9.25±2.842	0	2.9	3	1.7
N32	20.42±6.518ab	10	0.7333±0.3446ab	4	0.2667±0.1182a	0.00873±0.0037	2.6±0.245	0	1.4	2	0.6
NCo376	30.22±8.904ab	7	0.4667±0.2153ab	4	0.2667±0.1182a	0.02917±0.01321	4.833±1.108	0	2	3	1
mean	25.35	14.05	0.97	6.40	0.44	0.04	7.82	0.20	2.08	2.90	1.24
SD	11.38	10.67	0.73	5.18	0.37	0.03	3.72	0.41	0.66	1.07	0.67

Mean ± SE, values in a column followed by the same letter are not significantly different using the Sidak test (P = 0.05); *Standard errors could not be calculated due to only one recording ;[a] mean over all plants per pot (inoculations) for all replications per variety; [b] Total over all plants per pot (inoculations) for all replications per variety

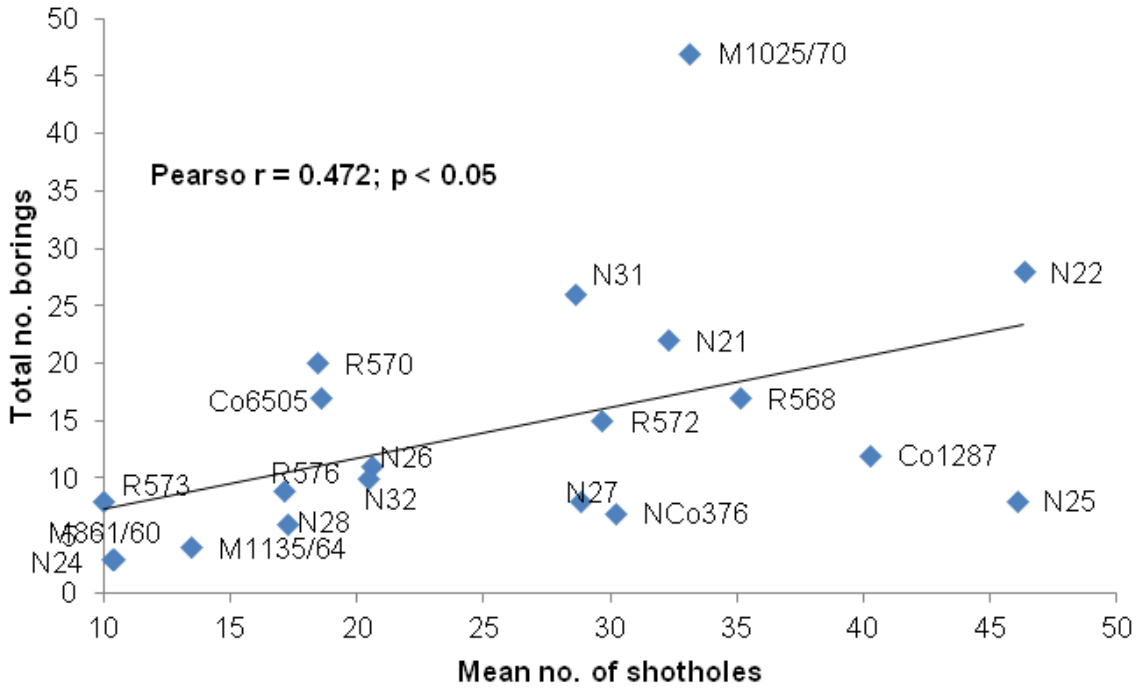

Figure 5.6 A significant correlation between total number of *Chilo partellus* borings and mean number of shotholes for 20 sugarcane varieties grown in Pot Trial Two (PT2 Pearson correlation r =0.472; p < 0.05).

5.3.3 Comparison of Pot Trial One versus Pot Trial Two

Sugarcane varieties N31, Co6505 and M1025/70 gave consistently high number of borings, above both trial means for both pot trials (labelled in red in Figure 5.7). M1135/64, N32, R573, N27, N28, N24 and Co1287 gave consistently low number of borings for both trials (labelled in blue in Figure 5.7). However, not all varieties performed consistently between trials, with R570 having no borings in the first pot trial and 20 borings in the second pot trial. Similarly, M861/60 had a high number of borings for the first pot trial and a low number of borings for the second pot trial (Figure 5.7).

Eleven sugarcane varieties were shown to perform consistently between both pot trials with respect to the total number of larvae recovered per variety (Figure 5.8). Again, M1025/70 and N31 gave a consistently high number of larvae for both pot

trials and N32, N28, N24, R573 and M861/60 gave consistently low numbers of larvae (Figure 5.8).

Sugarcane varieties having a consistently high number of mean shotholes/lesions for both trials were labelled in red and included M1025/70, N31, Co1287, R568, R572, N27 and N22 (Figure 5.9). Consistently low mean number of shotholes/lesions was seen for M861/60, N28, R570, M1135/64, N24, N32, R573 and N26 (Figure 5.9).

Sugarcane varieties to perform consistently for all of the above mentioned parameters across both pot trials were M1025/70 and N31, giving consistently high values, and M1135/64 and N32, giving very low values for all of the damage parameters (circled in Figures 5.7-5.9).

In Table 5.5 varieties are classified into resistant, intermediate, or susceptible groupings, based on the mean number of shotholes/lesions, total number of larvae recovered and the total number of borings for Pot Trial One and Pot Trial Two. With regards to the overall rating, two varieties were classified as susceptible, five as intermediate-susceptible, four as intermediate, one as intermediate-resistant and nine as resistant. The two susceptible varieties were M1025/70 and N31, while the nine resistant varieties were R570, R573, R574, Co1287, M1135/64, M861/60, N24, N28, and N32.

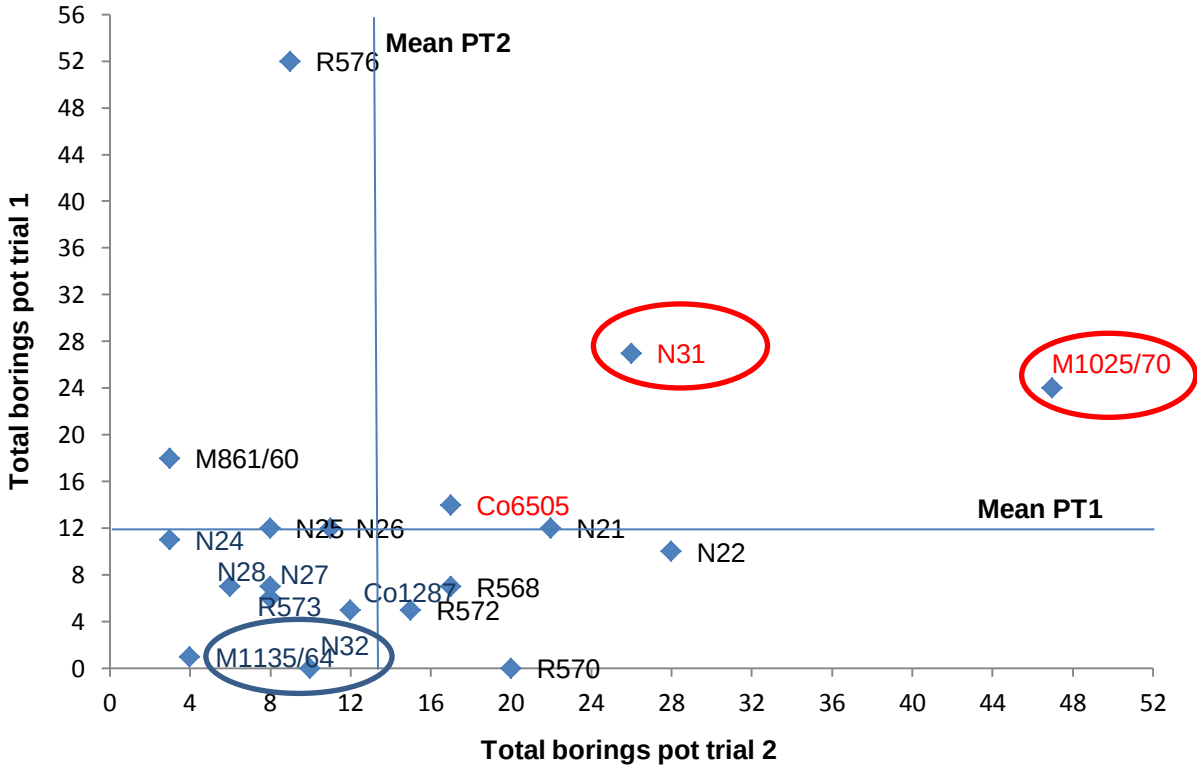

Figure 5.7 Total number of *Chilo partellus* borings per variety for Pot Trial One (PT1) versus Pot Trial Two (PT2)

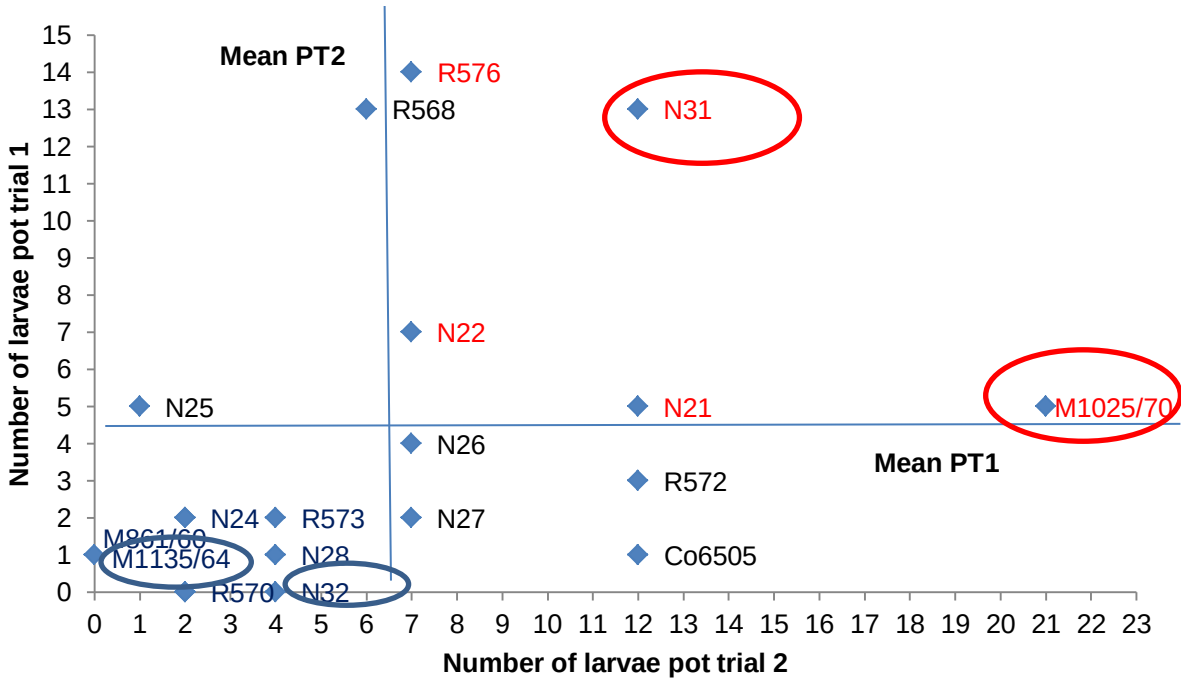

Figure 5.8 Total number of *Chilo partellus* larvae per sugarcane variety for Pot Trial One (PT1) versus Pot Trial Two (PT2)

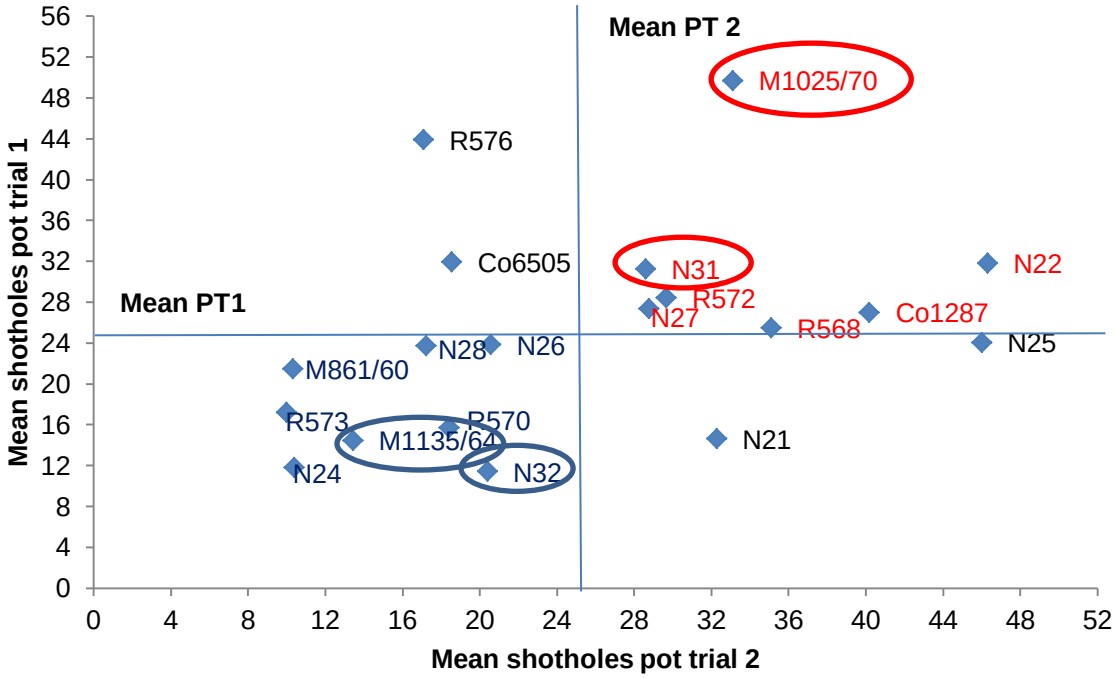

Figure 5.9 Mean number of shotholes/lesions made by larvae of *Chilo partellus* per sugarcane variety for Pot Trial One (PT1) versus Pot Trial Two (PT2)

Table 5.5 Subjective overall ratings based on the balance of the three statistically significant damage parameters for Pot Trial One and Pot Trial Two. R = resistant (Highlighted in peach); IR = intermediate-resistant; I = intermediate; IS = intermediate-susceptible; S = susceptible (Highlighted in yellow).

Variety	Borings Trial 1, Trial 2	Larvae Trial 1, Trial 2	Shot-holes Trial 1, Trial 2	Overall Rating
R 568	R,I	S,I	I,S	IS
R 570	R,S	R,R	I,I	R
R 572	R,I	R,S	I,S	I
R 573	R,R	R,R	R,R	R
R 574	R,-	R,-	I,-	R
R 576	S,I	S,I	S,R	IS
Co1287	R,I	R,R	I,S	R
Co6505	I,I	R,S	S,I	IS
M1025/70	S,S	I,S	S,S	S
M1135/64	R,R	R,R	R,R	R
M861/60	I,R	R,R	I,R	R
N21	I,S	I,S	R,S	IS
N22	I,S	I,I	I,S	IS
N24	I,R	R,R	R.R	R
N25	I,I	I,R	I,S	I
N26	I,I	I,I	I,I	I
N27	R,R	R,I	I,I	IR
N28	R,R	R,R	I,I	R
N31	S,S	S,S	I,I	S
N32	R,I	R,R	R,I	R
NCo376	-,R	-,I	-,I	I

5.3.4 Comparison between *Chilo sacchariphagus, Chilo partellus*, and *Fulmekiola serrata* ratings

Ratings obtained for *C. sacchariphagus* (from Conlong *et al*., (2004), and Nibouche and Tibere, (2010)) and *C. partellus* for Natal and Reunion varieties can be seen in Table 5.6. Variety N28 is resistant to both *C. sacchariphagus* and *C. partellus*. Variety N32 is intermediate-resistant to *C. sacchariphagus* whilst it was one of the most resistant to to *C. partellus*. Ratings for N21 and N22 are intermediate to *C. sacchariphagus* and intermediate-susceptible to *C. partellus* respectively. Varieties N25 and N26 are susceptible to *C. sacchariphagus* whilst intermediate ratings were obtained for *C. partellus*. The variety R570 is resistant to both *C. sacchariphagus* and *C. partellus*, and R576 is susceptible to *C. sacchariphagus* and rates as intermediate-susceptible to *C. partellus* in this study.

The correlation between the mean number of shotholes/lesions per variety versus the mean number of thrips per leaf whorl was poor where pearson r values of 0.037 and 0.381 were obtained for Pot Trial One and Pot Trial Two respectively (Figure 5.10).

Table 5.6 Comparison of resistance ratings for *Chilo sacchariphagus* and *Chilo partellus* for those varieties with data for both.

Variety	***C. sacchariphagus* total ratings for N varieties (Conlong *et al*., 2004)**		***C. sacchariphagus* ratings for R varieties (Nibouche and Tibere, 2010)**	***C. partellus* overall rating (Table 5.5)**
R570	-	-	R	R
N28	6	R	-	R
N32	19	I	-	R
N21	22	I	-	IS
N22	23	I	-	IS
N25	36	S	-	I
N26	44	S	-	I
R576	-	-	S	IS

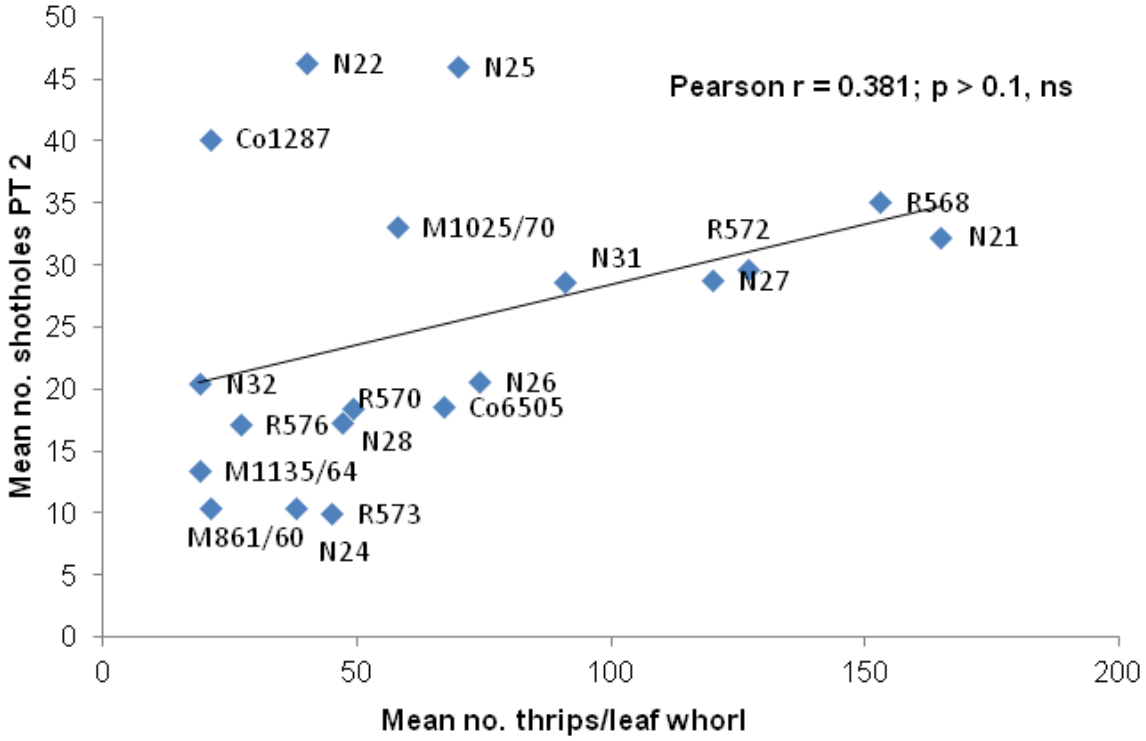

Figure 5.10 Correlation plot of the mean number of shotholes/lesions made by *Chilo partellus* larvae per variety from pot trial two (PT2) versus the mean number of thrips per leaf whorl for the same varieties, obtained from a field trial run by the South African Sugar Research Institute. (PT1 Pearson correlation r = 0.037; p > 0.1, not significant).

5.4 Discussion

The results from the pot trials, regarding the various damage parameters, indicated that the screening method used was able to discriminate between varieties for resistance to *C. partellus* larvae. Varieties differed significantly for all damage parameters recorded, except for mean tunnel length and mean larval weight (Appendices 5.2-5.11).

Although ten neonate larvae were inoculated into the whorl of each plant, the final number of larvae found to be established was relatively low across all sugarcane varieties. In the first pot trial, the highest total number of larvae found across all replications was 14 on Variety R576, and in the second pot trial, a total of 21 larvae were found for Variety M1025/70. A total of 150 larvae were inoculated per variety

per pot trial. Sharma (1997) recommended that five to seven neonate larvae should be used to inoculate plants, however, it is indicated from this study that higher numbers of larvae (30-40) should be used for inoculations for sugarcane varieties. The tougher leaf tissue and differing structure of the whorl of sugarcane compared to sorghum could be a contributing factor to the higher number of larvae required for inoculations.

No dead larvae were recovered from on or inside the plants or in the pots. The inability of larvae to feed and survive on the plant whorl of most of the varieties is a possible reason for low numbers of larvae being established. This could be due to chemical or physical properties of the leaf which inhibit larval growth (Kamala *et al*., 2012). Antixenosis effects of specific sugarcane varieties could also have had an effect on larval feeding initiation (Thayumanavan and Sadasivm, 2003). Low numbers of larvae found on R570, M1135/64, N32 and M861/60, in both pot trials, could indicate that these varieties have some form of resistance (antibiosis or antixenosis; constitutive or induced) against *C. partellus*. In a study conducted by Kumar (1995), it was found that the percentage of larvae recovered from resistant maize varieties was significantly lower than that of a susceptible variety. This could be due to antibiotic effects of certain chemicals in the plant tissues, or the inability of the larvae to feed on the plant due to structural features (Kamala *et al*., 2012).

The more susceptible sugarcane varieties had a higher larval population of *C. partellus*, as well as a higher mean tunnel length and number of borings. This concurs with results recorded by Nazir (2009) where maize varieties with higher *C. partellus* larval populations had longer tunnel lengths. The same can be observed for the number of shotholes/lesions on a specific variety, where a higher number of shotholes/lesions also indicate a higher number of larvae present on the plant and a higher tunnel length. It has been shown that young *C. sacchariphagus* larvae feed on the terminal leaves before entering the stalk and this in turn results in leaf lesions. These leaf lesions give a good indication of the borer populations on the plant (Nibouche and Tibere, 2009). Moreover, in a study conducted by Kumar (1997), it was found that there was a direct correlation between the number of borings (entry/exit holes) and tunnel length caused by *C. partellus* larvae on maize. Therefore, by counting the number of borings, the laborious exercise of dissecting the stalk to measure tunnel length can be avoided. The number of borings on a plant

is a good criterion for assessing resistance to borers as it indicates the suitability of the variety for the larvae to complete its growth and ultimately emerge from the stalk for pupation (Padmaja *et al*., 2012). The low numbers of borings in specific varieties could be due to the hardness of the stalk (Kumar, 1997).

On some sugarcane varieties larvae were recovered, but there was a low number of borings and little to no stalk damage. This was possibly due to the larvae being found on the outside of the plant (on the leaves or stalk), and therefore the larvae never in fact entered the stalk. This can be seen in the first pot trial for R573, N26 and N28, where the number of larvae recovered were two, four and seven, respectively, and the tunnel length was 0 cm for all of them. Even though a few borings were initiated, no tunnel damage was observed, suggesting that there was incompatibility of the larvae when attempting to feed inside the stalk. The same can be observed for the second pot trial for sugarcane varieties N32 and R573, where there was very little stalk damage. This suggests that there was some form of resistance in the stalk of the plant in these specific sugarcane varieties. The mechanical characteristics of the cane stalk are determined by the structure of plant tissues and properties of the cell walls such as lignins and silicate; and have been shown to play a part in resistance against *C. sacchariphagus* (Rochat *et al*., 2001). Larval weight also seemed to be less for all of these sugarcane varieties, suggesting antibiosis leading to slower growthrates. It has been found that *C. partellus* larval development is slower on resistant varieties (Kumar, 1997).

According to Conlong *et al*. (2004), the use of a non-destructive leaf rating system is useful in determining differences between sugarcane varieties for resistance to *C. saccharphagus.* A correlation with a stalk dissection method was shown. In this study statistically significant correlations can be seen for the mean number of shotholes/lesions and the total number of borings where a high number of borings were associated with a high number of shotholes/lesions. Therefore there is potential for the use of shothole assessments in the rapid mass screening of sugarcane genotypes for resistance to *C. partellus* within the plant breeding programme.

According to the mean leaf feeding scores used in this study, it is evident that none of the sugarcane varieties tested had complete resistance to *C. partellus*. However, varieties M1025/70 and N31 showed a high degree of susceptibility to *C. partellus*

larvae in both pot trials conducted, particularly in the first pot trial, where M1025/70 had the highest number of shotholes/lesions and borings compared to other varieties. Conversely, N32 gave the lowest leaf feeding score, along with the other damage parameters, for both pot trials, indicating it is the least susceptible variety to *C. partellus*. The role of leaves in resistance against stalk borers for sorghum and maize is well known (Pathak, 1990). The good correlation between the mean numbers of shotholes/lesions with the number of borings, suggests that the resistance mechanism of the leaves plays a vital role prior to the establishment of the larvae and ultimately determines their populations. In a study conducted by Afzal *et al*. (2009), it was found that the most contributing factors to the resistance of maize varieties against *C. partellus* were leaf trichomes, followed by stem diameter.

Conlong *et a*l. (2004) reported Variety N26 to have a *C. sacchariphagus* leaf feeding index of 30.2 and a resistance rating of 44 (highly susceptible). According to this investigation, N26 was intermediate in resistance rating to *C. partellus* and it gave fairly high numbers for all the damage parameters in both pot trials.

Similarly, varieties R576 and N22 were found to be susceptible and intermediate in resistance to *C. sacchariphagus* respectively (Conlong *et al*., 2004; Nibouche and Tibere, 2010). According to this study R576 and N22 are both intermediate-susceptible to *C. partellus*.

N28 was reported to be resistant to *C. sacchariphagus* by Conlong *et al*. (2004). This concurs with the results from the first pot trial conducted in this study, where N28 had only one larva found on the outside of the plant, with no tunnelling in any of the plants across all replicates. N28 seemed to be one of the more resistant sugarcane varieties across both trials and had below mean values for Pot Trial One and Two.

Conlong *et al*. (2004) reported N32 to be one of the most resistant varieties against *C. sacchariphagus*, and the pot trials conducted in this study suggest the same for N32 against *C. partellus*. N32 showed low amounts of damage for all damage parameters measured in the first pot trial and was also one of the less susceptible sugarcane varieties in the second pot trial, having a low number of larvae recovered across all replications.

According to a study conducted by Nibouche and Tibere (2009), R570 was the most resistant variety against *C. sacchariphagus* and should be used as a reference for resistance against this borer. In the trials conducted in this study, R570 was also seen to be one of the least susceptible varieties to *C. partellus*. In the first pot trial conducted, R570, together with N32, were the only two varieties to have no larvae recovered, zero borings and zero tunnelling. The number of shotholes/lesions was also low. In the paper published by Nibouche and Tibere (2010), it was confirmed that the resistance of R570 is due to a reduced establishment of larvae on the plant within the first 48 hours after infestation and laboratory bioassays revealed that it was due to larval antixenosis on the abaxial surface of the leaf sheath.

With regard to *F. serrata* numbers, correlations between the mean numbers of shotholes per variety and the mean number of thrips per leaf whorl for the same varieties gave poor Pearson r values for both pot trials. This implies that thrips would not be a reliable surrogate insect for *C. sacchariphagus* in host-plant resistance screening studies given that *C. partellus* may be of use as a surrogate for *C. sacchariphagus* (Table 5.6). However, when looking at specific varieties, interesting observations can be made. For example, varieties N21, R568 and R572 had the highest *F. serrata* numbers in a trial conducted by SASRI. All of these sugarcane varieties were shown to be susceptible or to have an intermediate rating to *C. partellus* in the pot trials. R568 was shown to be one of the more susceptible varieties to *C. partellus* according to both pot trials conducted in this study. M1135/64 had the lowest *F. serrata* numbers recorded. This concurs with the results from the pot trials conducted in this study. M1135/64 was shown to have little damage caused to it by *C. partellus* in both pot trials.

Results from this study indicate that R570, R573, R574, Co1287, M861/60, N24, N28, N32 and M1135/64 show the highest levels of resistance against *C. partellus.* A correlation with *C. sacchariphagus* ratings obtained from previous studies is also indicated in Table 5.6. Further trials should be carried out using these sugarcane varieties to determine the biochemical basis of resistance mechanisms of these sugarcane varieties. The involvement of plant secondary compounds which are known to impact on larval behaviour could also be investigated further.

5.5 References

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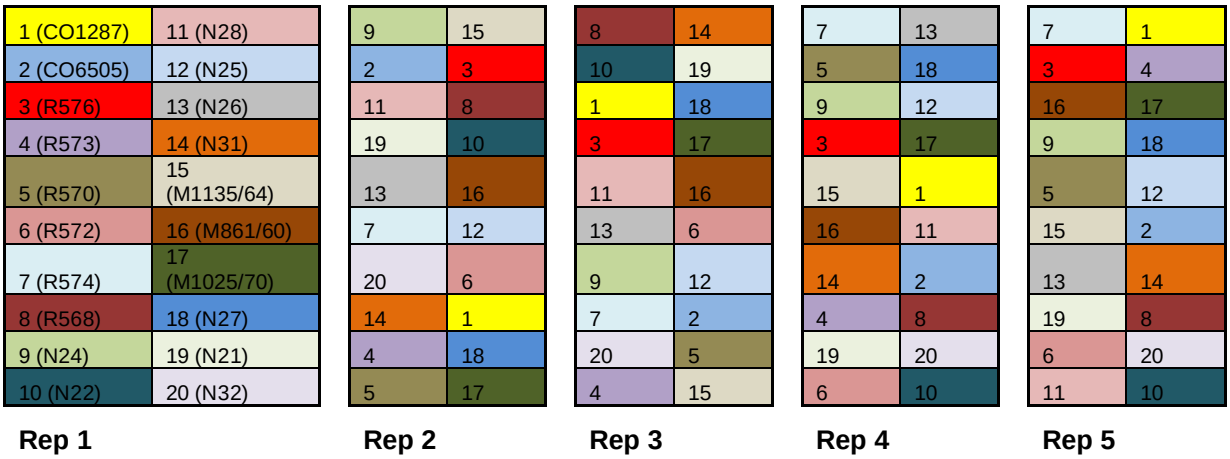
5.6 Appendix

Appendix 5.1 Layout of the pot trials for *Chilo partellus* resistance screening studies in the glasshouse



Appendix 5.2 Results for REML analysis of the number of shotholes for Pot Trial One

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	45.78	19	2.41	83.6	0.003

Appendix 5.3 Results for REML analysis of the number of borings for Pot Trial One

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	89.35	19	4.7	280	<0.001

Appendix 5.4 Results for REML analysis of larvae number for Pot Trial One

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	47.4	19	2.49	77.9	0.003

Appendix 5.5 REML analysis for larvae weight for Pot Trial One

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	28.06	16	1.72	14.4	0.153

Appendix 5.6 REML analysis for tunnel length for Pot Trial One

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	48.52	14	3.47	22.1	0.189

Appendix 5.7 REML analysis for shothole number for Pot Trial Two

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	52.81	19	2.78	76.4	<0.001

Appendix 5.8 REML analysis for number of borings for Pot Trial Two

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	84.36	19	4.44	76.2	<0.001

Appendix 5.9 REML analysis for number of larvae for Pot Trial Two

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	95.7	19	5.04	275	<0.001

Appendix 5.10 REML analysis for larvae weight for Pot Trial Two

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	28.5	18	1.58	53.2	0.101

Appendix 5.11 REML analysis for tunnel length for Pot Trial Two

Fixed	Wald	n.d.f.	F	d.d.f.	F pr
Variety	23.23	19	1.22	48.9	0.281

CHAPTER 6

FEASIBILITY STUDIES ON THE USE OF NEAR-INFRARED REFLECTANCE SPECTROSCOPY (NIRS) AS A RAPID SCREENING TOOL FOR EVALUATING AND PREDICTING FOR RESISTANCE TO *CHILO* SPP. (LEPIDOPTERA: CRAMBIDAE) AND *FULMEKIOLA SERRATA* (THYSANOPTERA: THRIPIDIA) IN SUGARCANE BREEDING PROGRAMMES

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Abstract

Eldana saccharina and *Fulmekiola serrata* (thrips) are serious pests of sugarcane in South Africa. The potential for an invasion by the borer *Chilo sacchariphagus* from Mozambique poses a great risk to the South African sugarcane industry. *Chilo partellus* represents a threat similar to the one once posed by *E. saccharina* before it adapted to feeding on sugarcane. *F. serrata*, *C. partellus*, and *C. sacchariphagus* all feed on the whorl of their hosts, and therefore similar resistance mechanisms of sugarcane varieties may act against them. Rating of sugarcane varieties for damage caused by these pests can be difficult and expensive, and it can take up to 15 years before varieties are released. This study describes the process for developing a rapid, non-destructive, on-site technique for predicting sugarcane varieties for resistance to pests such as *Chilo* spp. and *F. serrata.* The technique is based on near-infrared reflectance spectroscopy (NIRS), which can be used to examine the

interaction between sugarcane and its herbivores. Near infrared (NIR) reflectance spectra obtained from intact surfaces reflect biochemical and structural differences within the leaf, since NIR can penetrate up to 2.5 mm into plant material. Therefore spectral data was obtained from intact leaf surfaces from 21 selected sugarcane varieties using a portable handheld spectrometer. Correlations between NIR spectral data and reference values obtained for *C. partellus* and *F. serrata* were developed using partial least squares (PLS) regression with full cross validation. Validation plots were useful in discriminating between sugarcane varieties for either constitutive or induced resistance based on predicted and actual reference value data. Test validation was conducted on selected reference material using a validation set of five samples. Test validations gave fair results, with the best predictions observed for the mean number of shotholes per variety (R^2 of 0.75, SEP of 8.1). Results indicate that a larger calibration and validation sample set incorporating more sugarcane varieties is required for models to have an improved predictive ability. Reference data directly related to the surface of the leaf gave better model performance than reference data that were influenced by other factors.

Keywords: *Eldana saccharina*, *Fulmekiola serrata*, *Chilo sacchariphagus*, *Chilo partellus*, sugarcane, near-infrared reflectance spectroscopy (NIRS), portable handheld spectrometer, partial least squares (PLS) regression, calibrations, full cross validation, test validation

6.1 Introduction

There are over 1,500 insect species that attack sugarcane worldwide (ul-Hussnain *et al*., 2007). Stalk borers are some of the most serious insect pests of crops such as sugarcane (*Saccharum* L), maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) (Kfir *et al*., 2002). In South Africa *Busseola fusca* Fuller (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) are the two major stem borers on maize and sorghum, while *Eldana saccharina* Walker (Lepidoptera: Pyrialidae) and *Fulmekiola serrata* (Thysanoptera: Thripidae) (thrips) are the most serious pests of sugarcane (Kfir *et al*., 2002). Extreme changes in climate and increasing global trade results in the spread of pests and diseases more easily and causes them to establish in new, previously unaffected countries (Goebel

and Sallam, 2011). The potential for an invasion by the borer *Chilo sacchariphagus* Bojer (Lepidoptera: Crambidae) from Mozambique into South Africa poses a great risk to the South African sugarcane industry. Although it has not yet entered South African territory, studies on climatic conditions suited for the pest show that the coastline of KwaZulu-Natal and neighbouring river valleys, particularly northwards are suitable for the pest's establishment (Goebel, 2006; Bezuidenhout *et al*., 2008). *C. partellus* has adapted to sugarcane in North Africa and is present in the South African sugarcane agro-ecosystem (Assefa *et al*., 2009). *C. partellus* may represent a threat similar to the one once posed by *E. saccharina* before it added sugarcane to its list of host plants. *C. sacchariphagus* and *C. partellus* larva feed on the whorl of the plant before becoming top borers, while all life stages of *F. serrata* take place in the leaf spindle suggesting that similar resistance mechanisms may act against them. Among the few South African sugarcane varieties with known resistance or susceptibility to *C. sacchariphagus,* there appears to be a correlation between *F. serrata* and *C. sacchariphagus* rankings (Figure 1.9; Chapter One). With an increasing number of potential pests of sugarcane in South Africa, the need for a rapid and less costly method to screen varieties increases in importance.

The development of new varieties can take up to 15 years and is a resource intensive process (Purcell *et al*., 2010b). Screening for pest and disease resistance can only take place in much later selection stages when plant numbers are less, due to space and cost limitations (Rutherford, 1998; Purcell *et al*., 2010b). The use of field trials is also difficult due to variable pest populations, or lack of infection (Purcell *et al*., 2005). Near-infrared reflectance spectroscopy (NIRS) is a rapid, non-destructive and reliable technique that can be used in the field. NIRS will allow for earlier screening of varieties which will have a positive effect on the number of clones being brought forward to later stages in a selection programme, a reduction in the need for field trials, which will in turn allow for better resource management and generation of reliable data that can be used in future research projects (Purcell *et al*., 2005). Calibration of near-infrared spectrometers involves acquiring spectra of representative samples, reference analysis of samples using laboratory or traditional methods and model building using chemometrics (Table 6.1) (Blanco and Villarroya, 2002; Chen *et al*., 2002).

Table 6.1 The basic steps involved in near-infrared reflectance (NIR) model construction and their associated purposes (adapted from Blanco and Villarroya, 2002)

Step	**Purpose**
1 Select calibration samples	Should be representative of the component of interest, with a good range
2. Obtain reference data (traditionally/wet chemistry)	Obtain a value for the component of interest in an accurate manner
3. Obtain spectral data	Scan samples in a reproducible manner
4. Averaging and pre-treatments of spectra	To reduce unwanted effects such as scatter effects and particle size on spectra
5. Constructing a model (calibration) using multivariate methods	To determine the relationship between predicted data and reference data
6. Validation	Using independent samples to ensure the model accurately predicts the property of interest
7. Real time use in the industry	Predict unknown samples

NIRS has been applied in numerous industries, including the likes of the sugarcane industry. Rutherford and Van Staden (1996) developed near-infrared (NIR) techniques to predict for *E. saccharina* resistance by building a stepwise linear multiple regression model using surface stalk waxes. Purcell *et al*. (2003, 2004) also analysed sugarcane surface waxes using gas chromatography (GC) and spectroscopy and were able to differentiate between plant properties. In an experiment conducted by Purcell *et al*. (2010b), 31 sugarcane samples were used for a validation trial. NIRS was used to obtain the spectra from stalk bud tissue which were then pre-treated and analysed using chemometrics. The smut ratings based on NIR were compared to ratings from field trials. The results obtained were promising and showed good potential for using NIRS as an early screening method for resistance in sugarcane to smut (Purcell *et al*., 2010b). NIRS has also been used to scan intact sugarcane leaves in order to predict pre-challenge constitutive resistance to Fiji Leaf Gall Virus (Purcell *et al*., 2009). Pest resistant eucalypt species have also

been identified using NIRS, and it has been found that plant resistance to pests can be due to physical, chemical, or ecological components of plants (Henery *et al*., 2008). Physical traits may be leaf toughness or the presence of hairs, while chemical traits can be components within the plant tissues which act as toxins or antifeedants that effect insects (Purcell *et al*., 2005). Young *C. partellus* larvae feed on the leaf whorl during the seedling stage, after which the adult larvae leave the whorl to bore into the stalk of the plant (Kumar *et al*., 2006). Therefore, observed differences in sugarcane varieties in terms of resistance may in part be due to biochemical and physical characteristics of the leaf. Wax components on the surface of sugarcane stalks have been shown to be involved in resistance to *E. saccharina* using NIR (Rutherford and Van Staden, 1996). However sub-surface characteristics can also be probed using NIR, shown in a study whereby NIRS was used to relate spectra obtained from foliage samples of *Eucalyptus grandis* to damage caused by the beetle *Paropsis atomaria* (Coleoptera: Chrysomelidae) (Henery *et al*., 2008). It has been shown that NIR can penetrate up to 2.5 mm into plant material which suggests that NIR spectra should represent the biochemical and structural differences of the leaf (Purcell *et al*., 2010a).

With regards to the success of the above studies in constructing inferential methods to predict results from laboratory or traditional analysis conducted on sugarcane, we hypothesized that the same methods could be used to predict the performance of sugarcane varieties with respect to pest resistance. The main objective of this study was to develop fibre-optic NIR methods to predict resistance of sugarcane varieties to *Chilo* spp. *and F. serrata* using reflectance/transflectance spectra from intact leaves. Such a method would allow for rapid screening of sugarcane varieties for resistance to these pests, without the need for artificial infestation or the use of field trials.

6.2 Materials and methods

6.2.1 Reference data

A set of 21 selected sugarcane varieties were used to obtain reference data to build NIR calibrations and validation models. The sugarcane varieties were tested for their

resistance and susceptibility to *C. partellus* and *F. serrata* using different screening methods.

6.2.1.1 Artificial diet bioassays

Crushed leaf powder from 20 selected sugarcane varieties were incorporated into an established artificial diet of *C. partellus* using 8 ml vials (Lasec, Durban, South Africa). Each diet cell was inoculated with two neonate larvae (obtained from the South African Research Sugar Institute (SASRI) (KwaZulu-Natal, South Africa) which were allowed to feed on the diet for 27 days, after which larval weight and larval survival was measured. A rating score was developed based on larval survival percentage (Table 6.2). These parameters were used to compare sugarcane varieties for any constitutive resistance differences and were used as reference material in building NIR calibrations for predicting for *C. partellus* resistance (Table 6.3).

Table 6.2 Rating score based on larval survival of *Chilo partellus* fed on artificial diets incorporating sugarcane varieties

Survival (%)	Rating (1-6)
55-60	1
61-65	2
66-70	3
71-75	4
76-80	5
81-85	6

Table 6.3 Reference values from artificial diet bioassays used in building NIR calibration and validation models for predicting *Chilo partellus* resistance of sugarcane varieties

	Artificial diet bioassays		
Variety	**Mean larval weight (g)**	**Mean larval survival %**	**Survival rating score (1-6)**
NCo376	0.061	67.82	3
Co6505	0.048	75.8	5
M1135/64	0.065	62.5	2
N27	0.072	82.5	6
N24	0.075	59	1
R570	0.077	65.8	3
Co1287	0.078	70	4
N21	0.078	80	5
N22	0.079	79.1	5
N26	0.081	61.67	2
N32	0.083	71.7	4
R576	0.089	68.3	3
R573	0.096	85	6
N31	0.102	63.3	2
N25	0.110	80.8	6
R572	0.112	77.5	5
R568	0.113	70.8	4
M1025/70	0.115	80.8	6
N28	0.121	71.7	4
M861/60	0.123	74	4

6.2.1.2 Ovipositional experiments

Experiments were conducted whereby 20 selected sugarcane varieties were randomly planted into replicated trays and placed into BugDorm® rearing tents (BugDorm-2400, Megaview science Co. Ltd., Taiwan). *C. partellus* moths (SASRI, KwaZulu-Natal, South Africa) were placed into the individual tents and allowed to oviposit for five days. Egg number and egg batch number were counted per variety and used to determine any antixenosis ovipositional resistance differences between them. Egg number and egg batch number for the two experiments conducted were used as reference material in building NIR calibrations (Table 6.4).

Table 6.4 Reference values from *Chilo partellus* ovipositional experiments used in building NIR calibration and validation models

	Ovipositional experiments					
Variety	**Mean batch number Exp. 1**	**Mean batch number Exp. 2**	**Mean egg number Exp. 1**	**Mean egg number Exp. 2**	**Mean total batch number Exp. 1 and 2**	**Mean total egg number Exp. 1 and 2**
Co1287	3.6	6.4	99.8	134.6	24.5	586
Co6505	2	3.4	52	93.6	13.5	349.5
M1025/70	3	4.4	83	114.6	18.5	492.5
M1135/64	1.4	3.4	29.4	83.8	12	283
M861/60	2.8	4.4	69	110	18	465.5
N21	2.8	3.4	72.8	68	15.5	322.5
N22	3.8	2	73.8	35	14.5	272
N24	3.4	3.2	113.8	96.2	16.5	511
N25	4.6	3.2	75	79.4	19.5	386
N26	2.4	2.6	70	73.8	13	359.5
N27	5	2.4	103.8	69.2	18.5	432.5
N28	3.4	6	73.4	143	23.5	526
N31	3.4	4	62.8	96.4	18.5	398
N32	1.2	0.8	23.6	34.6	5	120.5
R568	5	2.2	115.6	48.4	18	410
R570	2.6	1.2	74.6	35.4	9.5	275
R572	2	4.6	42.4	91	16.5	333.5
R573	1.4	0.6	67	11.4	5	196
R574	1.4	3.8	26.6	94.6	13.5	303
R576	1.2	4.8	36.6	91.8	15	318

6.2.1.3 Glasshouse studies

Three plants of each of the 20 selected sugarcane varieties were planted into individual pots in a completely randomized design, replicated five times. The whorl of the plants was inoculated with 10 neonate *C. partellus* larvae. 30 days after inoculations, damage parameters were measured to compare sugarcane varieties for resistance or susceptibility and were used in building NIR calibration and validation models for predicting for resistance to *C. partellus* (Table 6.5). The pot trial was repeated twice.

Table 6.5 Reference values obtained from glasshouse trials for sugarcane varieties used in building NIR calibration and validation models

	Pot Trial 1			Pot Trial 2		
Variety	**Total no. borings**	**Mean number of shotholes**	**Total no. larvae**	**Total no. borings**	**Mean number of shotholes**	**Total no. larvae**
R570	0	15.71	0	20	18.43	2
N32	0	11.42	0	10	20.42	4
R574[a]	1	23.67	0	-	-	-
M1135/64	1	14.48	0	4	13.45	2
R572	5	28.46	3	15	29.68	12
Co1287	5	27	2	12	40.21	2
R573	6	17.2	2	8	10	4
R568	7	25.46	13	17	35.12	6
N27	7	27.36	2	8	28.8	7
N28	7	23.73	1	6	17.23	4
N22	10	31.85	7	28	46.33	7
N24	11	11.82	2	3	10.4	2
N21	12	14.64	5	22	32.31	12
N25	12	24.06	5	8	46.03	1
N26	12	23.85	4	11	20.61	7
Co6505	14	31.92	1	17	18.57	12
M861/60	18	21.5	1	3	10.35	0
M1025/70	24	49.72	5	47	33.14	21
N31	27	31.28	13	26	28.62	12
R576	52	43.92	14	9	17.11	7
NCo376[a]	-	-	-	7	30.22	4

[a]R574 was replaced by NCo376 in the second pot trial due to lack of planting material

6.2.1.4 *Fulmekiola serrata* trials

Thrips trials were conducted by the South African Sugar Research Institute (SASRI, KwaZulu-Natal, South Africa) by plant breeders to determine *F. serrata* numbers and ratings of sugarcane varieties (Table 6.6) (Joshi, personal communication). The ratings are based on the International Society of Sugar Cane Technologists recommended rating system, whereby highly resistant clones are given a rating of 1 and the most susceptible clones are rated at 9 (Hutchinson, 1970).

Table 6.6 Reference values obtained from *Fulmekiola serrata* trials used in building NIR calibration and validation models

Variety	Thrips ratings
R570	3
N32	1
R574	7
M1135/64	1
R572	7
Co1287	2
R573	3
R568	8
N27	4
N28	3
N22	2
N24	2
N21	9
N25	4
N26	4
Co6505	4
M861/60	2
M1025/70	4
N31	5
R576	2

6.2.2 Plant sampling

The same set of selected sugarcane varieties used to obtain reference data were used to obtain spectral data. Varieties were grown in a pot trial in a completely randomized design. Each pot (experimental unit) consisted of three plants of the same variety, replicated five times, resulting in a total of 15 plants per variety. The adaxial surface of the half unfurled leaf from the leaf spindle was used to perform scans, at two different positions, 20 cm above the leaf ligule. This position for sampling was established by Purcell *et al*. (2005). The leaf area is also where *C. partellus* larva initially feed and oviposits on the plant. Therefore, any resistance or susceptibility of a variety could be directly related to the preference of the larvae to the leaves on which it initially feeds (Purcell *et al*., 2005). Two scans were acquired for each plant, achieving a total of 30 scans per variety. This pot trial was repeated

twice, so two separate spectral data sets were acquired (viz. Pot Trial One and Pot Trial Two). A handheld (portable) Brimrose Luminar 5030 spectrometer (Brimrose Corp, MD, USA) with a fibre optic probe was used to acquire reflectance spectra, with near-infrared radiation from 1100 to 2500 nm at 1 nm intervals. Collected spectra were shown in the absorbance mode (log (1 / R)), where R is the reflectance from leaf samples.

6.2.3 Chemometric methods

6.2.3.1 Calibration

The 30 scans acquired per variety from each pot trial were averaged using WinISI (version 4.5, Infrasoft International) to provide a final spectrum for each pot trial. Spectra were averaged by co-adding individual spectra in order to improve the signal to noise ratio. Mean scans from the first pot trial were used for building calibration models using the reference material from the first pot trial, while mean scans from the second pot trial were used with reference material from the second pot trial to build calibrations. Scans from both pot trials (60 scans per variety) were also combined then averaged, and used to build models using reference material from the diet bioassays, ovipositional experiments and *F. serrata* trials. For chemometrics processing, Unscrambler® X (version 10.3 (32-bit), CAMO, Trondheim, Norway) was used. Mathematical pre-treatments applied to the averaged spectral data were Savitzky-Golay derivative smoothing (using the 1st derivative, polynomial order of 2 and a smoothing point of 5) (Savitzky and Golay, 1964) and standard normal variate (SNV) (Barnes *et al*., 1989) to determine their usefulness in removing unnecessary information from the spectra. Savitzky-Golay smoothing is a derivative method which removes overlapping peaks and corrects the baseline. SNV transformation accounts for the slope variation of the spectra as a result of scatter variation and particle size (Jørgensen, 2000). Averaged NIR spectral data (untreated and pre-treated) was analysed using partial least squares (PLS) regression. This technique seems to perform best for predictive purposes (Purcell *et al*., 2009). PLS includes both dependent and independent variables in the data analysis (Purcell *et al*., 2009). It involves a calibration step and a validation step whereby unknowns are fitted to the calibration. All calibration models developed were evaluated by means of full cross

validation. Unscrambler® X (version 10.3 (32-bit), accurately describes the process of full cross validation, which involves the same samples that are used for calibration and estimation. Samples are left out from the calibration data set one by one and the model is calibrated using the remaining samples. This process is repeated until all samples have been deleted from the calibration once. The values for the left-out samples are predicted and the prediction residuals are computed. Calibrations and cross-validations using treated and untreated spectral data sets, together with different reference material were judged based on their coefficient of determination for calibration and cross validation (R^2, defined as the proportion of variance in the reference data which is explained by the variance in the spectral data) (Williams, 2001), standard error of calibration (SEC, defined as the error due to differences between reference values and NIRS-predicted values within the calibration sample set), the standard error of cross validation (SECV, defined as the error due to differences between reference values and NIRS predicted values within cross-validation sets) (André and Lawler, 2003) and the root mean square error of calibration or validation (RMSEC or RMSECV, gives a measure of how efficient the calibration is) (Williams, 2001). The optimum number of factors (F) used for the PLS regression calibrations was determined by the RMSECV for cross-validation. The F with the smallest RMSECV was selected for PLS regression models (He *et al*., 2005). The data pre-treatment to give the best model performance based on these statistics were used to present results for each reference parameter obtained from the different resistance screening methods for *C. partellus* and *F. serrata*.

6.2.3.2 Test validation

Reference parameters which gave the better performing calibration models during the feasibility study were used for test set validation, and included survival rating from diet bioassays, mean number of shotholes from Pot Trial Two, mean batch number from Experiment One of ovipositional experiments and *F. serrata* ratings derived from field trials. Test set validation tests the calibration model on a subset of samples, which are not present in the computation of model parameters. The predicted Y-values are then compared to the reference Y-values, resulting in a prediction residual that can be used to compute a validation residual variance. The data for reference

material chosen for test validation was separated into a calibration subset using 15 of the sugarcane varieties and a validation set using the remaining five sugarcane varieties. The validation subset was selected to ensure a well-spaced range of reference values was chosen and are shown in Table 6.7. Statistics used to assess the model performance were the SEC, R^2, the standard error of prediction (SEP, defined as errors due to differences between reference values and NIRS-predicted values of spectra obtained outside the calibration set) and the ratio of prediction to deviation (RPD, Ratio of SEP to the standard Deviation (SD)) (André and Lawler, 2003).

Table 6.7 Validation data sets and their associated reference values

Screening method	Reference parameter	Varieties for test validation	Reference value
Diet bioassays	Survival rating	N22	5
		N32	4
		R570	3
		M1135/64	2
		R573	6
Pot trial 2	Mean number of shotholes	N22	46.33
		NCo376	30.22
		R576	17.11
		M1135/64	13.45
		N32	20.42
Oviposition Experiment One	Mean batch number	N22	3.8
		N32	1.2
		R570	2.6
		R573	1.4
		N28	3.4
Thrips trials	Thrips ratings	N22	2
		R568	3
		R572	5
		R573	8
		N31	7

6.3 Results

6.3.1 Calibration and full cross validation

Spectral data pre-treated with Savitzky-Golay derivative and SNV gave the best calibration performances across all the reference parameters and were therefore used to present results.

Calibration and cross-validation equations developed for the various reference parameters of sugarcane varieties from different screening methods were characterized by their R^2 values, SEC and SECV (Table 6.8). When building PLS calibrations using diet bioassay results as reference values, the R^2 values ranged from 0.19 for mean larval weight to 0.81 for the larval survival rating. The best calibration was observed when using survival rating as the response variable, which gave a SEC of 0.66. With respect to the first pot trial conducted, the R^2 values ranged from 0.44 to 0.59, with high SEC values for all the reference parameters. A fairly good calibration was observed when using the reference values for mean number of shotholes per variety obtained from Pot Trial Two, giving a R^2 value of 0.82 and a SEC of 4.85. PLS regression calibrations with R^2 values below 0.8 were observed when using the ovipositional experiment reference data. When using *F. serrata* numbers and *F. serrata* ratings as reference values in PLS regression calibrations, fairly good performance was observed with R^2 values of 0.83 and 0.78 and SEC values of 18.9 and 1.1 observed respectively. Low R^2 values and relatively high SECV values for cross validation were observed for all the reference parameters.

Calibration and cross validation plots provide a visual perspective of the outcome of this investigation for reference parameters which gave the better performing calibrations (Figure 6.1 to 6.4). In terms of the larval survival rating scores obtained from the diet bioassays, it was shown by the cross validation model that Variety N24 was predicted to have a high survival rating of almost 4 whereas its actual rating was 1 and N22 was predicted to have a rating of almost 2 that was much lower than its actual rating of 5. The majority of the varieties were predicted to have higher ratings than their actual ratings, with varieties N28, N26, R576 and M861/60 having predicted ratings close to their actual ratings (Figure 6.1). Cross validation models predicting the mean number of shotholes from Pot Trial Two showed sugarcane varieties N24, M1135/64, M861/60 and R573 predicted to have a high number of shotholes, whereas their actual numbers of shotholes were low. Sugarcane varieties N27, R568, NCo376 and R576 had similar predicted and actual ratings, but no sugarcane varieties were predicted one hundred percent accurately (Figure 6.2). Cross validation models predicting the mean batch number from ovipositon trials showed sugarcane varieties R568, M861/60, M1025/70 and R572 predicted to have

a lower mean batch number than their actual mean batch numbers, and varieties M1135/64 and R574 were predicted to have higher mean batch numbers than their actual mean batch numbers (Figure 6.3).

With respect to the cross-validation model results for *F. serrata* ratings, the correlation between predicted and reference values becomes negative. This could be due to the skewed reference data i.e most varieties are resistant with only a few being susceptible. When a susceptible one is left out for cross validation, the effect on the predictive model is too great. Many more varieties are needed and equal numbers of resistant, intermediate and susceptible should be used for calibration (Figure 6.4).

The reference parameters highlighted in red in Table 6.8 were used for test validation using PLS regression and external test validation sets as shown in Table 6.7.

Table 6.8 Summary of near-infrared reflectance spectroscopy (NIRS) calibration and cross validation results using PLS regression for different reference parameters of sugarcane varieties obtained from *Chilo partellus* and *Fulmekiola serrata* screening trials

							Calibration			Full cross validation		
Screening method	**Reference parameter**	**Factor Level**	**Min**	**Max**	**SD**	**N**	**R^2**	**SEC**	**RMSEC**	**SECV**	**RMSECV**	**R^2**
Artificial diet bioassays	Mean larval weight (g)	3	0.048	0.123	25	20	0.19	0.019	0.019	0.02	0.02	0
	Larval survival (%)	3	55.8	8.85	8.3	20	0.76	3.8	3.65	7.68	7.52	0.08
	Survival rating (1-6)	3	1	6	1.5	20	0.81	0.66	0.64	1.44	1.42	0.18
Pot Trial 1	Total borings	3	0	52	12.03	20	0.59	7.73	7.54	15.83	15.34	0.04
	Mean number of shotholes	3	11.4	49.7	9.91	20	0.59	6.33	6.18	13.39	13.04	0.06
	Total larvae per sugarcane varieties	3	0	14	4.48	20	0.44	3.37	3.28	6.16	5.99	0.2
Pot Trial 2	Total borings	3	3	47	10.67	20	0.75	5.33	5.2	13	12.6	0
	Mean number of shotholes	3	10	46.3	11.38	20	0.82	4.85	4.73	10.75	10.4	0.21
	Total larvae	3	0	21	5.18	20	0.75	2.61	2.55	6.38	6.16	0
Ovipositional trials	Mean batch number exp. 1	3	1.2	5	1.22	20	0.77	0.58	0.57	1.11	1.08	0.25
	Mean batch number exp. 2	3	1.2	6	1.56	20	0.53	1.07	1.01	1.9	1.84	0.01
	Mean egg number exp. 1	3	23.6	115.6	27.3	20	0.51	19.03	18.55	27.71	26.96	0.04
	Mean egg number exp. 2	3	35	114.6	34.28	20	0.56	22.8	22.23	41.77	40.46	0.01
	Total batch number mean exp. 1 and 2	3	5	23.5	5.1	20	0.79	2.3	2.25	6.05	5.84	0
	Total egg number mean exp. 1 and 2	3	120.5	586	115	20	0.72	61.32	59.76	136.66	132.17	0
Thrips field trials	Thrips ratings (1-9)	3	1	9	2.3	20	0.78	1.1	1.04	3.18	3.11	0.45*

SD = standard deviation; N = number of samples in the calibration set; SEC = standard error of calibration; RMSEC = root mean square error of calibration; SECV = standard error of cross validation; RMSECV = root mean square error of cross validation, R^2 = coefficient of determination. * correlation in cross-validation is negative (see Figure 6.4)

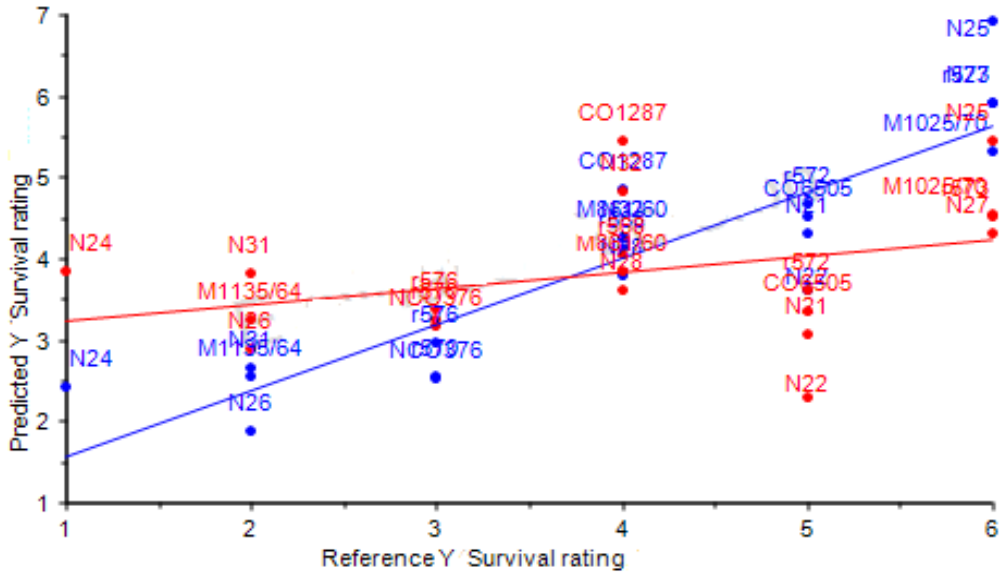

Figure 6.1 Calibration and cross validation plot of near-infrared reflectance spectroscopy (NIRS) predicted survival rating scores (1-6) of *Chilo partellus* versus measured survival rating scores (1-6) obtained during diet bioassays of selected sugarcane varieties. The blue line is the regression line for calibration and the red line is the regression line for validation.

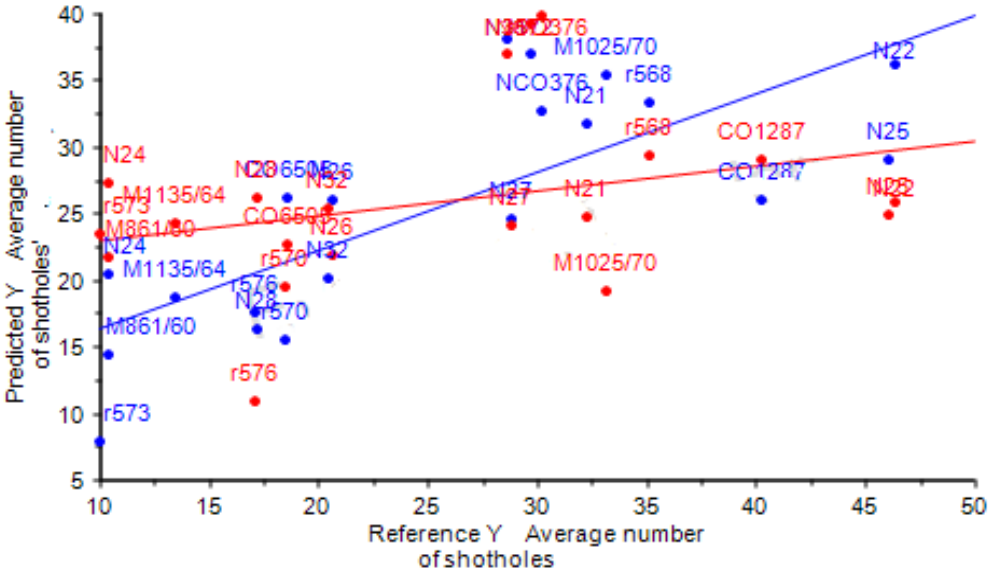

Figure 6.2 Calibration and cross validation plots of predicted mean number of shotholes caused by larvae of *Chilo partellus* per variety obtained from near infrared reflectance spectroscopy (NIRS) versus measured mean number of shotholes per variety obtained from the second pot trial analysis. The blue line is the regression line for calibration and the red line is the regression line for validation.

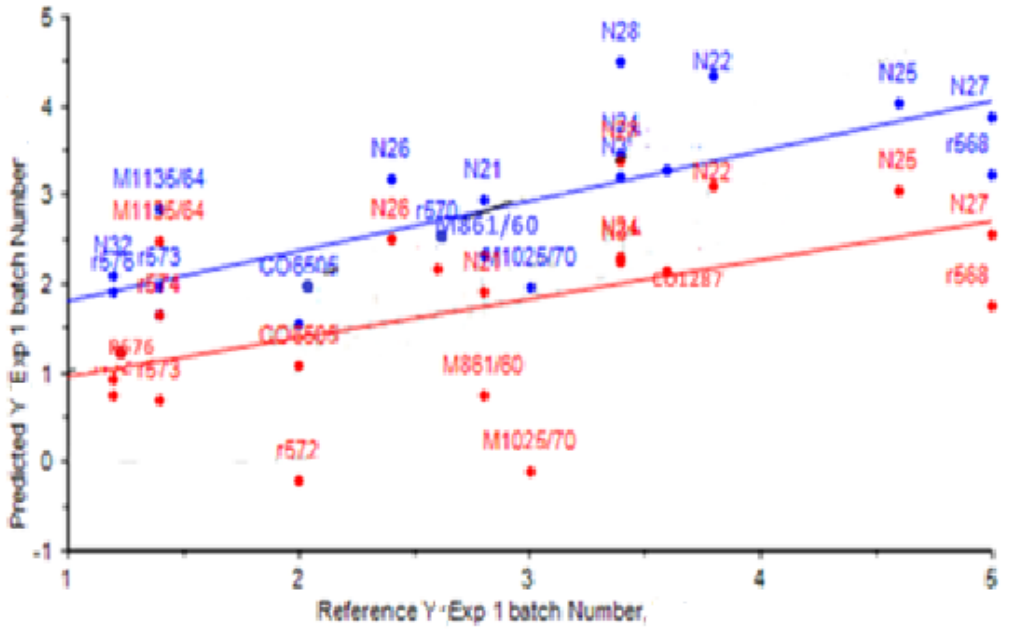

Figure 6.3 Calibration and cross validation plot of predicted mean number of batches of *Chilo partellus* eggs per variety obtained from near infrared reflectance spectroscopy (NIRS) versus measured mean number of batches per variety obtained from the oviposition experiment One. The blue line is the regression line for calibration and the red line is the regression line for validation.

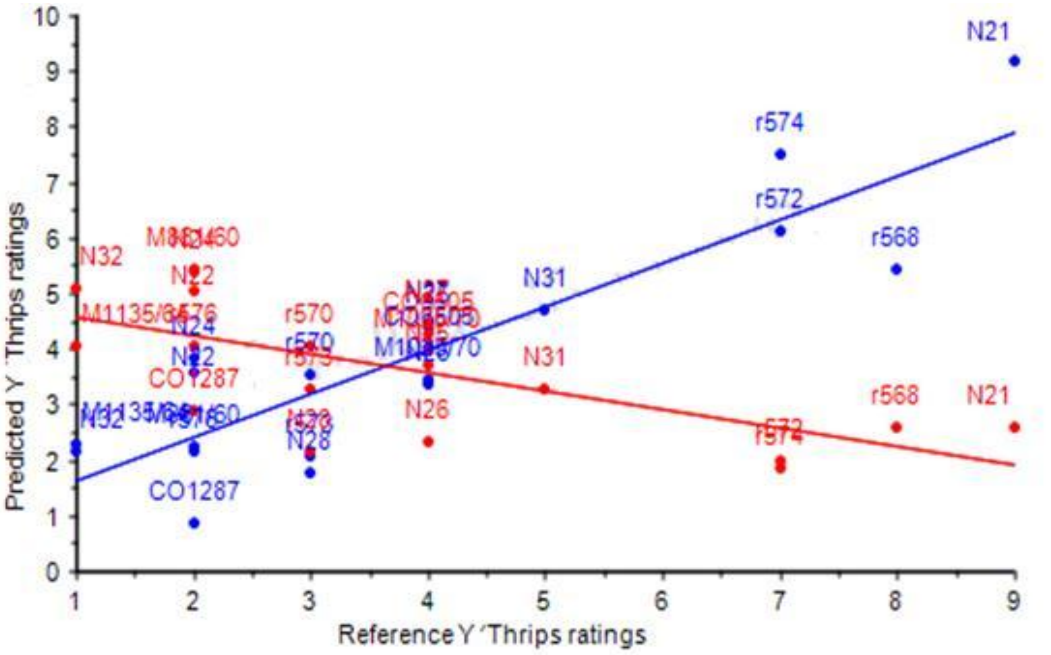

Figure 6.4 Calibration and cross validation plots of predicted *F. serrata* ratings obtained from near infrared reflectance spectroscopy (NIRS) versus measured *F. serrata* ratings (1-9). The blue line is the regression line for calibration and the red line is the regression line for validation

6.3.2 Test validations for selected reference data

The SEP, R^2 value and the RPD (Table 6.9) were used to assess the accuracy of calibrations by performing test validations on a subset of five sugarcane varieties, using the selected reference parameters based on their PLS regression models in the calibration feasibility studies. R^2 values of above 0.8 and fairly low SEC values were obtained for all the calibration models when using 15 samples in the calibration set (Table 6.9). However, the R^2 values for the test validations were not as good, with none of the models having an R^2 value of above 0.8. The highest R^2 value of 0.75 was obtained for the model predicting the mean number of shotholes per variety, while the SEC and SEP were 3.88 and 8.1 respectively. The highest RPD of 1.4 was also achieved by this model. The mean number of shotholes was predicted fairly well for sugarcane varieties M1135/64 and R570, but was predicted too low by the model for NCo376 and N22 and too high for N32 (Figure 6.6). The model used to predict survival rating of *C. partellus* larva obtained from artificial diet bioassays, gave an R^2 value of 0.63 and a SEP of 2.9. Sugarcane varieties M1135/64 and R570 with low measured survival ratings were predicted accurately by the model, whereas N22 was predicted to have a lower survival rating than its measured one (Figure 6.5). The test validation model for predicting batch numbers obtained during Experiment One of oviposition experiments gave and R^2 value of 0.63 and an SEP of 0.96. None of the varieties had batch bumbers predicted accurately by the model, with all the varieties having slightly higher predicted batch numbers than their actual batch numbers, with the exception of N28 which had a lower predicted batch number (Figure 6.7). Test validation models for predicting *F. serrata* numbers and *F. serrata* ratings were not as fit, with R^2 values of 0.28 and 0.3 respectively. All the sugarcane varieties were predicted to have higher *F. serrata* ratings than their actual *F. serrata* ratings, except for N22 which was predicted to have a lower *F. serrata* rating (Figure 6.7).

Table 6.9 Summary of test validation statistics for reference parameters of sugarcane varieties obtained in *Chilo partellus* and *Fulmekiola serrata* screening trials

Screening method	Reference value	Calibration				Test validation			
		SD	Nc	R^2	SEC	Nv	R^2	SEP	RPD
Diet Bioassays	Survival rating (1-6)	1.5	15	0.94	0.4	5	0.63	2.9	0.52
Pot Trial 2	Mean number of Shotholes	11.38	15	0.88	3.88	5	0.75	8.1	1.40
Oviposition Exp. One	Mean batch number	1.22	15	0.87	0.42	5	0.63	1.17	0.96
Thrips trials	Thrips ratings (1-9)	2.3	15	0.85	0.83	5	0.3	2.2	1.05

SD = standard deviation; Nc = number of samples in the calibration set; SEC = standard error of calibration, Nv = number of samples in the validation set; SEP = standard error of prediction; RPD = ratio of SEP to SD; R^2 = coefficient of determination

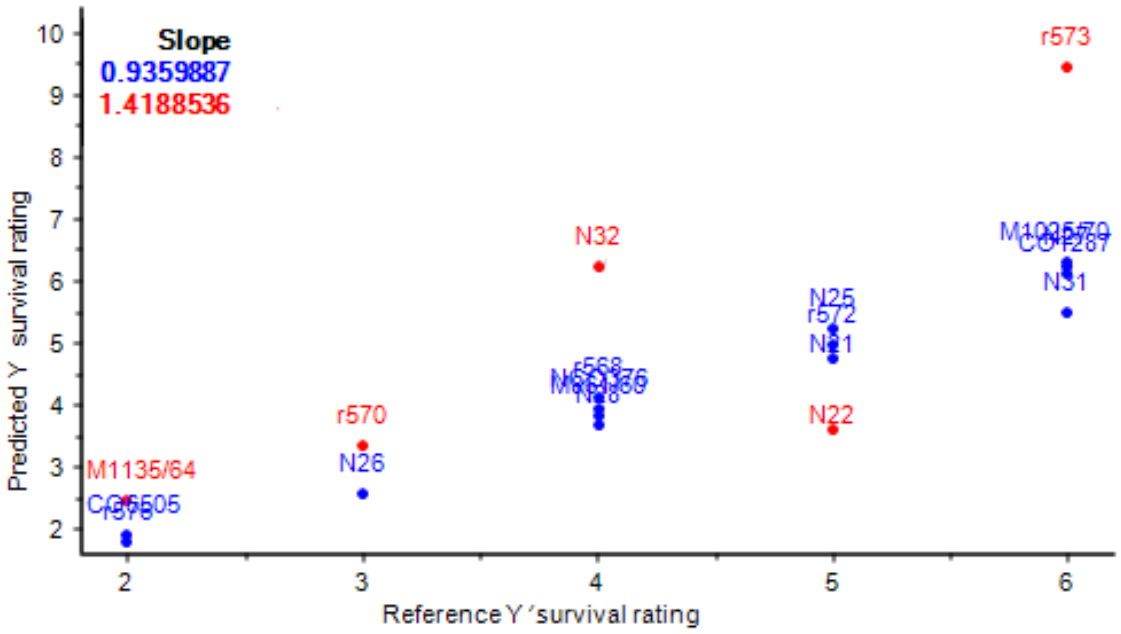

Figure 6.5 Scatter plot of near-infrared reflectance spectroscopy (NIRS) predicted survival rating scores (1-6) of *Chilo partellus* versus measured survival rating scores (1-6) obtained during diet bioassays of leaf whorl powders. Values for the external validation set are shown in red.

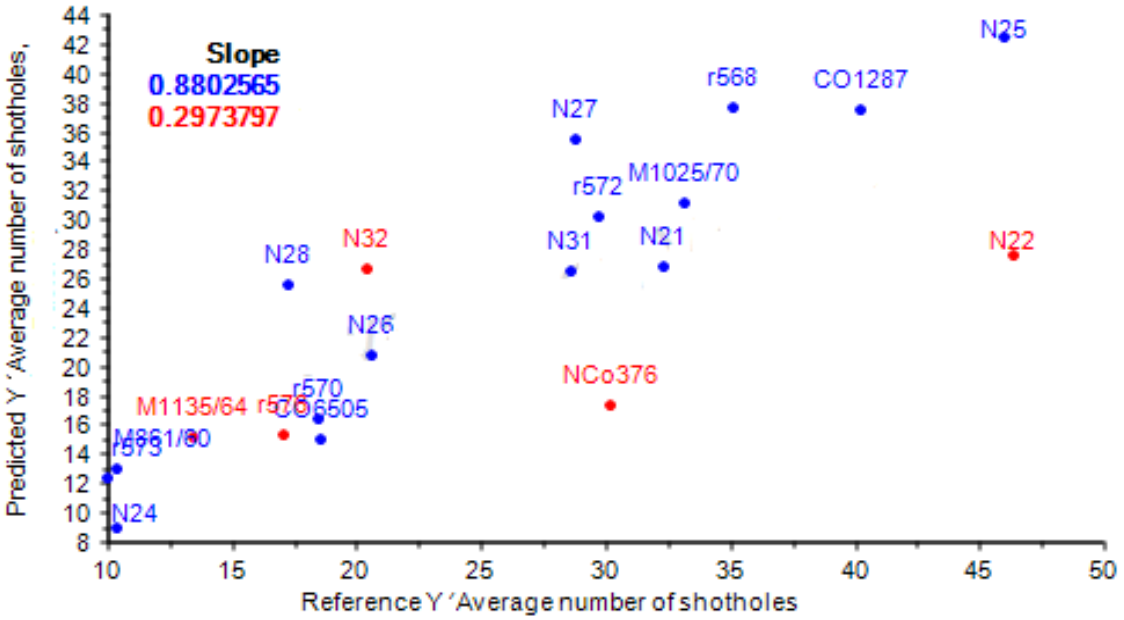

Figure 6.6 Scatter plot of predicted mean number of shotholes obtained from near infrared reflectance spectroscopy (NIRS) versus measured mean number of shotholes obtained from the second pot trial, caused by larvae of *Chilo partellus*. Values for the external validation set are shown in red.

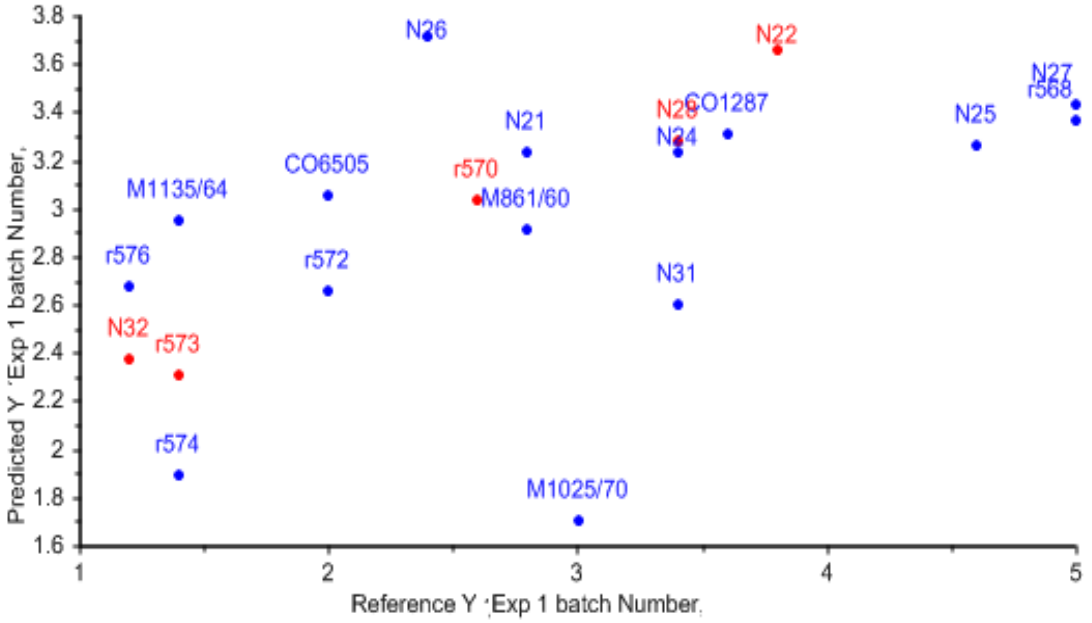

Figure 6.7 Scatter plot of near-infrared reflectance spectroscopy (NIRS) predicted batch number versus measured batch number obtained from *Chilo partellus* oviposition experiment one. Values for the external validation set are shown in red.

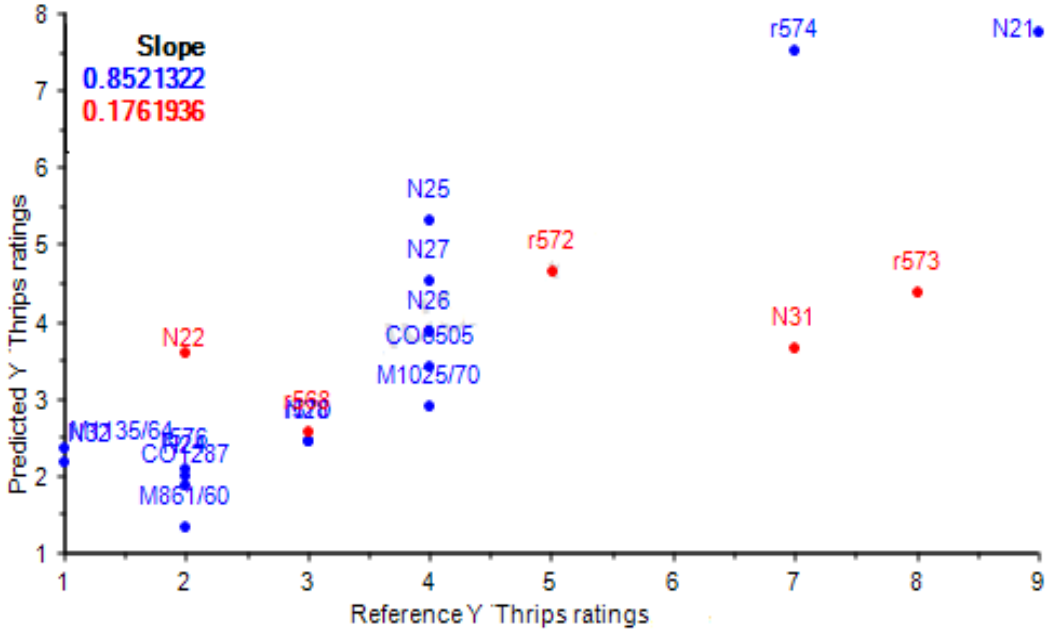

Figure 6.8 Scatter plot of near-infrared reflectance spectroscopy (NIRS) predicted *Fulmekiola serrata* ratings (1-9) versus measured *F. serrata* ratings (1-9) obtained in field trials run by SASRI. Values for the external validation set are shown in red.

6.4 Discussion

Observed differences in sugarcane varieties in terms of resistance may be due to biochemical differences in the leaves. This study aimed to predict differences between sugarcane varieties with respect to pest resistance based on NIR spectra obtained from their intact leaf surfaces and reference values obtained from various screening techniques. Previous studies have shown that NIR can penetrate up to 2.5 mm into plant material which infers that NIR spectra should represent the biochemical and structural composition of the leaf that could be related to sugarcane resistance to pests such as *C. partellus* and *F. serrata* which feed on them (Purcell *et al*., 2010a). Although the results from this investigation are preliminary in nature, some of the calibrations do show a degree of potential for the development of predictive models for *F. serrata* and *Chilo* spp. resistance in sugarcane.

Although the PLS regression calibrations were fairly good for certain reference parameters, with R^2 values higher than 0.8 achieved, results obtained from the

majority of the cross validation models (leave one out method) were discouraging. This is probably due to the calibration data sett being too small. When a sample is left out for cross validation, the effect on the predictive model is too great resulting in a prediction that differs greatly from that of the completely inclusive calibration model (Table 6.8).

Calibrations and cross validations for all reference values obtained during oviposition experiments were particularly poor, indicating that no correlation occurred between the spectral data and the measured values for ovipositing. This could be due to the lack of any significant differences between varieties for egg number and batch number found during oviposition experiments, suggesting poor quality reference data and consequently a poor predictive model. Perhaps the differences in sugarcane varieties with respect to ovipositing behaviour were a result of olfactory stimuli (Thompson and Pellmyr, 1991). Herbivore induced plant volatiles include terpenoids, benzenoids, and green leaf volatiles which are released from the plant to either attract or repel insects. It has been shown that ovipostion by herbivorous insects on plants can result in a change in volatile emissions which may deter further deposition of eggs (De Moraes et al., 2001; Fatouros *et al*., 2012).

Results from the test validations were more promising than those from the cross-validations for the selected reference parameters. The mean number of shotholes from Pot Trial Two used as a reference parameter was shown to have the best calibration and test validation performance, with 75% of the variation in the reference data being accounted for by the spectral data obtained from leaf surfaces. Perhaps it is because the number of shotholes is a direct measurement of damage caused to the leaves by larva and is therefore more closely related to spectral data taken from the leaves than other parameters measured. The mean number of shotholes is a good indication of susceptibility of sugarcane varieties to *C. sacchariphagus* and *C. partellus* and has been shown to be a non-destructive measure by which to rate sugarcane varieties (Conlong *et al*., 2004). Using this parameter to develop NIR models for predicting for resistance may be very useful in the future because it indicates that compounds in the leaf effect larval feeding and should be explored further. The mean number of shotholes was predicted to be fairly close to the actual number of shotholes for sugarcane varieties M1135/64 and R570, indicating that they

could possibly have a strong constitutive resistance against *C. partellus* larvae. Test validation models built using larval survival ratings of *C. partellus* from diet bioassays gave an R^2 value of 0.63 and a SEP of 2.9. Field based ratings are known to have an associated error of +/- 1 units, indicating that the SEP for survival rating is too high and is therefore unsatisfactory to be used for screening purposes (Purcell *et al*., 2009). Similarly, a high SEP of 2.2 was observed for the test validation model using *F. serrata* ratings as reference values. High SEP values could be attributed to a poor or skewed range of values within the sample set (Edney *et al*., 1994).

In general terms, for NIR calibrations to be of use in the prediction of unknown samples (e.g. for total wheat nitrogen (N) content), R^2 values for calibration and validation should be greater than 0.80. If R^2 values are between 0.7 and 0.8 then the calibration can be used for rough prediction or classification, while R^2 values less than 0.7 require further calibration development (Williams, 2001). The SEC and SECV should be as small as possible for good calibrations, while a large gap between SEC and SECV or SEP indicates that the sample sett is too small (Dardenne, 2010). Acceptable predictions are characterized by low SEP values and high R^2 and RPD values (Chen *et al*., 2002).

However, unlike analyses such as total N content, the determination of reference resistance ratings using live plant inoculation assays quantifies total resistance, whereas NIR scans of undamaged plant material can only be linked to constitutive preformed resistance. Since the inducible component of resistance is not accounted for, high R^2 values in calibration or validation should not be expected when calibrating against total resistance reference values determined in live plant bioassays. If an equal contribution of constitutive and induced resistance were assumed, then an R^2 in calibration of 0.5 would be reasonable.

Relatively low R^2 values are still useful in plant breeding programmes where the aim might be to discard all susceptible clones at an early stage in the selection process. Due to large numbers, conventional screening at early selection stages is not possible. According to calculations made by Schenk and Westerhaus (1993), clones predicted as susceptible (of the three groupings; resistant, intermediate, and susceptible) would include only 4% of actual resistant clones, based on a R^2 during

cross-validation in the region of 0.5 (Table 6.10). The selected population would be enriched for constitutive resistance.

Table 6.10 Relationship between R-squared and the classification of predicted samples into three groupings e.g. resistant, intermediate and susceptible (adapted from Shenk and Westerhaus, 1993).

R squared in calibration	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**
Predicted in correct group	43	47	51	55	59	63	68	74	81
Predicted in adjacent group	43	42	41	38	37	34	31	26	19
Predicted R when actually S (and vice-versa)	14	11	8	6	4	3	1	0	0

Results from this study suggest that an expanded calibration sett (i.e., more sugarcane varieties) would result in better calibrations. A calibration set should ideally be large enough so that all the variation (physical or chemical) within a population has been sampled (Rutherford and Van Staden, 1996). Incorporating more sugarcane varieties with known ratings or response variables into the calibration set would most likely improve the models. However, since there were only 21 sugarcane varieties with known ratings and damage parameters for *C. partellus* and *F. serrata* in this study, the calibration set could not be extended any further. The same problem was observed in a study by Smyth *et al*. (2008) when attempting to use NIRS to measure volatile compounds in wine. In a study conducted by Rutherford and Van Staden (1996), it was shown that by expanding the size of a model, the correlation coefficient began to stabilize. According to Williams (2001) the ratio of samples used for calibration to validation should be 3:1, and if calibration sample sets are too small, the calibration will be highly sensitive to the addition of new samples and will show large differences in predictions.

It was observed that some sugarcane varieties were predicted to have higher or lower ratings than their actual ratings within calibrations or in validation. For example, in Figure 6.6 the variety NCo376 is predicted to suffer less shothole damage than its actual reference value. This suggests a strong constitutive component of resistance, perhaps with a muted induced response resulting in more damage than predicted.

N28 on the other hand is predicted to suffer more damage than its reference value suggesting poor constitutive resistance. In this case the resultant lower damge level would be due to a strong inducible component (unseen by NIR scans of undamaged leaves). That NCo376 has strong constitutive resistance whilst N28 does not, is borne out by the results of the diet inclusion study described in Chapter 3.

Clearly there are a number of issues that still need to be addressed before NIRS calibrations and validations based on leaf material can be used as a technique for predicting resistance of sugarcane varieties to *Chilo* spp. and *F. serrata*. NIR spectra need to be reproducible, with respect to the plant age, the sampling position and environmental effects (Purcell *et al*., 2005). The SEP needs to be compared to acceptable errors when using field, laboratory and glasshouse methods, and these errors need to be established. The accuracy and precision of the reference method is highly important, and errors in these methods can be reflected in the calibration models developed (Edney *et al*., 1994; Williams, 2001).

According to this study, it would be worthwhile to include a larger number of sugarcane varieties into the calibration and validation sets with known reference values in order to obtain improved models. Partial validations were achieved using the mean number of shotholes. Reference material directly related to the leaf surface, such as the mean number of shotholes caused by insect feeding, larval survival in diets incorporating cane leaf powder, or *F. serrata* populations on leaves seem to be more ideal in building calibrations based on leaf scans. NIRS does offer the potential to be used as a rapid technique for discriminating between resistant or susceptible sugarcane varieties and for gaining some insight as to mechanisms of resistance. Obtaining scans from leaf surfaces is a non-destructive technique, and hundreds of leaves can be scanned in one day.

6.5 References

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DISSERTATION OVERVIEW

There is an increasing number of potential pests in the South African sugarcane industry, such as *Chilo sacchariphagus* Bojer and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) due to an increase in globalisation and climate change (Way *et al*., 2011). Before they develop into major pests of sugarcane in South Africa, it is desirable to determine the susceptibility of current sugarcane varieties to these pests. Since *C. sacchariphagus* is not yet present in South Africa, it is not possible to screen sugarcane varieties for resistance against it, unless under quarantine conditions. Not all research stations have quarantine facilities available to them in which to conduct such trials. To this end, surrogate insect resistance screening can be used, for example, where *C. partellus* is used as a surrogate for *C. sacchariphagus* in resistance screening studies. Both of these pests are top borers that initially feed on the whorl of the plant before boring into the stalk, and therefore similar resistance mechanisms may act against them. Similarly, *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae), is also a serious pest in the South African sugarcane industry and its entire life cycle is spent in the whorl of the plant (Way *et al*., 2006; Way *et al*., 2010). Among the few South African sugarcane varieties with known resistance ratings for *C. sacchariphagus*, there appears to be a correlation with *F. serrata* resistance ratings (shown in this thesis, Chapter 1, Page 35. Figure 1.9). Screening for resistance to pests and diseases is currently limited to selection at the late stages of a plant breeding programme, due to the cost of field screening for insect resistance, and logistical factors (Rutherford, 1998; Purcell *et al*., 2010). The application of new screening tools at earlier selection stages would result in cost savings, productivity benefits, and an increased number of resistant clones progressing to later selection stages (Purcell *et al*., 2005). Near infrared reflectance spectroscopy (NIRS) is a non-invasive, non-destructive method that has the potential to determine the relationship between sugarcane varieties and their attackers.

The investigations undertaken in this research dissertation were comprehensive, and included the development of rearing methods and an improved artificial diet for *C. partellus*, exploring the concept of insect surrogacy for resistance screening, the development of methods for screening sugarcane varieties for resistance to *C. partellus*, and obtaining preliminary indications as to whether NIRS could be used to

build predictive models for constitutive resistance to pests. The key outcomes of this project were:

- It was determined that *C. partellus* is present in the sugarcane agro-ecosystem in KwaZulu-Natal, and given that it has adapted to sugarcane in Ethiopia, it therefore has the potential to become a pest of sugarcane in South Africa. In varieties R572, R574, R576, N25, R568 and M1025/70 development through to the pupal stage was recorded in pot trials.

- A new and improved diet was established for *C .partellus* incorporating 6.5% cane leaf powder (as opposed to the previous standard of 2.5% cane leaf powder), and the incorporation of non-fat milk powder and whole egg powder. This diet was ideal for use in *C .partellus* artificial diet bioassays incorporating sugarcane varieties, in order to screen for the presence of any constitutive resistance differences between them.

- Sugarcane varieties M1135/64, N24, and R570 gave consistently lower larval weights and larval survival when they were incorporated into the artificial diet of *C. partellus*. This suggests that these varieties do not promote larval growth and development, and therefore have a higher constitutive antibiosis resistance to *C. partellus* larvae when compared to the other varieties. Conversely, varieties M1025/70, R573, and N25 gave higher average larval weights and larval survival when incorporated into the diet, which suggests that they have little to no constitutive antibiosis resistance against *C. partellus*.

- No statistically significant differences were observed between varieties for both egg number and batch number during oviposition studies using *C. partellus* moths. However, varieties N28, Co1287, and M1025/70 had the highest egg number and batch numbers consistently for both experiments, whereas N32 and R573 had consistently low egg numbers and batch numbers for both experiments.

- In glasshouse trials varieties N32, N24, N28, R570, R573, R574, Co1287, M861/60and M1135/64 show the highest levels of resistance against *C. partellus*, while M1025/70 and N31 show the highest susceptibility.

- Reviewing all the resistance screening methods together some inferences can be made for varieties as to their resistance mechanisms (Table 1). It is suspected that the variety R570 has strong constitutive antixenosis and antibiosis resistance against *C. partellus*, given that it rates as resistant in diet bioassay and in oviposition experiments.

- Varieties N32 and R573 were more susceptible in diet bioassays, but were among the most resistant varieties in oviposition and pot trial studies. This could indicate that they have more of an induced resistance component acting against *C. partellus*, rather than constitutive resistance.

- Varieties N28, R568, M861/60 and Co1287 were rated as susceptible in artificial diet bioassays and oviposition studies, but suffered little to no damage by *C. partellus* in inoculated live plant pot trials conducted in the glasshouse. This suggests that these varieties have low levels of constitutive antibiosis and antixenosis acting against *C. partellus*, but rather have high levels of inducible resistance.

- Varieties M1025/70 and N31 appear to have no resistance mechanisms acting against *C. partellus* moths or larvae, and were consistently highly susceptible.

- Intermediate-susceptible and susceptible varieties N21, N22 and N31 constitute biosecurity risks to the South African sugarcane industry in terms of the possibility that *C. partellus* might adapt to sugarcane through them, and due to their known susceptibility or likely susceptibility to *C. sacchariphagus* (M, Co and R varieties are not commercially grown in South Africa).

Table 1 Inferred resistance mechanisms of sugarcane varieties to *C. partellus* based on a series of resistance screening experiments involving variety comparisons in artificial diet, oviposition experiments, and pot trials (pink represents susceptibility; green indicates intermediate; and blue indicates resistance). '+' indicates variety is likely to be positive for resistance mechanism; '-'indicates the variety is likely to be negative for resistance mechanism.

	Diet Bioassays	**Ovipositon Experiments**	**Pot Trials (PT)**	**Resistance Mechanism against *C. partellus***	
Variety	**Mean Larval Weight as % of Control**	**Mean Total Batch No. Combined Experiments**	**Overall Rating**	**Constitutive**	**Induced**
Co6505	79.64	13.5	IS	+	-
M1135/64	107.24	12	R	++	+
N27	118.72	18.5	IR	+	+
N24	123.48	16.5	R	+	++
R570	125.78	9.5	R	+++	-
N21	128.57	15.5	IS	+	-
Co1287	128.57	24.5	R	+	++
N22	130.38	14.5	IS	+	-
N26	133.33	13	I	+	-
N32	135.47	5	R	++	+
R576	146.14	15	IS	+	-
R573	157.47	5	R	+	++
N31	168.23	18.5	S	-	-
N25	180.62	19.5	I	-	+
R572	184.47	16.5	I	-	+
R568	185.3	18	IR	-	++
M1025/70	188.88	18.5	S	-	-
N28	199.38	23.5	R	-	+++
M861/60	202.46	18	R	-	+++

- The overall performance of the NIRS models was promising to fairly good, given the small numbers of samples used. However, they are not yet robust enough to be used for screening purposes. For calibration models to be suitably robust, a much larger number of varieties, ideally 200 to 300, need to be screened for resistance to *Chilo* spp. and *F. serrata* before incorporating their NIR scans into calibration and validation sets.

- Reference material should be representative of the pest of interest, and should relate to the surface from which the spectra are obtained. NIRS does offer the potential to be used as a rapid technique for discriminating between resistant or susceptible sugarcane varieties, and the resistance mechanisms involved. Obtaining scans from leaf surfaces is a non-destructive technique, and hundreds of leaves can be scanned in one day.

- With respect to the NIR models built, it was observed that some sugarcane varieties were predicted to have higher or lower ratings than their actual ratings within calibrations or in validation. This can be used to infer mechanisms of resistance. For example, in Figure 6.6 the variety NCo376 is predicted to suffer less shothole damage than its actual reference value. This suggests a strong constitutive component of resistance, perhaps with a muted induced response resulting in more damage than predicted. N28 on the other hand is predicted to suffer more damage than its reference value suggesting poor constitutive resistance. In this case the resultant lower damage level would be due to a strong inducible component (unseen by NIR scans of undamaged leaves). That NCo376 has strong constitutive resistance whilst N28 does not, is borne out by the results of the diet inclusion study described in Chapter 3.

- The concept of surrogacy between *C. sacchariphagus* and *C. partellus* received some support in the various screening studies. For example, N28 and N32 were reported to be fairly resistant to *C. sacchariphagus* by Conlong *et al*. (2004). This concurred with the results from the pot trials conducted in the glasshouse trials, where N28 and N32 were among the more resistant varieties, having below average values for *C. partellus* damage parameters for both pot trials conducted. Variety R570 is fairly resistant to *C. sacchariphagus* according to Nibouche and Tibere (2009). In these studies, R570 showed relatively little damage from *C. partellus*, also indicating that it is one of the more resistant varieties (Table 5.6).

Conclusions and future research

From the research conducted in this study it was shown that it is often necessary to combine a number of screening methods in order to characterise the resistance mechanisms of a specific variety to a pest (Vercambre *et al*., 2001). The biochemical and physiological basis of resistance to *Chilo* spp. should be further explored which could yield improved methods for phenotyping resistance in plant breeding programmes (Nibouche and Tibere, 2010). Additionally, the role of certain secondary compounds that are known to have an effect on larval feeding and development could be investigated. The concept of insect surrogacy has been shown to be worthy of further research. Further studies on the panel of varieties used in this project could be carried out in a country where *C. sacchariphagus* is endemic, e.g. Mozambique, Reunion Island or Mauritius.

Although the future of NIRS as a screening tool for pest resistance is a positive one, it has been confirmed that the development of NIRS calibration models requires many samples of different varieties. Ultimately, the quality of NIRS models for predictions depends on the quality of the reference material and the spectral patterns used to generate the model. Critically, accurate, robust calibration models require a large enough sample sett to include the entire spectrum of variation of physical and/or chemical properties of the populations of interest (Rutherford and Van Staden, 1996). In the future, a larger number of sugarcane varieties should be screened accurately for a particular trait, and this population of varieties should be used together with optimally obtained spectra (correct sampling position and plant part) to obtain robust calibration models for predictions.

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