Near infrared analysis of sugarcane (*Saccharum* spp hybrid) bud scales to predict resistance to eldana stalk borer (*Eldana saccharina* Walker)

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DECLARATION

These studies represent original work by the author, which have not been submitted in any form for any degree or diploma to any University. Where use was made of the work of others it is duly acknowledged in the text.

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I, the undersigned, confirm that I supervised the candidate, N Coetzee, in the reading of this thesis.

P Shanahan

ABSTRACT

The eldana stalk borer (*Eldana saccharina* Walker) is the most serious pest of the Southern African sugarcane industry, and it is imperative that effective control measures are available to minimize economic damage. Because conventional control methods have had limited success, cultivar resistance is seen as the most viable method of controlling infestation. However, due to the space- and time-consuming nature of the present screening methods, only small numbers of cultivars can be tested relatively late in the Plant Breeding selection programme. Increased resistance in breeding and selection populations is therefore slow.

Buds are a preferred entry point of eldana larvae as they are softer than the rind that is present on the rest of the stalk surface. Preliminary results by other workers suggested that near infrared spectroscopy (NIRS) could provide a rapid screening method for the chemical profile in bud scales, the outer coating of buds and therefore the first contact point of an invading larva. If feasible, analysis of samples using this method could be done in the South African Sugar Experiment Station's (SASEX) stage two selection trials, providing an early indication of eldana resistance on large numbers of cultivars, without the necessity of separate trials. However, knowledge of how environments, position of bud scales on the stalk and age affect NIRS is required in order to determine the feasibility of the method. Planting of a trial with an identical set of genotypes across a range of environments, sampled at a number of ages, would provide the necessary information on environmental effects, whilst simultaneously providing the necessary range of samples to develop a calibration between bud scale chemical profiles and eldana resistance ratings. Inheritance patterns of the characteristics being measured is also required if they are to be used in a breeding programme.

The original work by Rutherford (1993) was carried out on only five calibration sets (a set of standard clones with relatively well-known eldana resistance ratings), and different sets were not comparable due to what was assumed to be environmental differences between calibration sets. One aspect of the current experiment was to examine more closely the effect of genotype x environment interaction (G x E) on the performance of the NIRS technique under a range of conditions. Two sites were chosen to represent the conditions encountered in trials carried out by SASEX. The crops were sampled at three ages,

representing the range of ages at which sugarcane is harvested in South Africa. Two locations on the stalk were also examined, top and bottom, for removal of bud scales, based on the assumption that aging of bud scales may affect chemical composition.

A new NIRSystems 6500 instrument was acquired during the course of this study. Data from the new instrument indicated that there were no longer differences between the different calibration sets, and therefore no longer differences between environments. Spectra for different samples were very close, the differences being of the same scale as those recorded with repeated measures of the same samples, or between the readings for the standard solvent solution. This led to the conclusion that the differences observed on the original NIRSystems 5000 instrument were due to instrument error, not environmental differences. More importantly, the different calibration sets were not comparable despite being similar to each other. Prediction from one calibration set to another was low.

These observations led to the conclusion that NIRS was not a suitable method for determining chemical compounds associated with tolerance of sugarcane genotypes to eldana borer. The original NIRS instrument was subject to error, and the small number of calibration sets included in the study led to the erroneous conclusion that NIRS was suitable for the prediction of varietal tolerance to eldana. With the acquisition of the new instrument, the errors generated by the old instrument became apparent. With the increase in number of calibration sets included in the study, it also became apparent that a global calibration covering all environments was not possible.

An analysis of the heritability of the chemical compounds associated with eldana resistance was also included in this study. A biparental progeny design of 24 crosses with 33 unselected offspring per cross was used. This trial would have been analysed once the calibration had been developed using the environmental trial, and it would have provided knowledge of the breeding behaviour of the chemical compounds associated with tolerance to eldana. Because the NIRS technique proved to be unsuitable for detection of chemical compounds associated with eldana resistance, the heritability of these chemical compounds could not be studied.

As the NIRS study did not produce data, the G x E interaction analysis and determination of heritability was applied to the bud scale mass data set. This study showed a relatively

low positive correlation between bud scale mass and resistance to eldana. The broad sense heritability estimate for bud scale mass from the G x E interaction analysis was 0.45, and the narrow sense heritability estimate from parent-offspring regression analysis was approximately 0.27, suggesting a low degree of genetic determination in bud scale mass. The G x E interaction analyses gave varying results depending on the method used. The ANOVA analysis suggested that ages, sites and years had an effect on bud scale mass, while deviation from maximum plot showed no significance for G x E interactions. The number and choice of genotypes selected as unstable also varied with the method used to determine the stability of individual genotypes. Regression analysis and rank order analysis revealed a number of unstable genotypes, whilst stability variance and ecovalence, which produced similar results, detected only two unstable genotypes. In the rank order analysis correction of data to remove genotype effect, reduced the number of unstable genotypes, suggesting that the G x E interaction effect was partially confounded with the bud scale mass of the genotypes. This was a more reliable method than the uncorrected rank order analysis, and would be the preferred analysis type of all those tried.

PREFACE

The experimental work described in this dissertation was carried out at the South African Sugar Association Experiment Station (SASEX), Mount Edgecombe, under the supervision of Dr PE Shanahan and Prof PL Greenfield, of the Disciplines of Plant Breeding and Crop Science respectively, in the Programme of Agricultural Plant Sciences.

These studies represent original work by the author that have not been submitted in any form for any degree or diploma to any University. Where use was made of the work of others it is duly acknowledged in the text.

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LIST OF ABBREVIATIONS

AMMI – additive main effects and multiplicative interaction

CV – coefficient of variation

FR – factorial regression

G x E – genotype x environment

H – Mahalanobis

HPLC – high performance liquid chromatography

MPLS – modified partial least squares

NIR – near infrared

NIRS – near infrared spectroscopy

PCA – principal component analysis

PLS – partial least squares

r^2 – coefficient of determination

REML – restricted/ residual maximum likelihood

SEC – standard error of calibration

SECV – standard error of cross validation

SEP – standard error of prediction

SEV – standard error of validation

LIST OF DEFINITIONS

Ratoon – The above ground components of the plant that regenerate from the root system after harvesting.

Seedcane – Sugarcane is propagated from stalks of the sugarcane clone, rather than by the more conventional method of true seed.

Tiller – Sugarcane forms many auxiliary stalks that are indistinguishable from the one central stalk. These will be referred to as tillers in this thesis. Numbers may vary from a few to over a hundred. Number of tillers has a large influence on yield.

GENERAL INTRODUCTION

Sugarcane (*Saccharum* spp hybrid) in South Africa is grown only in Kwazulu-Natal and Mpumalanga (Nuss, 1998), on an area of over 420 000 ha. Approximately 320 000 ha are harvested every year, producing on average 22 million tons of cane and 2.5 million tons of sugar. Roughly 72 % of this crop is produced by about 2 000 large-scale commercial growers, 15 % by 51 000 small-scale growers and 13 % by sugar milling companies. There are 16 mills in the industry, owned by three milling companies and one grower co-operative (Anon, 2002).

The eldana borer (*Eldana saccharina* Walker) is the most serious insect pest in the South African sugar industry, resulting in a total loss to the industry of about R200 million every year (Murray, 1997). Numerous methods of control have been attempted, from using pesticides to biocontrol using natural predators, with little success. The only effective method of controlling eldana populations that has been used to date is through limiting the age at which the sugarcane crop is harvested (Carnegie, 1974; Atkinson, 1984; Carnegie and Leslie, 1990). Since there is a high prevalence of eldana in older cane, reducing the time to harvest from approximately 18 months to about one year provides some protection against high larval population development, as well as reducing potential losses from excessive damage to the crop. However, this agronomic practice increases the long-term maintenance costs involved in crop establishment, harvesting, weeding, etc., while reducing the sucrose production per hectare per year. Genetic resistance is perceived as being the most effective method of overcoming the current need for early harvest age.

Present eldana screening methods require the planting of additional trials separate from the selection programme trials in which clones are evaluated for yield performance characteristics. Numbers of genotypes that can be evaluated are low because of limited space, so these trials can only be carried out at a late stage of the selection programme. Because of the small size and artificial environment of the trials, results are not as accurate as required for breeding and selection purposes, and the same clones must be tested a number of times before a reasonable estimate of resistance potential can be made. As a result, an alternative means of evaluating eldana resistance is needed. The ideal method would be rapid, usable in the selection programme rather than in separate trials, reliable and unaffected by environmental influences. Use of near infrared spectroscopy (NIRS)

provides the possibility of fulfilling those criteria. If successful, it would be more rapid and cost-effective than the establishment of separate trials, has the possibility of producing consistent results regardless of the environment the trial was conducted in, and could be done on a larger population than is done with the present screening method.

However, the use of NIRS in this regard is untried. While preliminary results suggested that NIRS might be able to detect chemical components of bud scales that are related to eldana resistance (Rutherford, 1993), the effects of environment, age of sugarcane, location of bud scales up the stalk, and crop year on the chemical makeup of bud scales are not fully known. The genetic control and inheritance of the chemical components that NIRS detects is also unknown. This information is vital if the technique is to be used effectively, not only as a screening method in trials, but also in the selection of parents in order to establish a high level of eldana resistance in the breeding population.

The objectives of this study were therefore to determine whether NIRS was capable of detecting the chemical components of bud scales associated with eldana resistance across a range of environments. If so, the effect of environment on the eldana-related chemical components would be studied, using the same trials that tested the NIRS technique. This trial consisted of calibration sets of 60 genotypes with relatively well-defined eldana resistance ratings, planted in three replications at two sites and sampled in plant and first ratoon crops, in order to test the environmental influence on the bud scale chemical components. In each crop, samples were taken at three ages, in order to determine whether there was an optimal age at which to evaluate the bud scale chemical components. Furthermore, samples from the bottom of the stalk were taken to determine whether degradation of the bud scale chemical components was different for different genotypes, and whether this contributed to the level of eldana resistance. A heritability study was also set up in order to determine the inheritance of any bud scale chemical components that could be linked to eldana resistance and detected by NIRS. The offspring of 24 crosses, and the parents, were planted together with the calibration set for reference, to be used for heritability estimation by parent-offspring regression analysis.

CHAPTER 1
LITERATURE REVIEW

1.1 A background to the South African sugar industry and the South African Sugar Association Experiment Station (SASEX)

The subtropical conditions in the South African production areas limit the growth potential of sugarcane, which is essentially a tropical crop. The South African Sugar Association Experiment Station (SASEX) has succeeded in producing cultivars that are better adapted to the less than ideal local conditions. Five research stations cover the regions found within the South African sugar industry, namely high altitude Midlands, irrigated North, high potential Coastal, low potential Coastal and Hinterland (Nuss, 1998). In the Midlands area, growth is quite slow due to the colder conditions, and a cutting cycle of approximately two years is necessary for economic viability. The Hinterland, having a climate affected by both high altitude and coastal conditions, has a cutting cycle of approximately 18 months (Nuss, 1998). The Coastal areas had a similar cutting cycle until the appearance of the stem borer eldana, at which time a reduction to approximately one year became necessary (Carnegie, 1974; Atkinson, 1984; McCulloch, 1989; Carnegie and Leslie, 1990). Because of good soils, heat and plentiful water, the irrigated and high potential areas can produce sufficient cane on the short one-year cycle in order for growers to remain profitable. On more marginal lands, however, conditions are too poor for cane to grow sufficiently well in the shorter cycle for optimal accumulation of sucrose.

The primary purpose of SASEX is to breed and select superior cultivars for release in the industry. Fertile seed is difficult to obtain under South African conditions, due to cool temperatures at flowering leading to male-infertile flowers, and incorrect photoperiod. There is therefore a glasshouse and photoperiod house at SASEX to facilitate the production of fertile flowers for the crossing program. These facilities allow the control of temperature, humidity, light and other factors that affect flower production, pollination and seed set, and are also used for manipulating the time of flowering so that flowering is spread across a number of months (Brett, 1974; Brett and Harding, 1974; Brett *et al.*, 1975; Nuss, 1977; Berding and Skinner, 1980; Nuss, 1980; Berding, 1981; Nuss, 1982).

Superior parents are chosen each year, based on disease and yield characteristics, and are grown in the glasshouse and photoperiod house. Flowering takes place between May and August. Three times a week, open flowers are checked for pollen fertility and then crosses are made according to potential positive combinations of disease and yield characteristics of the parents (Nuss, 1982). Once the crossing season ends, seed selections are made from the crosses that test positive for viable seed. Seedlings germinated from these crosses enter the selection programme. This programme consists of five stages, with the top clones from each stage being advanced to the next (Brett, 1954; Bond, 1988; Butterfield and Thomas, 1996). Of the 250 000 clones, derived from seed, that enter the programme each year, only one or two may exhibit commercial potential and be released to the industry. Twelve to fifteen years of testing are needed for each clone, depending on the cutting cycle of the area that it is being tested in. A long testing phase is needed, not only because of variability between years in both yield characteristics and disease trends, but also because initially seed cane is in short supply and plots are small, resulting in imprecise trials.

Disease resistance measurements are not carried out until late in the selection programme, although clones found with disease are discarded at all stages. Many cultivars are rejected later in the selection programme because of a previously undiscovered susceptibility to a commercially important disease. This represents a considerable waste of resources, which would be more effectively used testing disease resistant cultivars for other important agronomic traits (Butterfield and Thomas, 1996).

1.2 Sugarcane genetics

In the development of the modern commercial sugarcane hybrid, different *Saccharum* species were used. The so-called noble sugarcane, *S. officinarum*, provided the high sucrose content, while *S. spontaneum, S. barberi* and *S. sinense* provided good growing habits such as high tiller numbers, good ratooning ability, vigour and disease resistance (Panje, 1971; Irvine, 1977; Grisham *et al.*, 1992; Burner *et al.*, 1993). The dominant parent was *S. officinarum*, which was used in backcrossing to achieve commercial standards of sucrose as rapidly as possible. This process was accelerated by the tendency of *S. officinarum* and its early generation offspring to transmit the somatic complement of chromosomes rather than the customary reduced gametes, when used as the maternal

parent in a cross with the other species, *S. spontaneum, S. barberi* and *S. sinense* (Roach, 1968a; Roach, 1968b; Roach, 1971).

Genetically, sugarcane is a very complex crop. It is generally accepted that *S. officinarum* is an octoploid, (Burner and Webster, 1994) and recent developments confirm that *S. spontaneum* is a 5- to 16-ploid (as reviewed by Butterfield *et al.*, 2001). Chromosome numbers for both species range from 60 to 120 (Li and Price, 1965; Roach, 1968a), although some clones may fall outside this range. Modern commercial sugarcane is also a hybrid of different species with different chromosome numbers. While there is a certain amount of chromosomal imbalance during cell division, because of the different ploidies of the wild progenitors, most cell division is normal (Srivastava and Srivastava, 2001), and the sugarcane clones tend to be very tolerant of irregularities such as aneuploidy, probably because of the high copy number of each chromosome (Chen *et al.*, 1983; Burner, 1991; Burner and Legendre, 1993).

Even though the wild progenitors of sugarcane came from diverse sources, very few genotypes within each species were used in the original hybridisations (Roach, 1971). The genetic base is therefore very narrow, although inbreeding effects are negligible due to the polyploidy. In recent years there has been a move to widen the genetic base in breeding populations, primarily to search for new resistance genes and greater vigour in commercial cultivars (Heinz, 1980; Roach, 1986; Legendre, 1989; Roach, 1989).

The genetic complexity of sugarcane clones makes it difficult for the plant breeder to predict the outcome of different parent combinations. Inheritance patterns are difficult to discern, particularly for quantitative traits, due to the multiple copies of chromosomes present. Any trials undertaken to study inheritance must therefore be done with the knowledge that some of the fundamental assumptions made in the calculation of population statistics may be violated to an unknown degree. In particular, the underlying theory of most statistical analyses is based on diploid genetics, whereas sugarcane is a complex polyploid crop.

1.3 History of the eldana borer in sugarcane

Eldana (*Eldana saccharina*) is an indigenous pest in South Africa, and is usually found in a range of weeds and grasses (Carnegie *et al.*, 1976). Its true hosts are the larger Cyperaceae (Atkinson, 1979, 1980), particularly *Cyperus immensus*, where it feeds primarily in the inflorescence. It has been found in a number of crops, but because of its preference for older, mature plants it does not cause significant loss in seed yield (for example, the grain of maize), and is generally not regarded as a serious pest in other crops (Cochereau, 1982).

Eldana was first found in sugarcane in South Africa in the 1940's (Dick, 1945; Carnegie, 1974; Atkinson *et al.*, 1981; Heathcote, 1984), but for unknown reasons the outbreak was short-lived, and eldana was not subsequently seen in sugarcane until the 1970's (Carnegie, 1974; Smaill, 1978; Carnegie and Smaill, 1980; Atkinson *et al.*, 1981). The second outbreak into sugarcane was permanent and ongoing, with eldana spreading from the first outbreak in the Umfolozi area, to the rest of the coastal areas where sugarcane is grown. The only region where numbers are limited in present times is the higher altitude Midlands area, where the colder temperatures are believed to reduce the spread (Heathcote, 1984; Way, 1994).

Eldana was also first recorded in Swaziland in the early 1970's, where it has become an established pest in sugarcane (Carnegie *et al.*, 1976; King, 1989). However, it has only been found in sugarcane in Zimbabwe from the 1990's (Mazodze *et al.*, 1999). In the Ivory Coast, sugarcane is damaged by both the eldana borer and a number of *Sesamia* species, which also attack maize (Cochereau, 1982). *Sesamia* do more damage to maize than eldana, because they attack the young plant and affect yield production, while eldana attacks the mature plant. In sugarcane, *Sesamia* could cause as much damage as eldana if infestation levels were the same, but at present it is not a serious pest. Differences in biology and behaviour between eldana found in South Africa and west and east Africa have been noticed, suggesting that there may be more than one biotype within the species (Conlong, 1997). The borer tends to attack the upper portion of the stalk in west and east Africa, unlike the borer in South Africa, which prefers the lower portion of the stalk, although both share a preference for older cane.

Eldana has become the most serious insect pest in the South African sugarcane industry. Excessive numbers can reduce yield substantially, by as much as 1.5% in recoverable sugar for every 1% stalk bored (Smaill and Carnegie, 1979; Cochereau, 1982; King, 1989; Leslie, 1994), resulting in a total loss to the industry of about R200 million every year (Murray, 1997). Loss in yield is caused primarily by reduction of sucrose content due to consumption by eldana larvae, although decreases in mass of stalk are also possible in heavily infested cane. The biggest influence that eldana has had on the coastal production regions is to reduce the age at which sugarcane is harvested (Carnegie, 1974; Atkinson, 1984; Carnegie and Leslie, 1990). Because of its preference for older cane (Girling, 1971; Carnegie and Smaill, 1980; Carnegie, 1982; Paxton, 1982), eldana has caused a reduction in the harvesting period from a two-year to an annual cycle. This affects sucrose yield as sucrose accumulates preferentially in mature internodes, with little being stored in immature internodes. In younger cane, the immature internodes comprise a larger proportion to the mature internodes than in older cane, causing a lower sucrose yield on a per month basis (McCulloch, 1989). Expenses are also increased substantially with more frequent planting, maintenance and harvesting operations.

The size of the eldana population generally remains low due to harvesting at a young age. Drought years can, however, cause a rapid increase in eldana numbers due to the higher susceptibility of stressed cane to pest proliferation (Atkinson *et al.*, 1981; Carnegie, 1982; Heathcote, 1984; Carnegie and Leslie, 1990). Years with good yields also present a problem, as a larger proportion of the crop cannot be processed during the milling season. Some fields may be left for the next milling season (carry-over cane), and the increased age results in high infestation levels the following milling season (Carnegie, 1981; McCulloch, 1989). Eldana therefore remains an ongoing problem with major detrimental effects to the sugar industry, making the search for new control methods a top priority.

Eldana larval levels are monitored in the industry using two methods: eldana surveys and light trapping. Eldana surveys consist of taking a destructive sample of a representative number of stalks from the field, splitting them open and assessing the amount of damage, either by measuring the proportion of damaged stalk or by counting the number of damaged internodes, as well as making counts of the number of larvae and pupae found (Carnegie *et al.*, 1976; Smaill, 1978; Bond, 1988; King, 1989). This method is also used when evaluating selection programme trials. The second method of predicting infestation

levels in fields uses light traps to catch moths. This is not as good a method of estimating eldana damage as the survey method, as it is an indirect method of measuring eldana numbers. Light traps are also not usually placed in-field, due to accessibility problems and they are used mainly to determine when moth peaks occur in order to predict subsequent rises in larval numbers (Atkinson, 1982; Carnegie and Leslie, 1990).

1.4 Current status of the eldana borer work at SASEX

1.4.1 Present screening methods for determining eldana resistance

Two forms of trial assessment are used to measure eldana resistance levels in cultivars. Yield trials from the selection programme that are planted in areas where naturally high infestation levels can occur, are surveyed for eldana (Nuss and Atkinson, 1983). These trials usually have high infestation variability, due to variability of soil, water availability, etc. across the trial. The second type of assessment is conducted in pots (Nuss and Atkinson, 1983; Nuss, 1991). Clones are planted in drums under shadecloth to prevent natural infestation, together with reference cultivars with known responses to eldana. At the age of eight months, the cane is stressed by water withdrawal and artificially inoculated with eldana eggs. Infestation levels on all cultivars are then evaluated using amount of stalk damage, and number and mass of larvae and pupae. Pot trials have correlated well with field trials but are also subject to inconsistent results between trials due to unknown factors (Nuss and Atkinson, 1983; Nuss, 1991), thereby requiring a fair number of repetitions of trials in order to ensure accuracy of results. A further possible screening method involved the hypothesis that eldana moths showed a preference for different clones as oviposition sites, but this screening method was rejected as no discernible difference of oviposition between clones was detected (Nuss and Atkinson, 1983).

Pot trials require several separate trials, with fairly limited numbers of clones, and are therefore unsuitable for testing large numbers of clones from the early stages of the selection programme. At these early stages, information on resistance ratings would give the most selective gain (Nuss *et al.*, 1986; Bond, 1988). Only later stages, with lower numbers of cultivars, can be tested, resulting in a loss of resources and time on cultivars that are unsuited for release to the industry (Butterfield and Thomas, 1996).

1.4.2 Control methods for eldana borer

One of the most commonly used measures available for combating a pest is chemical control. Whilst not desirable environmentally, insecticide usage usually provides a means of controlling a variety of pests in many crops. Tests conducted by SASEX have shown that eldana larvae have slower growth rates and higher mortality when fed on an artificial medium containing insecticides (Heathcote, 1984). In the field, however, the effectiveness of the same insecticides decreases (Carnegie, 1982; Heathcote, 1984). Chemical control of eldana is inconsistent, affected by method and number of applications (Leslie, 2001). The inconsistent results may be caused by the rapidity with which the larvae bore into the stalk. Compared to other borers, eldana larvae spend very little time feeding on the exposed areas of the plant (Girling, 1971; Leslie, 1993). The eggs are also inserted under the leaf sheath, where the insecticides do not penetrate (Carnegie, 1974; Leslie, 1982).

A number of physical control measures have been attempted to control eldana numbers. Due to the eldana moth's preference for dead leaf material as oviposition sites, it was thought that the removal of dead leaves in a standing crop, a process known as pre-trashing, would reduce eldana numbers (Carnegie and Smaill, 1982; Leslie, 1994). Besides having no effect on the level of infestation in the field, pre-trashing also appeared to reduce yield. The killing of larvae in seedcane by chemical or hot water treatment of stalks has an effect on numbers of eldana in the subsequent crop (Carnegie *et al.*, 1976; Heathcote, 1984), but also affects germination of the buds, requiring replanting in the gaps. Burning before harvesting also helps control larvae levels in the following crop (Carnegie *et al.*, 1976; Atkinson, 1984), but is no longer environmentally desirable. A method currently under study is the use of indigenous plants as lures or repellents for eldana (Conlong and Kasl, 2000 and 2001).

Natural parasites are a biological solution to the problem of control. Many parasites and predators of eldana exist on the natural host plants of eldana, but there is a curious absence of these insects in sugarcane fields (Carnegie and Leslie, 1979; Carnegie, 1982; Cochereau, 1982; Leslie, 1982; Carnegie *et al.*, 1985). Differences between natural hosts and sugarcane are thought to be the cause. Not only is the stalk of sugarcane much harder than the natural hosts' to bore through, preventing parasites from reaching the eldana

(Carnegie *et al.*, 1985), but the sugarcane plant may also not carry the correct chemical signals to attract the parasites (Conlong and Kasl, 2000; Conlong and Kasl, 2001).

Cultivar resistance is the most viable control method available to date (Girling, 1971; Hensley *et al.*, 1977; Klenke *et al.*, 1986; Bond, 1988; Nuss, 1991). Resistance mechanisms range from physical structure to chemical composition of stalk components. Physical traits that act as deterrents include rind hardness and high fibre content. Both of these factors have good heritability in sugarcane, and could be useful in combating eldana infestation. However, both characteristics are undesirable to the grower and the miller (Davidson, 1968; Skinner, 1974; Gravois and Milligan, 1992). Cane cutter output drops drastically with hardness of stalks, and high fibre content is an unwanted complication in the processing of sugarcane in the mill (Gravois and Milligan, 1992).

Transgenic maize (*Zea mays*) plants exhibiting resistance to borers have been produced, and transgenic sugarcane plants are currently being studied for resistance to eldana. The resistance genes commonly used are from *Bacillus thuringiensis* and code for insecticidal proteins. The Bt genes have been used against insect pests of a number of crops, including tomatoes (*Lycopersicon esculentum*), cotton (*Gossypium hirsutum*), potato (*Solanum tuberosum*) and maize (Perlak *et al.*, 1993; Armstrong *et al.*, 1995; Fitt, 2001), and preliminary results with artificial medium have confirmed that the Bt gene is effective against eldana (Cassim *et al.*, 1999). The Biotechnology department at SASEX is currently working on inserting the appropriate Bt gene into sugarcane cultivars, and testing the expression of this gene through a number of ratoons (Cassim *et al.*, 1999). However, as this gene is patented, there will be additional costs associated with its use in commercial sugarcane production. Public opinion of transgenic crops is also not favourable and needs to be taken into account before this approach is implemented. The testing phase is also quite long, and the cultivar selected for transformation may no longer be commercially popular when testing is complete.

1.5 Plant defence mechanisms

The chemical composition of the plant is perhaps the most important aspect of resistance to insects. Exact mechanisms of resistance are unknown, and they may be too complex to classify completely. Some aspects have, however, been studied.

In cotton, gossypol is a pigment that is known to be highly toxic to animals (Bi *et al.*, 1999). Studies have suggested that gossypol may also be toxic to insects (Lukefahr and Martin, 1966), and that there is a good indication that gossypol content is strongly heritable (Lukefahr and Houghtaling, 1969), making it ideal in breeding for insect resistance. It may even be possible to produce plants with high vegetative gossypol content with low gossypol content seeds, so that the seeds can still be used as feed and extracted for their oil content (Bi *et al.*, 1999).

Others substances produced by the cotton plant, such as tannins, are also involved with the natural defence mechanisms against insects (Klocke and Chan, 1982; Lege *et al.*, 1992). Tannins are present in many plants, where they are considered the most important group of defensive compounds (Lege *et al.*, 1992; Bialczyk *et al.*, 1999). Resistance mechanisms are thought to be through unpleasant taste and inhibition of enzymatic activity in the insect's digestive processes, thereby decreasing digestibility of certain nutrients (Klocke and Chan, 1982; Lege *et al.*, 1992; Bialczyk *et al.*, 1999). Examination of various lepidopteran larval growth and survival rates on artificial diets confirms this (Manuwoto and Scriber, 1986). There is also the possibility that tannins have a direct toxic effect on some insects (Karowe, 1989). However, tannins are strongly influenced by environment, making them a less than ideal candidate in breeding programmes (Lege *et al.*, 1992; Bialczyk *et al.*, 1999).

Hydroxamic acid in maize seems to provide protection from a range of pests (Long *et al.*, 1977). It has a proven effect on the corn leaf aphid (*Rhopalosiphum maidis*) and, because there seems to be a positive relationship between aphid infestation levels and attack by the European corn borer (*Ostrinia nubilalis*), it probably works against the borer as well. Hydroxamic acid also seems to be associated with levels of infestation of stalk rot (*Diplodia zeae*) and Northern corn leaf blight (*Helminthosporium turcicum*), making it a good candidate for all round protection. Wheat (*Triticum* spp) and its relatives also utilize hydroxamic acid as a defence mechanism (Thackray *et al.*, 1990). Levels are highest in young plants and in the emerging leaves of all ages, giving protection at the most important development stage. Levels are, however, heavily influenced by environmental factors such as water availability and light intensity, making it unreliable under all conditions.

Lectins are present in a number of plant species, occurring primarily in the seeds. They have a negative effect on insect growth, possibly by inhibiting absorption of nutrients (Chrispeels and Raikhel, 1991). Tests using artificial diet confirm the deleterious effect of lectins on insects (Czapla and Lang, 1990).

Proteinase inhibitors have been found in the roots, leaves and seeds of several plant genera (Walker-Simmons and Ryan, 1977). They are wound induced, accumulating only when the plant is damaged by insects or animals. They are not, however, restricted to the site of the wound, and usually spread to undamaged parts of the plant as well (Green and Ryan, 1972).

It is also possible for a combination of compounds to work together to confer insect resistance. In young sorghum (*Sorghum bicolor*), cyanide is produced to provide protection for the most vulnerable stage of the plant's development. When the plant is older, and can survive some damage, cyanide production drops, and phenolics become the primary defence mechanism (Woodhead and Bernays, 1978). Interactions are not always favourable, however. In lucerne (*Medicago sativa*), saponins provide the mechanism for insect resistance, but only in the absence of sterols. Whilst not involved in plant defence, high levels of sterols seriously compromise the protection given by the saponins, causing plants with a resistant profile to be susceptible (Shany *et al.*, 1970). Plant breeders need to be aware of these types of interactions when trying to achieve resistant plant populations.

Some compounds are undesirable in a plant, as they attract insects rather than repelling them. Sulphur-bearing chemicals in onions serve as attractants to the onion fly, *Delia antiqua* (Soni and Finch, 1979), and rice (*Oryza sativa*) produces compounds that draw the brown planthopper, *Nilaparvata lugens* (Saxena and Okesh, 1985). Some plants will produce a chemical when wounded, attracting further attack by an insect pest, such as carrot fly (*Psila rosae*) on carrots (*Daucus carota)* (Cole *et al.*, 1988). A problem for the plant breeder is when a compound that confers resistance to one type of insect serves as an attractant to another. An example of this is the tannins, which make a good protection mechanism for many insects, but actually serve as a feeding stimulant to some (Manuwoto and Scriber, 1986).

There is little information on the mechanisms of defence to insect damage in sugarcane. Expression of several defensive proteins have been observed, but not examined in detail (Falco and Silva-Falco, 2001). Rutherford (Rutherford, 1993; Rutherford *et al.*, 1993) studied a range of these substances to determine if they were related to eldana resistance. These include tannins, lignin, bud flavonoids and surface waxes. Tannins and lignin seem to have little effect on eldana resistance (Rutherford, 1993). Surface waxes are the first chemicals that feeding larvae encounter, and they have been found to be a mechanism of resistance in a number of other plant species (Woodhead, 1982; Woodhead and Taneja, 1987; Bodnaryk, 1992; Woodhead and Padgham, 1988). In sugarcane, larvae have shown limited feeding on artificial diets containing surface waxes, suggesting that these may be a mechanism for eldana resistance (Rutherford, 1993). Smut (*Ustilago scitaminea*) resistance in sugarcane has been closely correlated to flavonoids found in bud scales, and there seems to be an inverse relationship with eldana resistance. The bud is a common entry point for eldana larvae (Atkinson, 1979), and therefore a good place to search for resistance factors. However, tests using bud scale extracts in artificial diets were inconclusive, showing an increase in feeding and survival on 'susceptible' diets, but no decrease in feeding or survival on 'resistant' diets compared to the control diet (Rutherford, 1993). Initial work by Rutherford (1993) did suggest correlations between chemical profiles of bud scales and eldana resistance, however. These studies were done on a limited number of sample sets of sugarcane genotypes grown in a limited number of environments, and it was therefore not known how the chemical compounds found in the bud scales would change in different conditions, and whether their detection by near infrared technology would remain reliable in different environments. Furthermore, little is known about the inheritance of the chemical composition of bud scales. Knowledge of heritability and environmental interaction effects is important if this method of resistance is to be used successfully in breeding and selecting resistant cultivars (Ladd *et al.*, 1974; Schoonhoven, 1982; Thackray *et al.*, 1990; Lege *et al.*, 1992). Evaluations of the inheritance of resistance to eldana in sugarcane (Bond, 1988), and other borers in sugarcane and maize (Bastos *et al.*, 1980) using analysis of variance, have shown promising results.

An important aspect in determining the resistance of sugarcane to eldana is finding a quick, easy method to identify the level of resistance, preferably without the confounding effect of environment. If bud scales on sugarcane contribute significantly to eldana resistance, and their chemical composition can be quickly and easily measured, then selection for a

specific chemical profile associated with eldana resistance would indirectly increase eldana resistance. A technique such as near infrared spectroscopy (NIRS), which may measure bud scale chemical profiles accurately enough for selection purposes, is ideal for this purpose, as it provides a quick, easy method of analysis that can be added to existing trials, precluding the need for separate trials (Rutherford *et al.*, 1993; Rutherford and van Staden, 1996).

1.6 An overview of near infrared spectroscopy (NIRS)

1.6.1 Background of NIRS technique

Absorption of light at a particular wavelength in the near infrared (NIR) region of the spectrum indicates a particular molecular bond. The spectral information is usually repeated a number of times within the NIR region, and since the absorbance bands involved become weaker by an order of magnitude each time, they represent a built-in dilution series. Wavelengths in the NIR region occur from 1100 to 2500 nm (Reeves, 1997; Givens and Deaville, 1999; Larrahondo *et al.*, 2001).

There are a number of instrument models available for NIRS analysis and whilst they differ in several respects, they all have the same basic components and method of operation. A source of radiant energy produces a primary light beam that passes through a device that provides wavelength discrimination. In older models, interference filters were used to give a few fixed wavelengths, but these were very limiting as knowledge of the relevant wavelengths was necessary prior to scanning. If unknown, a long, tedious process would be involved to determine the wavelengths best suited to a particular sample. Newer models with monochromators have the capacity to scan across the entire near infrared range at every sampling, thereby producing useful information even on unknown samples. The light from the wavelength discriminator is directed onto the sample. A photoelectric detector collects the resultant absorbed or reflected radiant energy. Restriction of wavelengths reaching the sample is necessary, as the detector responds to all wavelengths and sensitivity decreases with an increase of wavelengths. Placing the detector close to the sample minimizes loss of transmitted or reflected energy. Once the recording of reflection values is complete, the information is converted to the log of the reciprocal (Starr *et al.*, 1981; Givens and Deaville, 1999). The amount of reflected light is inversely proportional

to the amount of light energy absorbed at a particular wavelength, which is proportional to the concentration of the constituents that are optically active at that wavelength. Therefore the concentration of the constituents can be derived if the relationship between amount of reflected light and concentration is known. This knowledge is obtained by using a calibration set of known concentrations.

There are three types of NIRS scanning options available: reflectance, transmittance and transflectance. Reflectance is when the light beam is bounced off the surface of a solid, opaque sample (Baer *et al.*, 1983). For the majority of samples no preparation is required, although in some cases such as grains it is necessary to grind the sample prior to analysis. In transmittance, the absorption at different wavelengths as the light passes through a transparent sample is measured. In transflectance, the sample is transparent, but the instrument is set up for reflectance. In this case, the light passes through the sample and is then reflected back through the sample, usually using a ceramic plate. The wavelength absorption is then determined as for reflectance. In transflectance, all radiation is collected, including that radiation reflected within the sample. Therefore, transflectance provides a more reliable measure of absorbance of light scattering samples than transmittance, where the back-scattered radiation is not measured. In all three cases, the sample is moved slowly across the optics window and an averaged reading is obtained. This averaging is necessary, particularly in solid samples, because uneven particle size plays a large role in the accuracy of NIRS. New technology has made an additional piece of equipment available. Fibre optics allows the sample to be scanned outside the instrument itself, even allowing readings to be taken in the field. The fibre optic probe can scan solid, unprepared samples (Givens and Deaville, 1999). Since the NIRS technique is sufficiently rapid for process control, on-line sample presentation techniques have also been devised (Lee *et al.*, 1997; Givens and Deaville, 1999; Schaffler, 2001b).

1.6.2 NIRS calibration

NIRS instruments need calibration before they can be used for quantitative measurement. Many other measuring techniques also require calibration, but NIRS is unusual because an extensive set of calibrations must be carried out for the instrument to be of use (Shenk and Westerhaus, 1993; Velasco and Becker, 1998; Givens and Deaville, 1999). For many of the well-established applications, the manufacturers may supply instruments with

calibration equations in place, although even these require adjustments for instrument bias and local conditions (Hymowitz *et al.*, 1974; Osborne *et al.*, 1982; Shenk and Westerhaus, 1991c). A number of calibration methods are available for overcoming the problems inherent in NIRS. The main problems are the complex nature of NIRS spectra, where any peak of interest is almost always overlapped by one or more interfering peaks (Osborne *et al.*, 1982; Lee *et al.*, 1997; Givens and Deaville, 1999); and the strong interference caused by the scattering properties of the sample, particularly of that related to particle size. No one calibration method has proved to be superior in all applications, and the decision as to which to use must be based on what each achieves for a particular purpose (Shenk and Westerhaus, 1991a; Li *et al.*, 1996; Smith *et al.*, 1998). The calibration method must, however, be able to handle the use of multiple wavelengths.

The method of least squares is one of the common methods to develop a calibration equation. It is usually the practice in NIRS calibration to take the reference measurement as the dependent variable. This has the advantage of being consistent with the way multiple regression is used to fit equations involving several terms. Because it makes a difference which way round the regression is performed it is important to be consistent when carrying out any checks on the performance of the calibration.

Whatever mathematical solutions are used, the first step of the process is to perform a calibration experiment. This involves collecting a set of calibration samples, which should be representative of the population of samples that the procedure will eventually be used on (Osborne *et al.*, 1982; Wetherill and Murray, 1987; Shenk and Westerhaus, 1991a; Shenk and Westerhaus, 1991b), and subjecting the samples to analysis by the reference method for the constituent of interest, and by the NIRS instrument. From these data the calibration method attempts to find a pattern for predicting the reference analysis results from the spectral data. A minimum of 30 samples is usually needed for calibration.

The residual standard deviation and the correlation coefficient are usually used as measures of the goodness of fit of the predictions (Shenk and Westerhaus, 1991b). The residual standard deviation, or standard error of calibration (SEC), is a root mean square average of the errors about the fitted line and represents a typical discrepancy from the prediction (Lee *et al.*, 1997). The SEC is usually an underestimate of errors that will occur in the prediction of future samples (Lee *et al.*, 1997). The main reason for this is that the equation used for

prediction is a line of best fit for the particular samples in the calibration set, not for the population as a whole (Osborne *et al.*, 1982; Shenk and Westerhaus, 1991b). A different set of samples would produce different, although hopefully only slightly different, values. This adds to the prediction errors, especially for samples near the extremes of the calibration range. This problem is not serious so long as the calibration samples are numerous enough, well distributed over the range and representative of the population to be assessed, but even then SEC will be a little optimistic.

The coefficient of determination (r^2) measures the extent to which the fitted straight-line relationship explains the variability in the y-values (Griggs *et al.*, 1999). In fact, r^2, which lies between 0 and 1, is the proportion of the total variance in the y-values explained by the fitted line. It therefore depends on the spread of the y-values as much as it does on the goodness of fit, and can only measure the predictive ability of the equation relative to the range of y-values in the calibration. Therefore, by increasing the range, possibly outside that of interest, an apparently impressive correlation can be created, although the prediction errors actually get larger. In some circumstances, where the natural range is very wide and the NIRS measurement quite accurate, even a poor calibration can have a high correlation. In other situations calibrations with correlations far less than this may be perfectly acceptable because the relationship between the natural range and the precision of the reference method is quite different. The prediction error, of which SEP is the best indicator, is therefore more important than the correlation coefficient, which is difficult to interpret (Wetherill and Murray, 1987; Velasco and Becker, 1998; Velasco *et al*, 1999a).

It is important to plot the data and inspect the plot for evidence of deviation from a straight line, outlying observations and any other unusual features (Smith *et al.*, 1998; Velasco *et al.*, 1999b). A check for skewness involves fitting a straight line, with the two variables being the reference measurements as the observed, independent variable and NIRS predictions as the predicted, dependent variables. The slope will be equal to one when there is no skewness. Residual plots, i.e. observed minus predicted values, plotted against predicted values, usually give the best indication of abnormalities. If any outliers, i.e. observations suspiciously far from the line, are found then the data should be checked, possibly even by repeating the measurements. If an outlier is not discarded it may unduly influence the calibration (Lee *et al.*, 1997). However, if it is discarded, then a false, better calibration fit may be obtained (Smith *et al.*, 1998). The definition of an outlier is partly a

matter of statistics, but also partly a matter of judgement, but more than one or two per set of fifty is excessive, and may indicate a problem.

Validation with a set of samples not included in the calibration is an important step in calibration development. Samples that were not part of the original calibration must be predicted using the calibration equation, and compared to the reference method values (Lee *et al.*, 1997). Statistics similar to those used for examining the accuracy of the calibration fit, can be employed to check the accuracy of the prediction, with the advantage that underestimation of prediction errors will not take place. If the samples used in validation are from the calibration set, but were not used as part of the calibration development, the relevant statistic is standard error of cross validation (SECV). If the samples are from an independent set of samples, then the standard error of prediction (SEP) or standard error of validation (SEV) is used (Shenk and Westerhaus, 1991b). Because the samples were not part of the calibration set, the accuracy of the prediction will not be influenced by the link between the calibration samples and the unique equation formed using those samples. Errors will still be influenced by the set of validation samples used, but will be a better indication of that found in the population as a whole.

1.6.3 Benefits and pitfalls of NIRS

Conventional chemical analyses are usually time-consuming, expensive or hazardous, and use a considerable amount of laboratory space, equipment and technical expertise. However, NIRS can enable these determinations to be achieved with a single instrument that is compact, cheap to run, simple once calibrated and safe (Starr *et al.*, 1981; Givens and Deaville, 1999; Larrahondo *et al.*, 2001). Generally, NIRS gives a high signal to noise ratio (Shenk and Westerhaus, 1991c), and can sometimes be more accurate than the reference analysis method (Lee *et al.*, 1997). However, because the accuracy of NIRS depends partially on the reference method, the higher accuracy of the NIRS technique is not always evident.

NIRS analysis has a distinct advantage over conventional analysis in many cases because spectra can be obtained from intact, opaque, biological samples, thereby facilitating non-destructive sampling. Sample preparation in general is minimal, contributing to the faster speed and reduced chemical usage of the NIRS technique when compared to conventional

analyses. Care does, however, need to be taken that sufficient sample preparation is done to ensure the necessary uniformity of material to give accurate results (Givens and Deaville, 1999; Griggs *et al.*, 1999; Velasco *et al.*, 1999; Larrahondo *et al.*, 2001; Lee *et al.*, 1997).

The benefit of being able to analyse samples with NIRS without complicated sample preparation or separation into components, usually far outweighs the small loss in accuracy that may be encountered when compared to standard analytical methods. Although a relatively small amount of preparation work may be required, time-consuming separation processes can be dispensed with. Therefore, NIRS has been explored for a variety of purposes, including the study of both quality and quantity characteristics. The most common examples seem to be in the determination of food quality, both for humans and livestock (Starr *et al.*, 1981; Marten *et al.*, 1984; Shenk and Westerhaus, 1993; Smith *et al.*, 1998; Fonseca *et al.*, 1999; Griggs *et al.*, 1999). The use of NIRS for most cases of food analyses has met with success, but there are exceptions. Some components in a multiple analysis seem to occasionally interfere with others. In the analysis of rapeseed for oil and fatty acid content, for example, samples with low erucic acid must be analysed separately from those with high erucic acid. Low erucic acid seeds show a range of values under NIRS instead of low readings, and cause the combined calibration to consistently overestimate erucic acid content in high erucic acid seeds (Velasco and Becker, 1998; Velasco *et al.*, 1999a). The structure of the samples also has an effect. Simultaneous analysis of sugars in fruit juices also proved unsatisfactory because of the overlap of absorption peaks for the different sugars (Lanza and Li, 1984).

The rapidity of NIRS over conventional analytical techniques is advantageous in ongoing processes, where rapid information on the quality of each step is vital for a correct final product. In cheese making, for example, the decision to move on to the next step in the cheese-making process is usually based on the experience of the cheese-maker, as conventional chemical analyses take too long to be viable. Cheese-makers found NIRS useful, because of its speed and ease of use, in determining the moisture, fat and protein levels in each step. The relative inaccuracy of the lactose content measurement, probably due to particle size, is an acceptable trade-off for the benefit of receiving valuable information on the other characteristics of interest (Lee *et al.*, 1997).

A disadvantage of NIRS is that it is an indirect method, relying on a calibration curve for prediction (Givens and Deaville, 1999; Larrahondo *et al.*, 2001). This requires samples covering the range of possible values of the constituent of interest to be run through the instrument, and a calibration equation devised using the known laboratory values of the constituents (Shenk *et al.*, 1993). New unknown samples can then be predicted using this equation, with occasional checks to ensure that no bias is being introduced over time (Starr *et al.*, 1981). In certain cases, it is not possible to develop a calibration equation that covers all possible samples. In the compositional analysis of whey powders, for example, a universal calibration is not possible, and whey from different sources must be calibrated and analysed separately (Baer *et al.*, 1983).

Probably one of the most serious problems in NIRS is due to the use of too small a sample size for the setup of the calibration equations. A calibration based on a small sample size may incorrectly show a better fit between conventional analysis and NIRS than actually exists, but will then be unable to accurately predict samples not included in the original calibration (Shenk and Westerhaus, 1993). An example of this problem was seen in an attempt to analyse sunflowers for oil quality characteristics in a breeding programme (Velasco *et al.*, 1999b). While the calibration performed well within the batch of samples used to create it, prediction of new batches of sunflower were not as accurate, possibly due to an environmental factor. However, because the requirement for the breeding programme was merely to provide enough accuracy to detect the lowest quality sunflowers, in order to discard them from the breeding programme, the NIRS technique was still acceptable.

Generally, NIRS only detects organic substances, because it can change the energy levels of the chemical bonds in organic molecules. Inorganic materials are not usually determined by NIRS, unless they influence the spectra of other materials that do absorb in the NIR region (Reeves, 1997). Low concentration levels of a particular constituent, even if organic, are also usually difficult to quantify, as the discrimination of NIRS is not great enough to detect low levels of elements in a sample (Albrecht *et al.*, 1987; Dumoulin *et al.*, 1987; Huijbregts *et al.*, 1996).

Accuracy of the NIRS technique is influenced by a number of factors in the sample. Particle size determines scatter of light off the sample, and this light represents a loss in absorption not directly related to the sample composition (Sverzut *et al.*, 1987; Macnab

and Gagnon, 1996; Griggs *et al.*, 1999; Larrahondo *et al.*, 2001). The problem of particle size is difficult to overcome, and is influenced by the loading of the sample into the sample holder as well as the actual particle size. The sample needs to be thoroughly mixed and homogenized to ensure consistent particles sizes are presented to the instrument. A sample that has been poured into the sample holder will be stratified and have an uneven presentation to the instrument (Osborne *et al.*, 1982). Similarly, packing the sample down influences the density of particles. Particle size is a lesser concern in liquid samples, although dissolved matter may also refract light away from the receptor.

Water content plays a role in absorbance spectra because water absorbs in the near infrared region. Samples containing too wide a range of water contents may not be predicted accurately, especially if the samples used for calibration did not contain a similar range of moisture variation (Shenk and Westerhaus, 1991b; Givens and Deaville, 1999; Griggs *et al.*, 1999). Difficulties will also be experienced if the absorption due to water is far greater than that due to the constituent of interest (Dull and Giangiacomo, 1984; Lanza and Li, 1984; Dumoulin *et al.*, 1987; Iizuka and Aishima, 1997). If the amount of water lost from the sample during preparation varies, the spectra produced will have a random element that will impact on prediction (Davies *et al.*, 1985). In addition, the temperature of all samples must be standardised as this affects the density of the samples, which will influence the readings taken by the NIRS instrument (Hymowitz *et al.*, 1974; Shenk and Westerhaus, 1991c; Schaffler, 2001a).

Variation due to instrument error can be a particular problem. Modern instruments tend to suffer less from this random variation than older models (Shenk and Westerhaus, 1985; Dalal and Henry, 1986). In one study of protein and moisture analysis in wheat, there was a bias from year to year that made the use of NIRS impractical (Osborne *et al.*, 1982). Subsequent work on a later model instrument showed no such bias, providing a workable alternative to conventional analysis (Osborne, 1983).

Often, more than one type of problem needs to be overcome for NIRS to be a viable alternative to the reference analysis method. In analysis of herbage, dry ground samples cost more to prepare, and there is an inconsistent alteration of the chemical composition of the samples during the drying process. Fresh samples are less accurate, due to variable moisture content, and more difficult to collect, but are cheaper and quicker to analyse. The

decision of which sample preparation to use would depend on the purpose of the sampling, whether cost and speed were an issue, and whether compositional changes were acceptable (Griggs *et al.*, 1999).

In some cases, no matter what strategies are employed to overcome the drawbacks of NIRS, the technique will be unable to provide a feasible alternative to conventional analytical methods. In the NIRS analysis of haemoglobin in blood, for example, the light-scattering nature of the samples is just too great to be overcome (Macnab and Gagnon, 1996). The analysis of inorganic substances, such as essential mineral concentrations in turfgrass, also tends to be unsuccessful (Rodriguez and Miller, 2000).

1.6.4 Previous applications of NIRS technology in sugarcane

Samples of cane stalks entering a mill need to be analysed quickly and accurately, as the economic value of the rest of the delivery depends on the quality of the sampled cane. Sucrose is the primary constituent that needs to be determined, but other factors affecting mill performance are also important. These include non-sucrose components of the cane juice, fibre and moisture. Use of NIRS technology is an ideal alternative to conventional analysis as it is far quicker and requires less toxic chemicals. Many countries have investigated the use of NIRS in the analysis of cane quality components, including Columbia, South Africa, North America, France, Fiji and Australia. Earlier results with less accurate instruments were disappointing (Ames *et al.*, 1989; Berding *et al.*, 1989; Schaffler *et al.*, 1993), but the findings with new, more precise instruments are proving satisfactory (Habib *et al.*, 2001; Larrahondo *et al.*, 2001). Some constituents, particularly those that are inorganic or present in lower levels in sugarcane, do tend to have a poorer prediction potential (Meyer and Wood, 1988; Schaffler, 2001a; Schaffler, 2001b).

The analysis of sugarcane samples is useful not only to the sugar manufacturing industry, but to the sugarcane breeders as well. Early selection stages include large numbers of clones under evaluation, and a limiting factor has always been the need to analyse a large number of samples. Because of this, much work has been done to improve the predictive ability of the NIRS technique with regards to determination of sucrose content. Shredded cane has been the primary choice of sample, as it requires minimum preparation time. Much progress has been made, aided by improvement in the instrument itself (Meyer and

Wood, 1988; Berding *et al.*, 1989; Berding *et al.*, 1991a; Berding *et al.*, 1991b; Amaya *et al.*, 2001).

Fertiliser costs are one of the major expenses in sugarcane farming. Applying the exact amounts of nutrients required potentially represent a considerable savings, as well as having benefits to the environment. Estimation of nutrient quantities required by a crop is best done with soil and leaf sample analysis. Soil sample data provide information on organic matter content, nitrogen levels and nitrogen mineralisation potential, enabling better nitrogen recommendations to be made (Meyer, 1989). Leaf sample data supply information on the nutrient status of the crop, particularly nitrogen, enabling precise quantities of fertiliser to be applied (Meyer, 1983; Larrahondo *et al.*, 2001). Both soil and leaf samples need to be processed rapidly, in order that the grower receives the results timeously. As NIRS is far more rapid than the conventional analyses, it has proved to be ideal for this purpose.

Trials that test for the resistance of sugarcane to pests are resource intensive and tend to be unreliable (Nuss and Atkinson, 1983; Nuss, 1991). A technique that can be applied within existing trials and that is unaffected by environment would be greatly beneficial to the plant breeding programme at SASEX. For this reason, NIRS was evaluated as a possible technique for prediction of eldana resistance. Whilst resistance to eldana is conferred by the interaction of a number of mechanisms, some unknown, the principle components seem to be the fibre, lignin and tannin content of the stalk, the composition of the wax layer on the exterior surface of the stalk, and the composition of the bud scales. Initial studies have shown that NIRS has the potential to predict these components (Rutherford *et al.*, 1993; Rutherford and van Staden, 1996), with the added benefits of sample-taking from within existing trials and rapid prediction. However, very little is known about the effect of environment on these factors, particularly the chemical composition of the waxes and bud scales. In order for NIRS to be suitable, it must be usable across different environments, crops and years. Knowledge of the changes that occur in the chemical composition of the stalks would be beneficial in evaluating the effectiveness of NIRS. Older stalks also show degradation of both the wax coating and the buds, particularly on the lower part of the stalk. This needs to be examined in order to determine not only the best sampling strategy for NIRS, but also to understand the effectiveness of these factors in conferring eldana resistance. An important criterion for breeders is the inheritance of these

components of eldana resistance. In order to achieve maximum benefit in the breeding programme, knowledge of inheritance patterns of these resistance mechanisms is required.

1.7 Techniques for data analysis

1.7.1 Introduction

If bud scale chemical composition contributes to eldana resistance, then the effect of the environment on bud scale chemical profiles needs to be determined, not only to determine if there are effects on the prediction accuracy of NIRS, but also to know whether the resistance is consistent across all conditions. A resistance mechanism that is reduced during low rainfall, for example, would be ineffective because that is when it is most needed. In sugarcane, little is known about the inheritance of eldana resistance in general. If the contribution of bud scale chemical composition to eldana resistance is significant, then the heritability of this aspect of resistance also needs to be determined, in order to fully utilise "bud scale resistance".

1.7.2 Genotype x environment interaction analysis

Selection programs are often complicated by the effect of different environments on genotypes. Genotype x environment (G x E) interactions are important as they affect the performance of genotypes across sites or years, and have an effect on the choice of selection method for determination of top genotypes (Tai *et al.*, 1982; Milligan *et al.*, 1990; Tesemma *et al.*, 1998). Significant deviations associated with environments and G x E interactions have been detected using NIRS in other crops, so it is important to investigate this possibility in sugarcane applications (Smith and Kearney, 2000).

Total variance can be divided into that due to genotypes, environments, interaction between genotypes and environments, and error. Environments can be further sub-divided into sites and years, which can be of interest in determining whether it is more important to have more trials in fewer years, less trials in more years, or some optimum balance of both (Denis *et al.*, 1997; Robert, 1997; Basford and Cooper, 1998). In sugarcane, an additional complication is that ratoons are confounded with years (Jones *et al.*, 1993). Decline in yield due to ratooning differs between genotypes, and may also confound G x E

interactions (Rice and Brown, 1985). The G x E interactions for yield characteristics have been found to be significant in sugarcane (Kang *et al.*, 1984).

Genotypes are generally evaluated on the basis of mean of performance, which may lead to erroneous conclusions if G x E interactions are large (Kang and Martin, 1987; Pham and Kang, 1988; Shafii *et al.*, 1992). The G x E interaction properties of genotypes are specific to those environments in which the genotypes were tested, and to that group of genotypes that were tested (Dyke *et al.*, 1995; Hohls, 1995), so the environments should be as representative of the growing area of the crop as possible (Robert, 1997).

There are two types of G x E interactions (Hohls, 1995; Hohls *et al.*, 1995; Wu and Stettler, 1997; Basford and Cooper, 1998; Tesemma *et al.*, 1998; Wagoire *et al.*, 1999). Quantitative interactions change the absolute differences between genotypes without changing the relative ranks between genotypes. These interactions are of less interest to the plant breeder, as they do not affect the overall choice of which genotypes are superior. Qualitative interactions are the changes that are important to the breeder as they alter the relative ranks between genotypes across environments. These interactions require the breeding of genotypes for specific environments rather than genotypes that perform well in a number of environments. There are four main types of analysis for G x E interaction analysis: the analysis of components of variance, stability analysis, multivariate methods and qualitative analysis techniques (Hohls, 1995).

The design of trials for G x E interactions estimates must be carefully considered. Since variances are of interest rather than means alone, trial designs such as lattices, that confound genetic and environmental differences among means, are not suitable. When blocking is necessary to control environmental variation, the replications-in-blocks design or a blocks-in-replication design is generally satisfactory (Dudley and Moll, 1969).

In the analysis of variance components, the effects of genotype, environment and G x E interactions are estimated by comparing the observed and expected mean squares of the analysis. While the relative importance of the G x E interactions can be estimated, the causes and nature of the interactions cannot be determined (Shafii *et al.*, 1992; Hohls, 1995). Even if fairly extensive environmental data is included in the study, all aspects of

the environment cannot be measured, and there will be some unknown factors influencing the analysis.

Stability analysis uses the technique of joint regression analysis to elucidate the patterns of response of genotypes to different environments. This widely used technique involves the regression of genotype means on an environmental index (Powell *et al.*, 1986; Westcott, 1986; Hohls, 1995; Hohls *et al.*, 1995; Basford and Cooper, 1998), and has shown significant interaction effects in sugarcane (Tai *et al.*, 1982). However, this form of stability analysis depends on a linear relationship between genotype and environments. Deviations from the linear form can cause serious problems in the interpretation of results if not detected (Powell *et al.*, 1986; Hohls, 1995). The G x E interaction effect in sugarcane seems to have a large non-linear component (Tai *et al.*, 1982; Bissessur *et al.*, 2001a). Another problem is the definition of the environmental index. The most commonly used value is the mean of the genotypes grown in that environment. This causes a confounding effect between the environmental index and the genotype means in the regression, as the environmental index is based on particular genotypes (Dyke *et al.*, 1995; Hohls, 1995). This limits the interpretation of the data to only those environments and genotypes used in the study. One alternative is to use a set of control genotypes at each site (Tan *et al.*, 1979). However, because of limited resources an insufficient number of genotypes may be included, giving an environmental index susceptible to error. Alternatively, commercial fields in adjacent areas may be used as an indication of the potential trial site (Jones *et al.*, 1993). A further problem with joint regression analysis is the possibility of outliers exerting a strong influence on the regression equation, and consequently on the G x E interactions estimate (Westcott, 1986; Hohls, 1995). These outliers must therefore be identified and removed from the analysis.

Multiple regression on environmental variables may be useful in determining the underlying causes of differences between responses to environments (Saeed and Francis, 1984; Westcott, 1986; Denis *et al.*, 1997). They also provide an alternative to the environmental index calculated from the genotype means. However, these weather variables may be unpredictable and have different effects at different stages of the crop.

The definition of a stable genotype in regression analysis is a major problem in stability analysis (Jalaluddin and Harrison, 1993; Dyke *et al.*, 1995; Hohls, 1995; Tesemma *et al.*,

1998). One definition is that a genotype that has low variance across environments is stable. The advantage of this is that the measure of stability is not dependent on the other genotypes in the analysis. However, this type of stability measure does not provide an indication of the pattern of response of the genotype across different environments. Also, stability of this kind gives no indication of mean performance of the genotype, and a genotype may be stable but poor-yielding. An alternative measure of stability is to use the interpretation of regression coefficients. A stable genotype has a slope parallel to the mean response of all genotypes (Finlay and Wilkinson, 1963). A problem with this definition of stability is that it is dependent on the genotypes and the environments used in the stability tests. An alternative definition of stability, also calculated from the regression analysis, is the residual mean squares from the regression model. A stable genotype is defined as one with a low deviation from regression mean square. The main problem with this stability measure is that it does not take into account the regression coefficient. Another argument against it is that the regression coefficient is not predictive, as the environmental index is dependent on the genotype means. Therefore, deviations mean squares should only be used as a measure of goodness of fit of the linear regression equation. The use of control or standard genotypes as a measure of stability has also been used, on the basis that new genotypes should be more stable than known standards (Basford and Cooper, 1998).

Multivariate analysis is useful in stability measurements as the response to environment is rarely univariate (Westcott, 1986; Hohls, 1995). Through multivariate analysis, genotypes and environments with similar responses can be clustered, thereby summarising the data more effectively, and allowing extrapolation to a much wider range of environments than included in the experiment. Several clustering techniques are available, differing in the way in which they measure similarity and the clustering strategy undertaken. Unfortunately, these differences lead to different classifications of the same data, depending on the options used. Also, a structure may be forced on the data where no structure exists. Principal component analysis (PCA) has also been attempted for studying G x E interactions. However, the results are difficult to interpret. Geometric methods, including principal coordinate analysis, biplots and additive main effects and multiplicative interaction (AMMI) model, have been tried in order to overcome the problems with stability statistics. Biplots can be produced using various statistical options and are particularly useful for visualising analytical results (Gauch and Furnas, 1991; Tai, 1999; Yan *et al.*, 2000). One advantage of the clustering techniques is that one environment from

each cluster can be chosen to be representative of that cluster for future trial use, thereby minimising the use of resources (Robert, 1997).

The AMMI model incorporates both additive and multiplicative components into the analysis, which is an advantage, as main effects tend to be additive and interactions effects are usually non-additive. Main effects are estimated with an analysis of variance, followed by PCA on the residual portion in order to determine interaction effects (Gauch and Furnas, 1991; Shafii *et al.*, 1992; Hohls, 1995; Vargas *et al.*, 1998). An alternative is to use canonical variate analysis to estimate the multiplicative terms in the model (Tai, 1999). The AMMI model seems to provide better accuracy in sugarcane trial analyses than other statistical models (Bissessur *et al.*, 2001a). Significance tests for the interaction effects are complicated by the difficulty of assigning degrees of freedom to the multiplicative terms (Tai, 1999). The AMMI model is also affected by unbalanced data, where genotypes are not represented across all environments (Frensham *et al.*, 1998). Also, AMMI assumes that genotypes and environments are fixed effects, and therefore that G x E interactions effects are fixed. However, it is widely acknowledged that G x E interactions should be regarded as random (Gogel *et al.*, 1995; Frensham *et al.*, 1998). Other examples of multiplicative models include: the genotypes regression model, the sites regression model, the completely multiplicative model, and the shifted multiplicative model (Cornelius and Crossa, 1999).

Mixed models overcome the fixed effects problem, by allowing the fixed and random effects to be specified. They also allow the separation of G x E interactions from plot error variance and can therefore accommodate heterogeneity of plot error variance. Mixed models also allow the inclusion of genotype characteristics (e.g. plant type, maturity) and environment (e.g. soil type, rainfall) information in the analysis, enabling causes of G x E interactions to be studied (Frensham *et al.*, 1998).

Partial least squares (PLS) and factorial regression (FR) are techniques that allow the use of environmental and genotypic variables for studying G x E interactions (Balfourier *et al.*, 1997; Vargas *et al.*, 1998; Vargas *et al.*, 1999). Factorial regression models are ordinary linear models that explain G x E interactions by various environmental variables, and have the advantage that these influences can be tested statistically. However, if there are many explanatory variables, analysis and interpretation becomes difficult. The PLS regressions are more useful for these cases. The PLS analyses create linear combinations of the

explanatory variables and there is no limit to the number of explanatory covariables that can be used. The PLS regression models are not linear so the standard significance tests cannot be used, but there are alternatives available.

Components of variance analysis, joint regression analysis and multivariate analysis cannot distinguish between qualitative and quantitative interactions, so qualitative methods have been tried (Hohls, 1995). These test the change of rank order in two-way tables, and are particularly useful for comparing standard and new genotypes. Correlations can test for consistency of ranking of genotypes across environments (Vogel *et al.*, 1993). There are also procedures that separate the G x E interaction component into a component due to heterogeneity of variance and a component due to lack of correlation of genotype ranks between environments. The second component is the one of interest, as it is likely to complicate selection by causing changes of rank (Wu and Stettler, 1997; Basford and Cooper, 1998).

Since G x E interactions that affect the rank are most often of interest, stability estimates based entirely on rank may also be of interest (Huehn, 1990; Hohls *et al.*, 1995). If genotypes were ranked within each environment, then a genotype would be considered stable if it had similar ranks across different environments. Several statistical methods are available for measuring similarity of ranks across environments. These include the mean of the absolute rank differences of a genotype over several environments, variance of the ranks, and sum of the absolute deviations of the ranks from the maximum stability (Huehn, 1990; Hohls *et al.*, 1995). To remove the effect of genotypes from G x E interactions the data may be adjusted before the ranks are calculated, using the difference between the marginal mean of each genotype and the overall mean. The ranks will then reflect only the interactions effects (and the mean error which is assumed to be zero). The confounding effect of genotypes will be removed. These nonparametric measures of stability have some advantages. They are not as heavily influenced by outliers, and there need be no assumptions about the distribution of the data. The stability parameters are easy to use and interpret, and repeatabilty, an important requirement for the breeder, may be higher. Also, removal or addition of genotypes, or groups of genotypes, is not likely to cause great variation in the estimates.

Methods that further partition G x E interaction variance into components assignable to each genotype are useful to the breeder (Galvez, 1980; Kang and Miller, 1984; Kang and Martin, 1987; Pham and Kang, 1988; Denis *et al.*, 1997; Frensham *et al.*, 1997). These include ecovalence and stability-variance, where genotypes are considered stable if the interaction component assignable to that genotype is significantly less than or equal to experimental error.

An alternative for measuring stability in genotypes, particularly when the number of genotypes is low and there are few replications, is to use the deviations of the plot means from the maximum plot yield in that environment (Sumith and Abeysiriwardena, 2001). Genotypes with the lowest mean deviation and non-significant variance in deviations are deemed the most stable as they have the highest yield across environments. An analysis of variance on the plot deviations from the maximum plot yield among all plots allows a test of significance on the variance of deviations to be carried out, giving an indication of specific adaptability.

Trials across multiple environments are usually non-orthogonal, and the restricted maximum likelihood (REML) technique, also called the residual maximum likelihood, provides a useful alternative for data analysis (Harville, 1977; Swallow and Monahan, 1984; Robinson, 1987; Gilmour *et al.*, 1995; Gogel *et al.*, 1995; Frensham *et al.*, 1997; Basford and Cooper, 1998; Morrell, 1998). A REML analysis can handle unbalanced data from large data sets. The REML method takes into account the loss of degrees of freedom resulting from estimating fixed effects. It can also take into account heterogeneity of error variances between environments.

For other methods of analysis, where the assumption is that error variances are equal for all trials, the problem of unequal plot error variance may be overcome by the use of weighting (Hohls *et al.*, 1995; Frensham *et al.*, 1997; Frensham *et al.*, 1998), or by transforming the data (Frensham *et al.*, 1998).

A problem with genotype evaluation of disease resistance is to rank genotypes tested across different environments on a common scale. Ranking within a trial is relatively straightforward, provided the disease developed, but ranking between sites and seasons is complicated by differing levels of the disease from environment to environment. One way

to overcome this is to use standard or control genotypes, with known disease ratings, within each trial (Dyke *et al.*, 1995).

An inappropriate statistical model may show non-significance for interaction effects while a more suitable one may show important and significant interactions (Bissessur *et al.*, 2001a). Also, the use of more than one technique may give a better result than the use of one method only. For example, regression analysis can be used to determine the extent of G x E interactions, i.e. quantitative, and then a suitable qualitative analysis can determine the type of interactions present (Hohls, 1995). Alternatively, cluster analysis can classify genotypes and environments, followed by AMMI to analyse the interactions (van Oosterom *et al.*, 1993).

Repeatabilty of stability statistics across different environments is very important to plant breeders, and should be the primary determining factor in deciding which statistical method to use (Sneller *et al.*, 1997). Repeatability can be measured by calculating stability in different subsets of the environments available, ranking the genotypes by these stability estimators, and then correlating the rankings across the different subsets (Jalaluddin and Harrison, 1993). Alternatively a repeatability measure can be estimated as a function of the variance components and the number of locations and replications (Milligan, 1994), allowing a plot of repeatability against number of locations to be used to determine the optimum number of locations. However, in practice repeatability with all types of stability measures tend to be low (Virk *et al.*, 1985; Pham and Kang, 1988; Jalaluddin and Harrison, 1993) because of temporal effects.

1.7.3 Heritability analysis

Heritability is important to the plant breeder as it provides information on the amount of genetic progress that it is possible to make in the population under study. Knowledge of the inheritance mechanisms of a character of interest allow the breeding and selection strategies of a crop to be planned (Dudley and Moll, 1969; Talbert *et al.*, 1983; Milligan *et al.*, 1990; Diz and Schank, 1995; Tancred *et al.*, 1995; Gibson, 1996; Yin *et al.*, 1996; Rahman and Saad, 2000). Knowledge of covariances with other characters of interest is also important in planning a breeding strategy as negative associations with other characters can make improvements in both characters difficult (Milligan *et al.*, 1990). An

ideal character would have high heritability and low environmental influence (Gravois and Bernhardt, 2000). Improvement to a population also requires a high variability of the character in the initial population (Hogarth, 1968; Hogarth *et al.*, 1981; Vogel *et al.*, 1993).

Heritability estimates are affected by various genetic and environmental influences. They are dependent on the population used for the estimation, and it is therefore important that the population be as representative as possible of the entire population under consideration. This is particularly difficult when little is known about the characteristic and its incidence in the population. Environmental variance will influence the measure of heritability as well. Environmental influences mask the genetic effects, so it is important to have uniform conditions within a heritability trial. Even with all conditions being ideal, the evaluations of heritability must be made bearing in mind that the heritability only pertains to that population under those conditions (Milligan *et al.*, 1990; Rowe and Brink, 1993; Falconer and Mackay, 1996; Wall and Corgan, 1999).

An important component of a heritability trial is the size of the population that needs to be tested in order to give the results statistical significance, while minimising the use of resources. Determination of the number of individuals is usually difficult because some knowledge is needed of certain population parameters such as the variance of the characteristic being measured, information that will not be available until the trial is completed. However, it is usually possible to estimate these parameters based on certain assumptions about the population, and then taking the worst-case estimate to ensure that the required accuracy is achieved in the trial. When testing progeny in order to evaluate the performance of a cultivar as a parent, there are two considerations. One is the total number of offspring that will be tested, and the other is the number of crosses that will be included in the test. If a high number of crosses are tested, the number of parents evaluated is high, giving a better representation of the complete breeding population, but with decreased accuracy of heritability estimate. If the number of crosses is low, the accuracy is increased, but the number of genotypes evaluated is decreased (Wu *et al.*, 1978).

One way in which to determine the optimum number of offspring needed to evaluate a cross effectively, is to set up a once-off trial with a large number of individuals, and then use different sample sizes of random individuals. Determining the optimum number of individuals can then be done visually or statistically. The coefficient of variation (CV) for

different sample sizes will give an indication of optimum sample size for that particular characteristic visually (Wu *et al.*, 1978). Alternatively, the optimum sample size can be determined statistically (Robertson, 1957; Wu *et al.*, 1978; Cotterill and James, 1984), which is less subjective, and is therefore preferable to a visual assessment. A separate trial, however, still represents a drain on resources and a delay in setting up the actual trials. Calculation of sample size from empirical or historical data would be preferable (Wu *et al.*, 1978).

The type of mating design depends on a number of factors, including mode of reproduction, limitations imposed by the crop being studied, time, and the facilities available. The biparental design has a disadvantage as no parent is used as both a male and female, which means that maternal effects cannot be measured. It allows more parents to be evaluated, but the information obtained is less precise. However, diallel crosses are difficult to make with sugarcane due to incompatibility, male sterility, and inability to emasculate male flowers (Hogarth, 1968; Hogarth, 1971).

Heritability is estimated from the degree of resemblance between relatives. When using a biparental cross design, various methods can be used to calculate heritability. The most common method is the regression of offspring on one parent or offspring on the average of both parents (Vogel *et al.*, 1980; Casler, 1982; Falconer and Mackay, 1996). The covariance between one parent and its offspring, or mid-parent and offspring, is an estimate of one-half of the additive variance in a population. The covariance between offspring and parent is divided by the phenotypic variance of the parent to obtain the regression coefficient. If the regression coefficient is calculated using offspring and one parent, the regression coefficient is multiplied by two in order to obtain an estimate of narrow sense heritability. If the mid-parent value is used, the regression coefficient is the estimate of narrow sense heritability. The choice of which parent to use depends on the population under study. Factors such as maternal effects and the chances of misidentification of the male parent need to be taken into consideration (Casler, 1982). The mean of several offspring is usually used as the offspring value, but each individual offspring value can also be used, with parental value being repeated for all offspring. The results from these two techniques seem to be similar (Tancred *et al.*, 1995).

It is also possible to calculate heritability based on the resemblance between full siblings, but this is a more complicated case with bias due to dominance and epistatic effects, as well as an environmental component that can be quite large and difficult to eliminate (Casler, 1982; Falconer and Mackay, 1996). For this reason, full sibling analysis is rarely used. However, if both methods are available, both may sometimes be obtained, and then a pooled estimate calculated. This estimate has the potential to be more accurate than either of the original estimates (Hill and Nicholas, 1974). Heritability can also be calculated using variance component estimates (Vogel *et al.*, 1981; Talbert *et al.*, 1983; Swallow and Monahan, 1984).

The different methods do not necessarily measure the same things. For example, variance and regression methods only agree when all gene effects are additive. The type of sampling unit, whether plant, plot, or the mean of several plots, also plays a part in the sampling error and magnitude of the heritability estimate. Comparisons between heritability estimates must bear this in mind (Johnson *et al.*, 1955).

The estimation of heritability by these techniques depends on certain assumptions being made about the population under study. These assumptions include diploidy, absence of environmental correlation between relatives, linkage equilibrium, random mating, absence of inbreeding and absence of dominance or epistatic effects (Dudley and Moll, 1969; Vogel *et al.*, 1980; Tancred *et al.*, 1995). These assumptions are important so that the heritability estimate will be unbiased. If these assumptions are violated, bias may be introduced. Several of these assumptions may be and usually are violated in sugarcane. Studies suggest that influence of these violations on estimates of heritability seem to be small for some characteristics, but may cause large biases for others (Hogarth, 1977; Hogarth *et al.*, 1981).

The assumption of random mating usually does not apply in breeding programs as the population has usually undergone some selection. However, if selection intensity has been weak to moderate, and if a fairly large number of genotypes were used to form the breeding material for the next generation, the bias caused by the use of selected material would not be very great (Hogarth, 1968). Also, because the breeding population is the population of interest, randomly chosen parents from this population should meet the criteria of random matings (Hogarth, 1968). One difficulty with this selected population,

however, is that some of the parents may be closely related. Parent-offspring regression has an advantage because the estimates obtained are valid both when the parents are selected on some basis and when they are chosen at random from a population (Vogel *et al.*, 1980; Casler, 1982).

Sugarcane cultivars are complex polyploids, and inheritance follows a complicated pattern even for characters that show simple Mendelian inheritance as in diploid species (Natarajan *et al.*, 1967; Hogarth, 1968). The effect of variable numbers of chromosomes on quantitative genetics theory must be taken into account when evaluating heritability in sugarcane. A study of genetic relationships in sugarcane also seems to indicate the partial preservation of original parental species associations (Brown *et al.*, 1969). This is probably due to the segregation of whole chromosomes from the wild species as intact units. This type of transmission would be a major violation of the assumptions underlying genetic theory, and would cause problems with the interpretation of heritability.

The assumption of no maternal effects is also probably invalid in sugarcane, as the effect of both parents is quite noticeable is some studies (Natarajan *et al.*, 1967). Other researchers, however, believe that reciprocal differences in crosses are due to accidental selfing, not maternal effects (Wu *et al.*, 1980). Accidental self-pollination can cause a serious bias when estimating variance components (Hogarth *et al.*, 1981). Either alternative is a violation of the assumptions made for a population under heritability studies. In some cases, maternal effects in sugarcane were determined to be negligible, from whatever cause (Hogarth, 1977).

If inbreeding over a number of generations is present in the population under study, heritability estimates may be biased upwards (Smith and Kinman, 1965; Fernandez and Miller, 1985). Various adjustments are available, using measures of inbreeding in the population (Fernandez and Miller, 1985; Gibson, 1996). Inbreeding in sugarcane does not, however, seem to have a large effect on yield, possibly because of the polyploid nature of the crop (Ethirajan *et al.*, 1977; Cassalett *et al.*, 1996).

Heritability in the narrow sense deals with the additive portion of the genetic variance, as this is the portion that has the most effect on selection efficiency. The presence of dominance and epistatic effects makes the prediction of the heritability estimate less

accurate. Dominance and epistatic effects do seem to have an influence in some sugarcane heritability studies (Hogarth, 1977), in some cases at least equal to the additive effects (Hogarth *et al.*, 1981). For certain characteristics, such as sucrose content, non-additive effects vary in different studies from negligible to important, while for others, such as yield, non-additive effects were repeatedly found to be significant. However, other studies have found that additive effects were larger than non-additive effects for most characteristics in sugarcane (Hogarth, 1971). Because of this uncertainty it is vital to investigate the importance of non-additive effects in any new characteristics evaluated in sugarcane heritability studies. A number of ways of measuring the effect of non-additive effects have been proposed, including an alternative form of analysis using second order regression equations in orthogonal bivariate polynomials (Gimelfarb, 1986), and a trial design that includes selfed crosses in a biparental design (Smith *et al.*, 1990).

Environmental influences can also affect the heritability estimate, either increasing or decreasing it depending on whether the environmental effects increase or decrease the covariances between parents and offspring. The interactions effect (G x E) can be reduced to zero if the parents and offspring are randomised within the trial with respect to each other (Vogel *et al.*, 1980; Vogel *et al.*, 1981), while the environmental bias can be overcome by regressing progeny means from one environment onto parent means from another environment (Casler, 1982; Talbert *et al.*, 1983; Fernandez and Miller, 1985; Tancred *et al.*, 1995), or by analysing heritability under multiple years and locations (Dudley and Moll, 1969; Vogel *et al.*, 1981; Gibson, 1996). The analysis of heritability across years and locations will also be beneficial if there is low repeatability of heritability estimates across years and environments (Yin *et al.*, 1996). If all progeny from a cross are not grown adjacent to one another, some protection against environmental correlations will be possible (Tancred *et al.*, 1995). A covariance adjustment to remove the G x E interaction is also possible (Casler, 1982). If variance among plants of a single genotype can be measured, this can be equated with environmental variance and can be subtracted from the variance among genotypes in order to remove some of the environmental effects from the heritability estimates (Johnson *et al.*, 1955; Bissessur *et al.*, 2001b).

While environmental correlation may decrease within-family variance, within-family correlation may cause an overestimation of within-family variance, thereby masking the differences between families (Hogarth, 1977; Talbert *et al.*, 1983; Smith *et al.*, 1990; De

Sousa-Vieira and Milligan, 1999). In sugarcane, competition between neighbouring plants in a family can be important when calculating heritability estimates (Hogarth, 1971).

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1 Bud scale removal

Auxillary buds occur at the nodes of sugarcane stalks, each with a band of root primordia (Figure 2.1). Shoots germinate from these buds when the apical bud of the stalk is damaged, or when the stalk is planted. Each bud is protected by a bud scale, which covers the bud completely, and protects it from environmental damage. Bud scale shape varies between genotypes, but there are generally two overlapping "ears" on each bud scale, which may be joined at the base of the bud scale.

When removing the bud scale from the sugarcane stalk, only the bud scale must be removed. No tissue from the bud or surrounding stalk area must be included in the sample. All of the bud scale must be removed down to the base of the scale, where it connects to the stalk (Figure 2.2).

2.2 Preparation of samples

The method for preparing bud scales for NIRS analysis was based on that developed by Rutherford (1993). Quantities of extractants listed in the method were for 0.2 g samples, and were adjusted according to fresh mass of sample. A number of solutions were prepared:

Solution 1: methanol, chloroform and water in a ratio of 8:4:3.

Solution 2: chloroform and water in a ratio of 4:1.

Solution 3: a buffer and methanol in a ratio of 3:2. The buffer consists of 50mM phosphate buffer made up to a pH of 8 in water.

Three bud scales were removed from the top three nodes of each of seven fresh stalks of sugarcane. These bud scales were then weighed to determine the fresh mass of the sample. The bud scales were placed in a glass vial, to which 8 ml of Solution 1 was added, and then stored in a freezer for two days. This ensured adequate time and conditions for release of the extracts of interest.

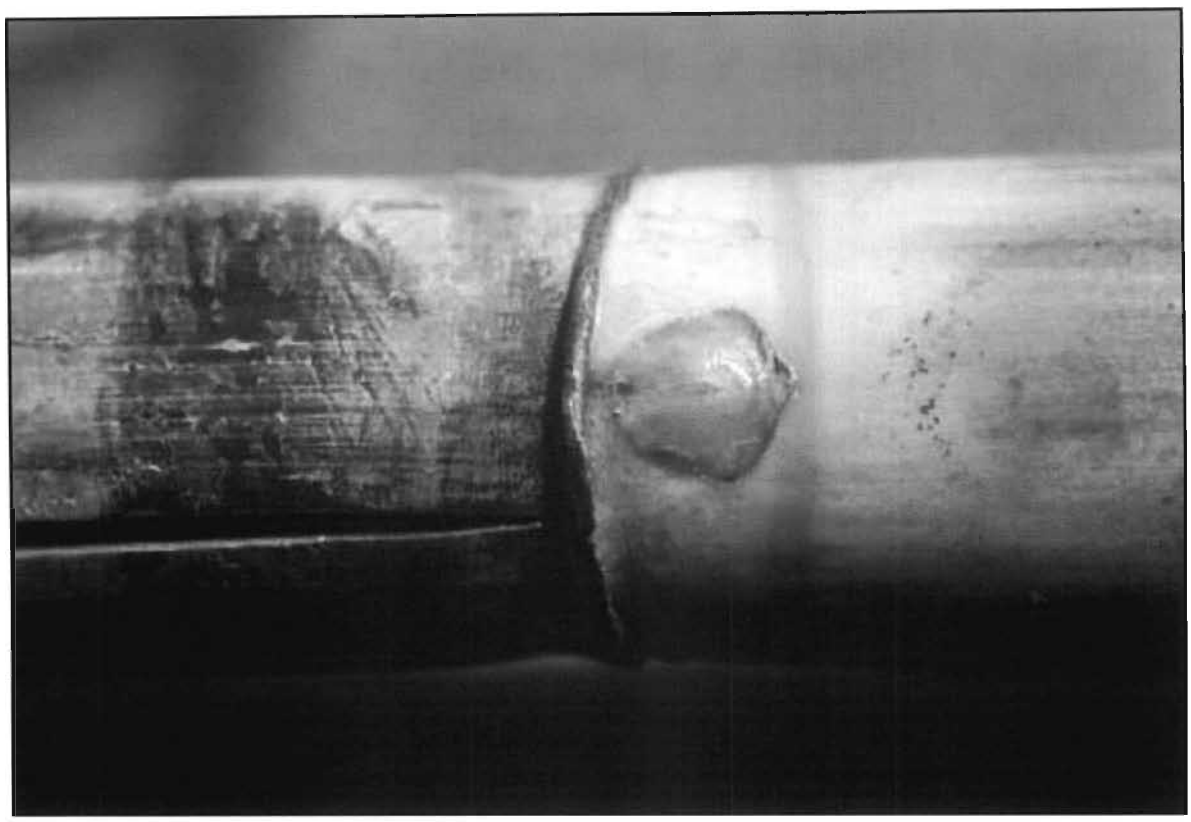

Figure 2.1: A sugarcane bud with an intact scale, located within the root primordial band at a stalk node

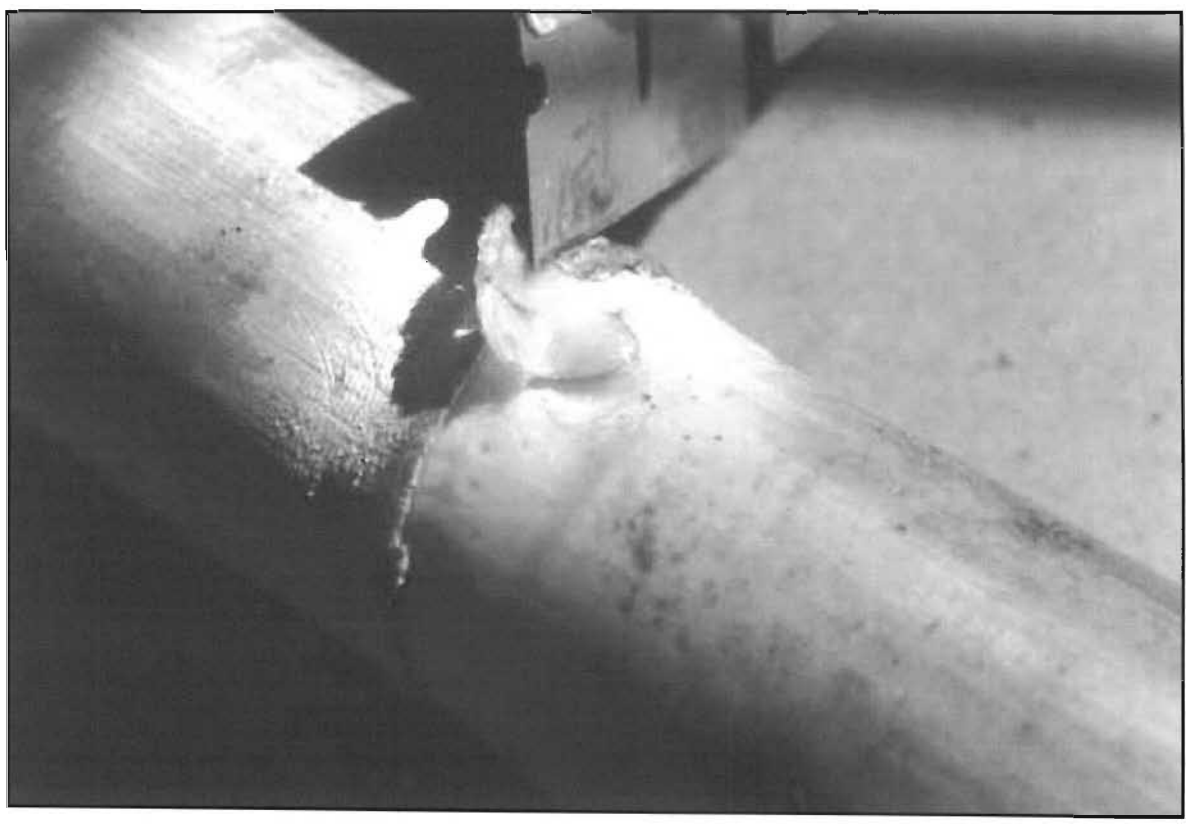

Figure 2.2: The sugarcane bud scale removal process, with a bud scale partially cut away and lifted to reveal the bud underneath

After two days, the bud scale extracts were further processed, simultaneously concentrating the extracts and preparing them for storage if necessary. To begin, 4 ml of Solution 2 was added to each vial, followed by 2 ml of Solution 3. The resulting solution was then mixed thoroughly. The layers were left to separate for an hour. The top layer contains the chemicals of interest. Initially, 5 ml of the top layer were removed and transferred to a test tube. A further 1 ml of Solution 3 was then added to the remaining solution in the vial, thoroughly mixed, and the layers allowed to separate as before. A further 3 ml of the top layer could then be removed and transferred to the same test tube as in the first removal. The bottom layer in the vial could then be discarded. To each testtube was added 20 µl of 2 M hydrochloric acid, and the mixture was vortexed thoroughly. The liquid was evaporated to dryness under a stream of N_2 gas at 60 °C in a water bath. The N_2 gas prevented oxidation of the sample. Once dried, the samples could be stored in a freezer until the NIR spectra could be read.

To prepare the samples for NIRS reading, the extracts in the test tubes were redissolved in 400 µl methanol and 600 µl water per 0.2 g fresh mass of bud scales. The methanol was added first, as addition of water first caused damage to the sample. After each addition, the test tube was vortexed to ensure that the sample was thoroughly dissolved and mixed. The sample was then decanted into a 1.5 ml eppendorf tube and the particulates spun down. To obtain the NIR spectrum, 750 µl of the sample was placed in a 1 ml NIRS quartz cuvette and placed in the sample holder in the NIRS instrument.

2.3 High performance liquid chromatography procedure

Once run through the NIRS instrument, some of the sample sets were run through HPLC to obtain profiles to compare with the NIR spectra and eldana resistance ratings. The 12 and 16-month samples sets from the plant crop were run through the HPLC column.

For HPLC the extract was redissolved at the rate of 1 ml g^{-1} in 60:40 HPLC eluents A:B (given below) and particulates were spun down in microfuge tubes. HPLC conditions were as follows:

Column	C18 Brownlee ODS-224 220 mm x 4.6 mm
Temperature	30°C
Flow rate	1 ml min^{-1}
Detection	345 nm
Eluents	A = pH 2 water (meta-phosphoric acid), B = 7:5 acetonitrile: MeOH
Gradients	10 % B for 10 minutes. 15 % B for 10 minutes. 35 % B for 30 minutes. 80 % B for 10 minutes. Re-equilibrate.

2.4 Rating system used for eldana resistance measurements

At SASEX, the international format for rating sugarcane diseases is used for rating eldana damage, namely the 1-9 scale. Genotypes are rated as 1 if immune, a 3 if resistant, a 5 if intermediate, a 7 if susceptible and a 9 if very susceptible. In selection programme trials, ratings are subjective, depending on the perceived severity of pest damage or disease infection. In disease or eldana trials, however, statistical analyses of the levels of disease infection or pest damage can be carried out. The following formula is used to determine the rating on the 1-9 scale:

$$\text{Rating} = \frac{(x/\bar{x} \times 100) - 100}{100 \times (s/\bar{x})} + 5$$

where x is the disease level or level of eldana infestation or damage for the clone, $\bar{x}$ is the mean of the trial and s is the standard error of the trial.

2.5 Near infrared analysis

At initiation of the project, an NIRSystems 5000 NIRS instrument was used. This instrument scanned the range of wavelengths from 1100 – 2500 nm, covering the NIR wavelength range only (Figure 3.1). This instrument was subsequently replaced by a NIRSystems 6500 instrument, which included the visible light spectrum, resulting in a range from 400 nm to 2500 nm (Figure 3.2). Samples from plant cane, 12 and 16 month top of stalk samples were run on the NIRSystems 5000 instrument, while for the 16 month base of stalk, 20 month and all ratoon samples the NIRSystems 6500 instrument was

available. All calibration development and results were therefore carried out on the more accurate NIRSystems 6500 instrument.

The NIRS instruments worked on a transmittance analysis, with a quartz cuvette for sample presentation. A total of 32 scans were run on each sample, and averaged to provide a more accurate reading. Readings were treated with a log 1/Transmittance transformation before being presented to the instrument operator. The NIRS instruments came with their own built-in software (listed below), capable of producing and verifying calibrations, as well as providing various graphical options for visualising data.

Calibration development by the NIRSystems software (explanations provided from the NIRS manual, © Infrasoft International, version 4.00, 1996) has a range of options including stepwise regression, principal component regression and partial least squares regression. Stepwise regression is the simplest option, where individual wavelengths are added or subtracted to the model until a desirable level of accuracy is achieved. The regression procedure begins with the calculation of correlations of predicted values with all wavelengths available. The most highly correlated wavelengths are then added to the equation. A number of iterations allows further highly correlated wavelengths to be added, and wavelengths to be removed if their correlations subsequently decrease in the equation. The statistic calculated to represent the accuracy of the equation is the standard error of calibration (SEC). Overfitting is possible with this form of analysis. Overfitting may be tested for by watching for an increase in the standard error of prediction (SEP), a statistic that is obtained by attempting to predict samples not included in the calibration. A standard error of validation (SEV) can also be calculated if a second set of samples is available. The final equation form of a chemical component will be $Y = a + b_1X_1 + b_2X_2 + \ldots + b_iX_i$ where X_i represents the absorbance value at a particular wavelength and b_i is a weighting factor for that wavelength. The value of each term in the equation can be evaluated from the size of its variance ratio (partial F).

The principal component analysis (PCA) method is a data reduction method, reducing the data points, in this case wavelengths, to a set of independent variables called principal components or eigenvectors. Wavelength information is used to compute the eigenvectors or loadings. Loadings are weights assigned to each wavelength and the weights are added up to give a score. Terms are produced in an iterative process as information is extracted

from the sample scores. The first term represents the largest source of variation among the scores.

Partial least squares (PLS) is another regression method that can be used to produce calibration equations. PLS is similar to PCA except that when the loadings are calculated they include information from all the X variables (absorbance readings at the different wavelengths) as well as the Y variable (reference values from conventional determination methods). The PLS is usually better than PCA because of the inclusion of the Y information in the regression. A further improvement is offered in modified PLS (MPLS), where the residuals, obtained after each factor is calculated, are standardised (divided by the mean residual value) before calculating the next factor. Both PCA and PLS have the benefit of being protected from overfitting by cross validation errors (SECV). Because only independent sources of information are being fitted to absorption data rather than coefficients and wavelengths, each sample in the calibration file can be tested for prediction accuracy.

Within the calibration equation development system are a number of options available for transformation of the data. Derivatives to various degrees are possible, as is a noise adjustment.

The NIRS software provides a facility for testing the validation of calibration equations with a separate set of samples, regardless of the mechanism used to produce the calibration equation. This analysis provides the standard errors needed to verify the good performance of a calibration equation, including SEP, SECV and bias. This step is important, as it is possible to develop a calibration equation that is only useful for predicting the samples within the set that was used to develop the calibration equation. Good prediction of an entirely new set of samples is the best test for performance of the calibration equation.

The PCA can be used for a further application. A neighbourhood Mahalanobis (H) distance can be produced from the principal components in order to determine the best samples to be used for a local calibration. The H distance is a measure of the distance in multidimensional space of each observation from the mean centre of the observations, taking into account all the variables being considered (Manly, 1986). This is especially useful if there are large differences between sample groups. A global library needs to be

created in order to cover all variations in sample groups and then local calibrations are formed for each new sample group run, based on the samples that are most similar to the ones being analysed.

The H distances can be used to graphically display the sample groups. A display of one sample group on a three dimensional graph gives an indication of the variation within that group, and can help determine whether some samples should be removed from a highly represented area. In addition, graphs of histograms of H distances are available in order to evaluate the scatter within a set of samples, and to earmark possible outliers. The greatest benefit can be obtained, however, with the plotting of multiple sample groups on one graph. This allows a visualisation of the amount of overlap between sample groups, even if only in three dimensions, and an indication of the effective cover of a global library. An example is shown in Figure 2.3. The sample group represented by the white crosses is spatially separated from the red and blue sample groups. The white sample group cannot therefore be used for predictions in the red and blue sample groups, or vice versa. The red and blue sample groups, because they overlap, can be used to predict from one group to the other. In the development of a global library for prediction purposes, the spaces between the sample groups would need to be filled with other sample groups, until all new sample groups fall into an area already covered by previously scanned samples.

NIRS software (© Infrasoft International, version 4.00, 1996) provides an additional feature known as frequency space devolution, which breaks down the overall absorbance into a series of peaks. It is assumed that these peaks exist, and that the combination of absorbances of these peaks gives the overall outline of the absorbance, even though the individual peaks are not differentiable. The software attempts to predict the presence and size of these peaks iteratively in order to provide more information. Peak locations are adjusted with the aid of the fourth derivative. If the estimated composite curve peaks do not match the observed spectra, then the peak location is adjusted using the fourth derivative rather than the peak of the observed spectra, as these may include the effects of neighbouring peaks. Peak height and width are also adjusted using the fourth derivative.

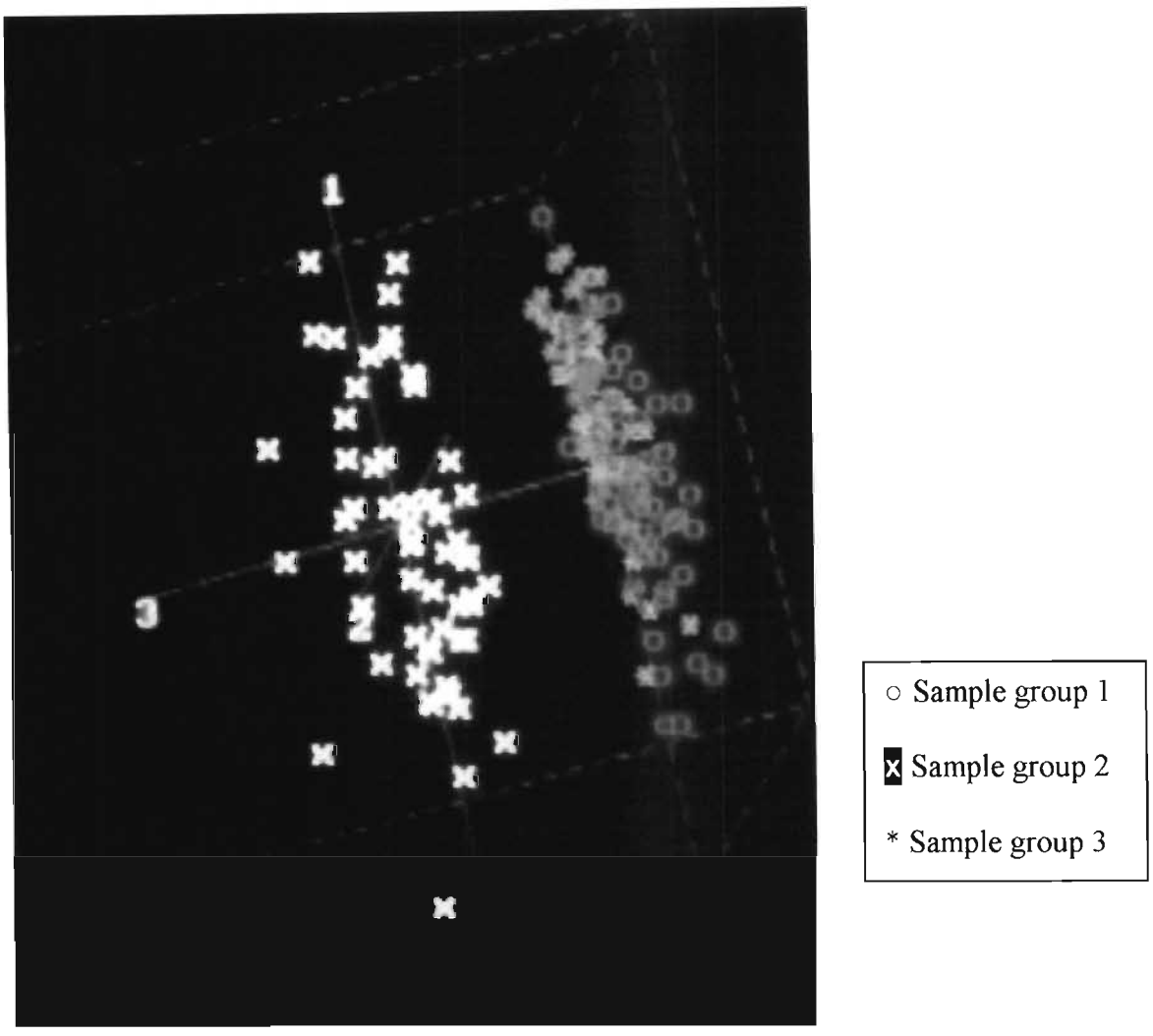

Figure 2.3: A graphical representation of PCA H distances between sample groups, for evaluation of the predictive potential between groups, where calibrations developed in one group cannot predict values in other groups that are spatially separated. Prediction is theoretically possible between overlapping groups

CHAPTER 3
CALIBRATION TRIAL

3.1 Introduction

Previous work (Rutherford, 1993; Rutherford *et al.*, 1993) at SASEX suggested that NIRS was a feasible method for predicting eldana resistance in sugarcane. Rutherford (1993) used a sample set of 60 genotypes to establish the calibration. However, only a small number of environments were included in Rutherford's evaluation, and prediction between samples sets from different environments was poor.

There were two aspects to the current calibration trial. The first was to develop a global library of samples to overcome environmental influences on the reading of samples. The second was to evaluate the effect of environment on bud scale chemical profiles. The sample set used in this study was the standard set of 60 clones used for the previous study (Rutherford, 1993), each sample set being from a different environment or time. The inability of a calibration set to predict eldana resistance ratings from one sample set to another was assumed to be due to the environmental influence on the spectral readings. This environmental influence is different from the environmental influence that may lead to actual differences in chemical profiles of bud scales. It can be caused by differences in sampling conditions, differing laboratory conditions at the time of reading the spectra, different moisture levels in the samples, etc. This problem is usually overcome with the use of a global set of samples representing all possible conditions that may be experienced during sampling of bud scales. The global library is developed by taking samples from a range of environments, at different times, until the full range of all possible samples is included. This is achieved when any new samples that are subsequently added are similar to ones already in the calibration. Similarity is based on PCA H distances, which reflect whether samples are statistically similar to each other, regardless of what the actual quantities of chemical constituents in the samples are. Part of the checks that are run on an already developed calibration are to ensure that samples coming in for analysis are valid in that there are similar types of samples already in the calibration. Once the global library is developed, then the environmental impact on the chemical composition of the bud scales can be evaluated, using the calibration to predict the amount of chemicals in the bud scales.

This second aspect of the calibration trial would determine if the resistance mechanism that was being measured by NIR scanning of the chemical components of bud scales was consistent over different environments. A clone that is resistant in all environments is preferable to a clone that is only resistant part of the time. If the chemical profile of the bud scales changed over different environments, then it would not be a worthwhile aspect of eldana resistance to study, regardless of whether the NIRS calibration worked well or not.

If bud scale profiles prove to be an environmentally independent form of eldana resistance, then the NIRS calibration can be used to discard clones from the breeding programme. In this case, the aim is not to identify the clones with good eldana resistance, but just to have sufficient accuracy to discard the clones that are susceptible. This would result in a savings in resources, from not carrying poor clones forward in the selection programme.

The standard set of 60 genotypes used in the original work by Rutherford (1993) was grown under a range of conditions, in order to both establish a global calibration and to test the environmental impact on bud scale composition. Because previous work had only been done on bud scales removed from the top section of the stalks, it was also relevant to determine the levels of the compounds of interest in the lower section of the stalk. The lower section of the stalk is the preferred entry site of the eldana larvae, and buds are frequently damaged or degraded in this area, being older than those at the top of stalks. Resistance of genotypes may be related to the degree of breakdown of chemicals in older material more than the original composition of these chemicals.

3.2 Materials and Methods

A set of 60 cultivars with known eldana ratings was used for the calibration set. These cultivars, together with their eldana ratings, are listed in Appendix 1. This set was planted in December, 1996. It was planted at two sites, one on the Coast at Shakaskraal (Appendix 3), the other in the Midlands at Bruyns Hill (Appendix 4). These are two of the research stations that were owned by SASEX at the time of the project. The trials were both planted as a randomised block of three replications, with 1 m single row plots.

A bud scale sample was taken at each site at twelve, sixteen and twenty months. At the sixteen and twenty month sampling dates, an additional sample was taken from the lower portions of the stalks, in the same manner as the standard sample. A sample consisted of taking seven representative stalks from each line, from different stools, excluding the end stools. The two sites were then cut back and allowed to ratoon. Each site was sampled again at the three different ages, in the same manner as in the first season.

3.3 Data analysis – calibration

A data analysis of the spectra was carried out with the NIRS software, in which calibrations were developed between NIRS absorbance values and eldana ratings with the various methods available, as outlined in section 2.4. Validations were carried out with the NIRS software.

Additional analyses were carried out in Microsoft Excel. Data from the NIRS instrument were imported. Each set of samples was standardised to have a mean of zero and a standard error of one, using the transformation $z = (x - \bar{x})/s$ where $\bar{x}$ is the mean of the sample set and s the standard error of the sample set. Correlations between sample sets were calculated.

Absorbance spectra were broken down into individual peaks using the frequency space devolution methods available in the NIRS software. These peaks were imported into Microsoft Excel. The locations of the peaks and their amplitudes were correlated with eldana resistance estimates to determine if there was a relationship.

When comparing absorbance values of the NIRS instrument (or HPLC peak areas), with the eldana ratings, the values of the absorbance values or peak areas were converted to the 1-9 scale, to be comparable to the eldana ratings. This was done using the following formula:

$Rating = (x - min) / ((max - min) / range) + offset$

where x is the absorbance value or peak area, *min* is the minimum value for that sample set, *max* is the maximum for that sample set, *range* is the range found in that sample set, and *offset* is the amount by which the lowest value must be shifted from zero. So for the disease ratings, the range is eight, i.e. 1-9, and the offset is one.

3.4 Data analysis – genotype x environment

3.4.1 Analysis of variance

In stability analysis, an analysis of variance gives an indication of the size and importance of the various factors and their interactions. It is the first step in determining the behaviour of a particular characteristic under different environmental conditions.

The analysis of variance for the calibration had the following model:

$$Y_{ijklm} = m + G_i + S_j + Y_k + A_l + (GS)_{ij} + (GY)_{ik} + (GA)_{il} + (SY)_{jk} + (SA)_{jl} + (YA)_{kl} + GSY_{ijk} + GSA_{ijl} + SYA_{jkl} + GSYA_{ijkl} + E_{ijklm}$$

where Y_{ijklm} is the *m*th observation of the *i*th cultivar in the *j*th site in the *k*th year at the *l*th age, and m is the mean. G_i, S_j, Y_k and A_l are the main effects of genotype, site, year and age respectively. The six terms thereafter are the first order interactions. The next three terms are the second order interactions, followed by one third order interaction. E_{ijklm} is the experimental error. The higher order interactions are grouped into the error term as they are difficult to interpret, and may improve accuracy in the experiment.

The expected variance components for the different factors and their interactions can be calculated according to the formulae in Table 3.1.

Significant interaction effects in the analysis of variance will indicate the need for further analysis using other techniques, as mentioned below. The value of V_G, obtained by subtraction, provides an estimate of broad-sense heritability.

Variance components can also be analysed using a mixed model, where varieties are set as fixed effects and environments as random effects. The REML model allows greater efficiency in analysis as it takes into account the loss of degrees of freedom resulting from estimating fixed effects. It can also take into account heterogeneity of error variances between environments.

Table 3.1: Sample ANOVA table for G x E interaction analysis, demonstrating the technique for estimating Expected Mean Squares using first order interactions (higher order interactions are included in the residual)

Source	df	Expected mean squares
Genotypes	(g-1)	$rsyaV_G + ryaV_{GS} + rsaV_{GY} + rsyV_{GA} + V_E$
Sites	(s-1)	$rgyaV_S + ryaV_{GS} + rgaV_{SY} + rgyV_{SA} + V_E$
Years	(y-1)	$rgsaV_Y + rsaV_{GY} + rgaV_{SY} + rgsV_{YA} + V_E$
Ages	(a-1)	$rgsyV_A + rsyV_{GA} + rgyV_{SA} + rgsV_{YA} + V_E$
G x S	(g-1)(s-1)	$ryaV_{GS} + V_E$
G x Y	(g-1)(y-1)	$rsaV_{GY} + V_E$
G x A	(g-1)(a-1)	$rsyV_{GA} + V_E$
S x Y	(s-1)(y-1)	$rgaV_{SY} + V_E$
S x A	(s-1)(a-1)	$rgyV_{SA} + V_E$
Y x A	(y-1)(a-1)	$rgsV_{YA} + V_E$
Residual	gsya(r-1)	V_E

3.4.2 Regression analysis

Regression analysis is still the most widely used technique for estimating individual genotype effects across environments (Finlay and Wilkinson, 1963). The environmental index in this trial would be the mean of all the genotypes. Various stability definitions could be used to determine the performance of the clones. Low variance across environments would indicate that the clone performs similarly across all sites. This stability measure is independent of other genotypes, but it gives no indication of the pattern of response of the clone. Examining the slope of the regression in relation to the mean response of all clones would indicate if the clone has an above average performance in all environments, but this only gives a stability indicator for those environments. Because of the absence of standard or control genotypes for bud scale extract work, using the definition of stability as being better than the control, could not be used in this trial.

3.4.3 Qualitative or rank order analysis

Because a clone should be resistant across all environments, it is important to test for qualitative environmental influences. A change from resistant to susceptible under certain conditions would make that particular characteristic undesirable to breeders. Tests that determine rank-order changes would be beneficial in the calibration. In this case a genotype is determined to be stable if its rank is similar over environments. The following calculations were used (Huehn, 1990):

x_{ij} = phenotypic value of the ith genotype in the jth environment (i = 1,2,...,K; j = 1,2,...,N). In this two-way table with K rows (genotypes) and N columns (environments) one ranks the K phenotypic values x_{ij} within each column, i.e. each environment separately (lowest value = rank of 1 and highest value = rank of K). Let r_{ij} be the rank of genotype i in environment j. A number of stability estimates were calculated.

$$(1)\ S_i^{(1)} = \frac{2\sum_{j=1}^{N-1}\sum_{j'=j+1}^{N} |r_{ij} - r_{ij'}|}{N(N-1)} \qquad \text{(Equation 1)}$$

which is the mean of the absolute rank differences of a genotype i over the N environments, where $r_{ij'}$ is the rank in the $(j+1)$ environment

$$(2)\ S_i^{(2)} = \frac{\sum_{j=1}^{N} (r_{ij} - \bar{r}_{i.})^2}{N - 1} \qquad \text{(Equation 2)}$$

with $\bar{r}_{i.} = \sum_{j=1}^{N} r_{ij}/N$

$\bar{r}_{i.}$ can be interpreted to be the expectation of each r_{ij} under the hypothesis of maximum stability (equal ranks)

$$(3)\ S_i^{(3)} = \sum_{j=1}^{N} \frac{|r_{ij} - \bar{r}_{i.}|}{\bar{r}_{i.}} \qquad \text{(Equation 3)}$$

which is the sum of the absolute deviations of the r_{ij}'s from mean stability, $\bar{r}_{i.}$, expressed in $\bar{r}_{i.}$ units

Since genotypes are ranked separately within each environment the environmental effects have no influence on this type of stability. However, differences among genotypes would have an effect on the stability measures and may lead to differences in stability among genotypes when there is no genotype-environment interaction. To avoid this the x_{ij} values were corrected using (Huehn, 1990):

$$x_{ij}^{*} = x_{ij} - (\bar{x}_{i.} - \bar{x}_{..}) \qquad \text{(Equation 4)}$$

where $\bar{x}_{i.}$ is the marginal mean of genotype i and $\bar{x}_{..}$ is the overall mean.

The ranks can then be calculated using the corrected values. Approximate tests of significance, based on the normal distribution, are available for the first two stability parameters, $S_i^{(1)}$ and $S_i^{(2)}$ (Huehn, 1990):

$$Z_i^{(m)} = [S_i^{(m)} - E(S_i^{(m)})]^2 / V(S_i^{(m)}),\ m=1,2 \qquad \text{(Equation 5)}$$

would have an approximate chi-squared distribution with one degree of freedom.

The means $E(S_i^{(m)})$ and variances $V(S_i^{(m)})$ may be computed from the discrete uniform distribution (1,2,…,K), using the following formulae:

$$E(S_i^{(1)}) = (K^2 - 1)3K]$$

$$E(S_i^{(2)}) = (K^2 - 1)/12$$

$$V(S_i^{(1)}) = (K^2 - 1)\,[\,(K^2 - 4)(N + 3) + 30\,]\,/\,45\,K^2\,N\,(N - 1)$$

$$V(S_i^{(2)}) = (K^2 - 1)\,[\,2\,(K^2 - 4)\,(N - 1) + 5\,(K^2 - 1)\,]\,/\,360\,N\,(N - 1)$$

(Equations 6)

3.4.4 Stability variance and ecovalence

The stability-variance G x E technique (Kang and Miller, 1984) allows the partitioning of G x E interactions into components assignable to each genotype, where genotypes are

considered stable if the interaction component assignable to that genotype is significantly less than or equal to experimental error. Stability variances were calculated as follows:

$$\hat{\sigma}_i^2 = [1/(s-1)(t-1)(t-2)] \times [t(t-1) \sum_j (\mu_{ij} - \bar{\mu}_{i.})^2 - \sum_i \sum_j (\mu_{ij} - \bar{\mu}_{i.})^2]$$

(Equation 7)

where $\mu_{ij} = Y_{ij} - \bar{Y}_{.j}$, $\bar{\mu}_{i.} = \sum_j \mu_{ij}/s$, s = number of locations, t = number of clones, Y_{ij} = trait value of *i*th clone in *j*th location, $\bar{Y}_{.j}$ = mean of all clones in *j*th location.

Another method of partitioning G x E interactions into components for each clone is ecovalence (Kang and Miller, 1984), which was calculated as follows:

$$w_i = \sum_j (\mu_{ij})^2 - 1 / s (Y_{i.} - Y../t)^2 \qquad \text{(Equation 8)}$$

where $Y_{i.}$ = sum of the *i*th clone over all s locations, and $Y..$ = grand sum.

Ecovalence sum of squares for each clone must be divided by (t-1)(s-1)/t to obtain a mean square, which allowed testing of its significance in the same manner as the stability variance.

3.4.5 Deviation of plot mean from maximum plot

Stability was also measured by using the deviations of the plot means from the maximum plot yield in the environment (Sumith and Abeysiriwardena, 2001):

$$d_{ijk} = Ymax_j - Y_{ijk} \qquad \text{(Equation 9)}$$

where d_{ijk} is the yield deviation of the i^{th} clone in the k^{th} replication in the j^{th} environment from the maximum plot yield recorded in that environment, $Ymax_j$ is the maximum plot yield recorded in the j^{th} environment and Y_{ijk} is the yield of the i^{th} clone in the k^{th} replication in the j^{th} environment.

The mean over replications for each clone in each environment was also calculated:

$$\bar{d}_{ij.} = Ymax_j - \bar{Y}_{ij.} \qquad \text{(Equation 10)}$$

Mean deviation across environments for each clone, D_i, would estimate the average superiority of that clone:

$$D_i = \sum_{j=1}^{n} \bar{d}_{ij.} / n \qquad \text{(Equation 11)}$$

where n = number of environments.

An analysis of variance was carried out on the plot deviations based on Table 3.2:

Table 3.2: Sample analysis of variance of the plot deviations from the maximum response, for use in stability analysis

Source	**df**	**SS**
Total	npq-1	$\sum_{j=1}^{n}\sum_{i=1}^{p}\sum_{k=1}^{q} d^2_{ijk} - (d_{...})^2/npq$
Reps/Environment(E)	n(q-1)	$\sum_{j=1}^{n}\sum_{k=1}^{q} d^2_{.jk} - \sum_{j=1}^{n} d^2_{.j.}$
Clone(V)	p-1	$\sum_{i=1}^{p} d^2_{i..} - (d_{...})^2/npq$
E(V)	p(n-1)	$\sum_{j=1}^{n}\sum_{i=1}^{p} d^2_{ij.} - \sum_{i=1}^{p} d^2_{i..}$
Variance of $v_1(v_1^2)$	n-1	$[\sum_{j=1}^{n} d^2_{1j.} - (\sum_{j=1}^{n} d_{1j})^2/n\ ]/q$
.		
Variance of $v_p(v_p^2)$	n-1	$[\sum_{j=1}^{n} d^2_{pj.} - (\sum_{j=1}^{n} d_{pj})^2/n\ ]/q$
Pooled error	[p-1][n/(q-1)]	$\sum_{j=1}^{n}\sum_{i=1}^{p}\sum_{k=1}^{q} d^2_{ijk} - \sum_{j=1}^{n}\sum_{i=1}^{p} d^2_{ij.} - \sum_{j=1}^{n}\sum_{k=1}^{q} d^2_{.jk} - \sum_{j=1}^{n} d^2_{.j.}$

where E is the environmental effect, V is the clonal effect and E(V) is the environmental within clonal effect.

The variance of E(V) supplies a parameter that indicates the existence of differential genotypic variances across environments, and is equivalent to the G x E interaction term in the standard analysis of variance. The E(V) variance can be partitioned into components, each of which corresponds to each clone. This v_i becomes the stability parameter for each genotype.

Genotypes with the lowest mean deviation and non-significant variance in deviations are the most stable as they have the highest yield across environments. An analysis of variance on the plot deviations from the maximum plot yield among all plots allowed a test of significance on the variance of deviations to be carried out, giving an indication of specific adaptability.

3.5 Results and Discussion – Calibration development

The results obtained from the NIRSystems 5000 in the original studies (Rutherford *et al.*, 1993; Rutherford and van Staden, 1996) indicated an inability to predict eldana resistance in one sample set (the set of standard clones used for calibration development taken from one particular environment) using a calibration developed from another sample set. Analysis of the different sample sets indicated very little statistical overlap between sample sets, based on PCA analysis with the NIRS instrument's software. Samples within a sample set also showed wide differences in their base lines. It was assumed that environment had a large effect on the sample sets, i.e. strong G x E interactions, and that a global library, representing as many types of sample sets as possible, was needed in order to produce a calibration that would be robust with regard to environment. Part of the present study was to use the samples obtained to begin to develop a global library, and to determine if it was possible to develop a complete global library that would be able to evaluate any new samples on the basis of samples already in the calibration.

In the present study, wide differences were also observed between sample sets read on the NIRSystems 5000, based on PCA H distances and graphic evaluation (Figure 3.1). Calibration and validation between sample sets was not possible, with correlations being extremely low (in the order of 0.001) for both PCA and stepwise regression calibrations. The same genotypes were used in each sample set, so the effect of G x E interaction was assumed to be large.

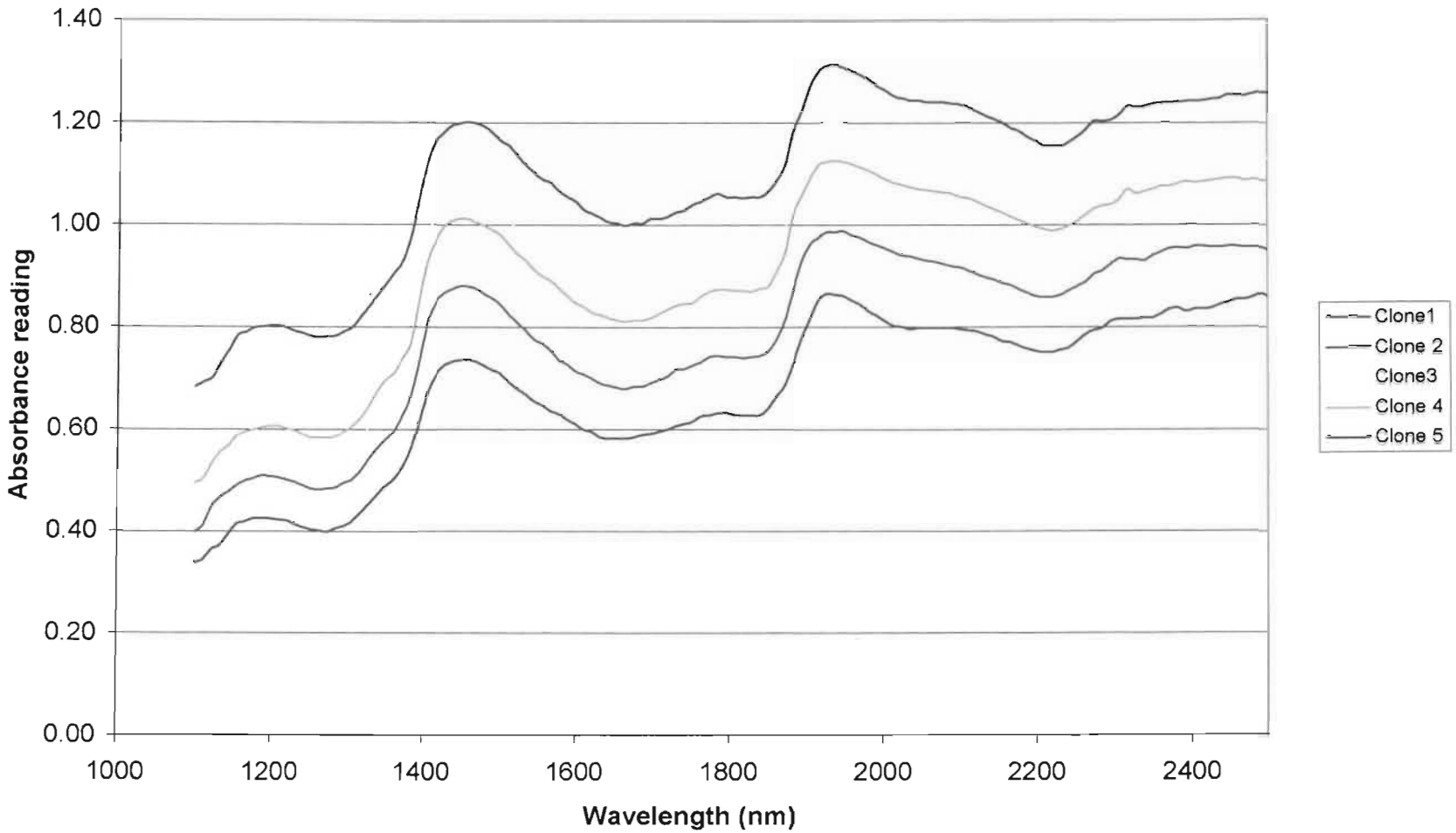

Figure 3.1: Example of NIRSystems 5000 spectra of extracted sugarcane bud scale chemical components, showing baseline shifts between samples within one sample set

Examination of wavelengths selected for stepwise regression calibration equations highlighted another problem. Wavelengths chosen from each sample set differed from those chosen from other sample sets, sometimes by only a few nm, other times having no relationship with other sample sets. The wavelengths that differed only slightly could have been caused by the baseline shifts that were observed even within sample sets, but the wavelengths that had no commonality between sample sets were of concern as they appeared to be random.

With the acquisition of the new NIRSystems 6500 instrument, the results were very different. Samples within a sample set all had the same baseline, a necessity for development of a good calibration equation. Analysis of the H distances between sample sets showed that all sample sets overlapped coordinately, based on PCA analysis with the NIRS instrument's software. This meant that all sample sets were comparable with each other, and that environmental differences did not alter the spectra obtained from bud scale extracts. It did not mean that the environment did not alter the amounts of the relevant chemicals within the bud scales, which still had to be determined, but merely that the ability of the NIRS instrument to detect bud scale profiles was not affected by the source of the bud scales. It was therefore concluded that the differences between and within sample sets on the NIRSystems 5000 instrument was due to instrument error, not environmental differences, and that a global calibration would not be possible on this instrument. The statistical overlap of sample sets on the NIRSystems 6500 instrument eliminated the need for development of a global set of samples, covering all possible sampling conditions, as all samples already overlapped as required.

However, the correlation between different sample sets was still low. None of the calibration techniques available on the NIRS software provided a calibration equation that could predict the values of another sample set. Use of derivatives and noise reduction did not improve the situation. Wavelengths selected by stepwise regression also still differed between sample sets despite the lack of baseline shifts on the new instrument. For this reason, it was decided to further evaluate the data from the NIRSystems 6500 instrument in Microsoft Excel.

In Microsoft Excel, the data was standardised, as in Chapter 3.3, to try to improve correlations between the different sample sets. However, no improvements were observed.

A study of the areas of the spectra where differences between genotypes were visible, showed that differences between spectra for different genotypes were very small. Figure 3.2 shows the spectra of four different genotypes and two blanks over the whole wavelength range of the instrument. Figures 3.3 to 3.6 show enlargements of the regions of the spectra where differences are most noticeable. The differences between genotype spectra in Figures 3.3, 3.5 and 3.6 are all parallel and may only be a base shift, rather than the presence of a peak. In Figure 3.4, however, there seems to be the presence of a small peak superimposed on the larger background peak, possibly suggesting the detection of a chemical bond that differs between samples. However, the differences between the spectra are small compared to the background signal. More importantly is the behaviour of the spectra of the blanks. The blanks contained the solvent mix of methanol and water in the same ratio as for the bud scale samples, and were read in at the same time as the sample sets to which they were compared. These blanks sometimes showed higher absorbances than some of the spectra, something that should not be possible if absorbance was increased on the basis of compounds in the samples. In Figure 3.4, the likeliest area for evaluation, the spectrum of one of the blanks had one of the highest absorbances.

The areas where the largest peaks are visible in the spectra correspond to the absorbance areas of methanol and water, the solvents used in the experiment (Figure 3.2). The breakdown of the spectrum of each clone into individual peaks using the option available in the NIRS software (Chapter 2.4), allowed the possibility of separation out of the peaks caused by the chemicals of interest (Figure 3.7), as well as allowing for the removal of any baseline shifts that may be present. Amplitude and position of peaks for each clone were compared with other clones within a sample set to determine if there was any correlation with eldana ratings of those clones, and then compared across sample sets. The exact location of peaks was inconsistent across different spectra, suggesting that the process was not precise enough for good results. However, the identification of peaks was approximated manually, and reasonable consistency of peak identity was noted for different clones. The sizes of these peaks were then compared to the eldana resistance ratings for each clone. No relationship with the sizes of any peaks at any wavelength was observed across sample sets.

With the original work on the NIRSystems 5000 instrument, it was assumed that the differences between the spectra of different clones within a sample set were due to

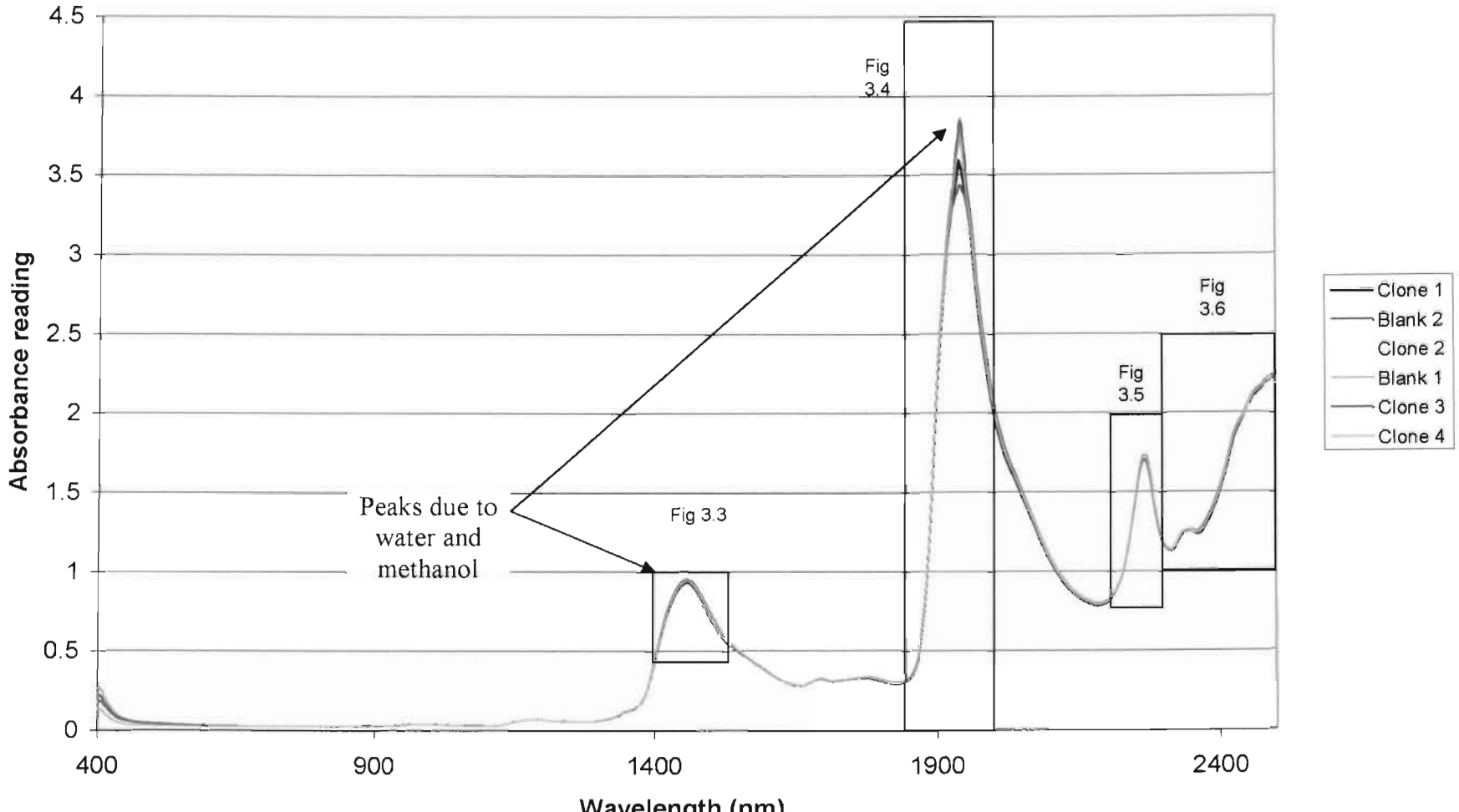

Figure 3.2: Example of NIRSystems 6500 spectra of extracted sugarcane bud scale chemical components, showing small differences between samples within one sample set

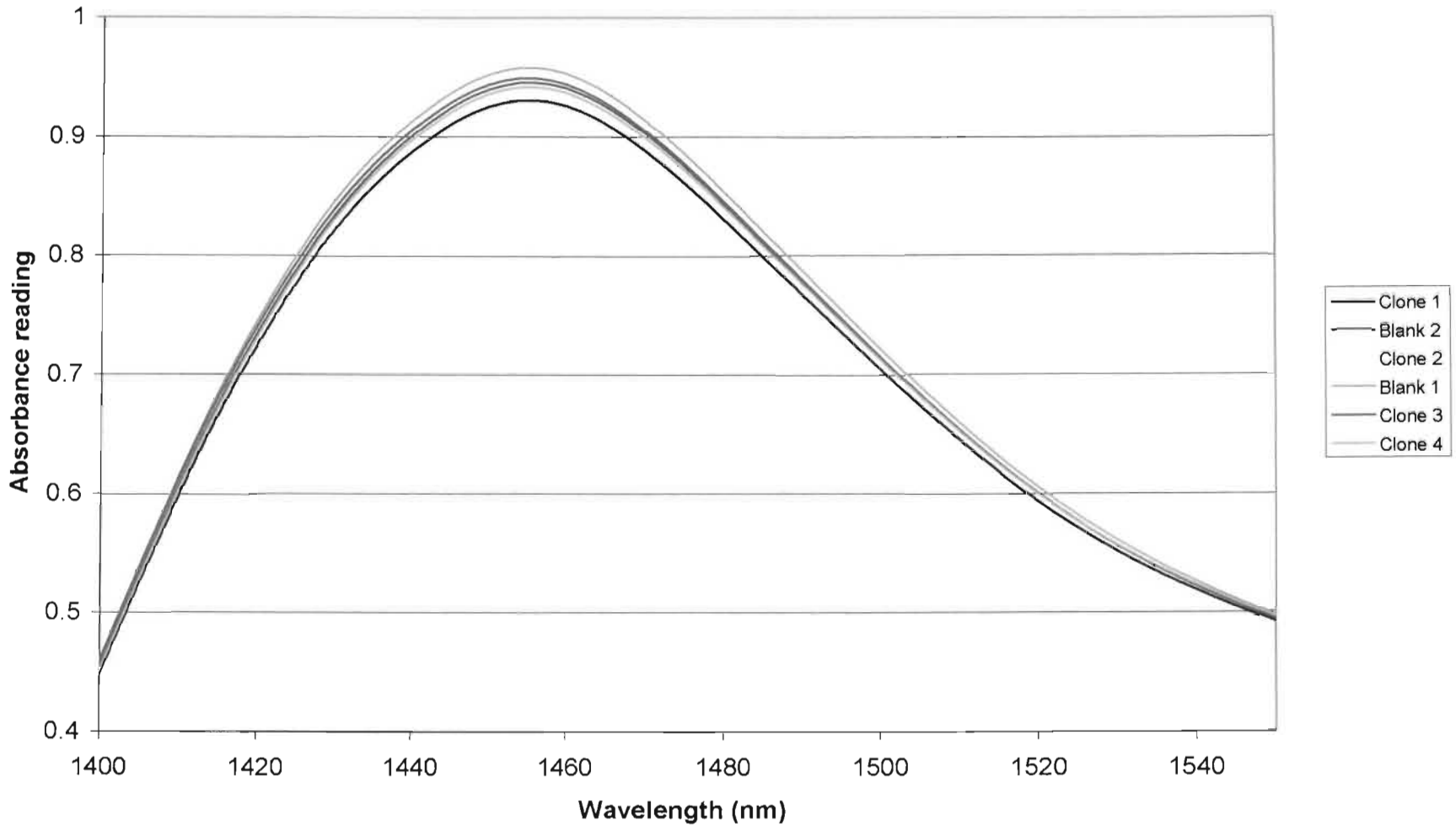

Figure 3.3: Enlargement of the graph area of the sugarcane bud scale extract absorbance peak at 1460 nm

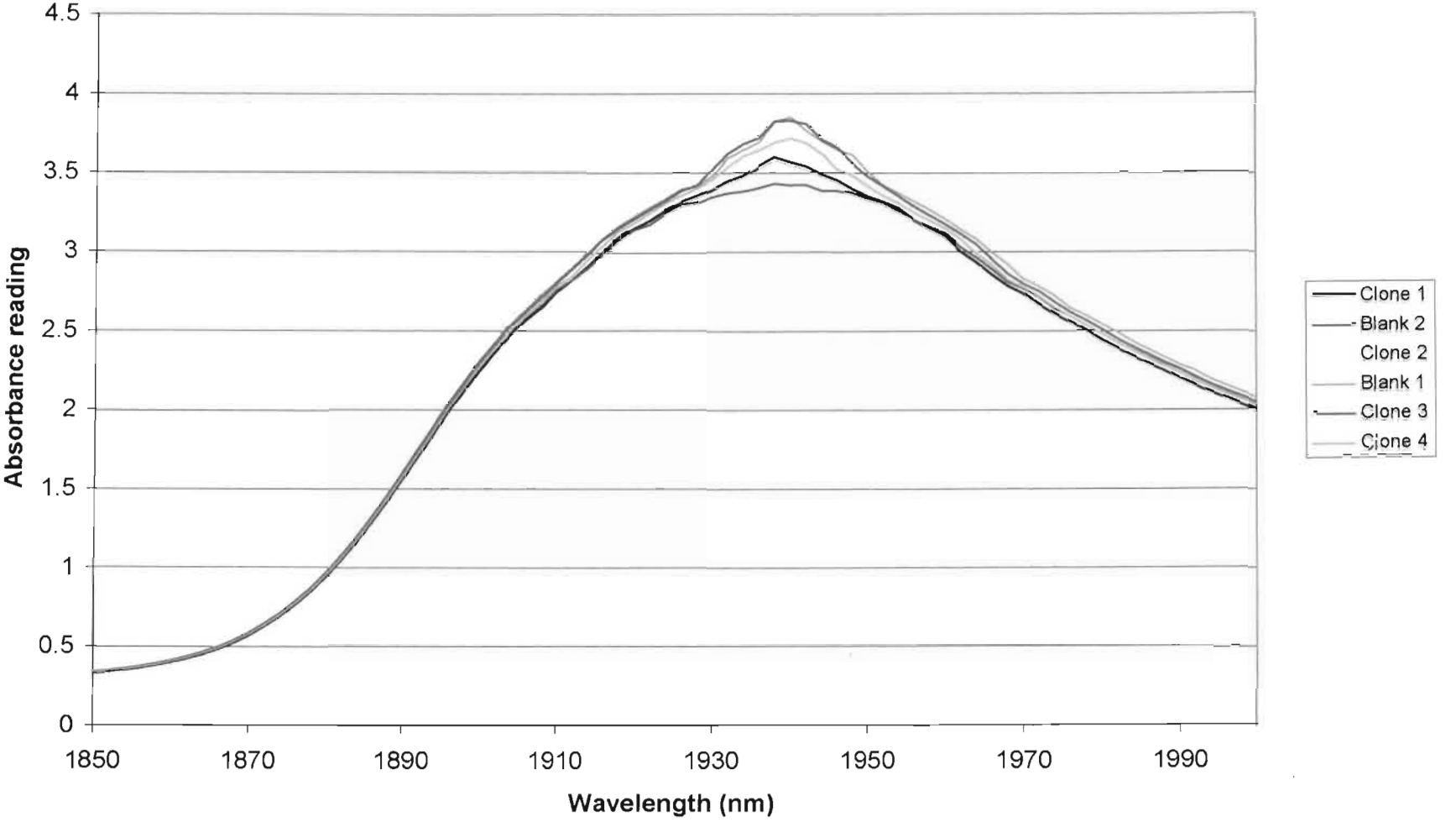

Figure 3.4: Enlargement of the graph area of the sugarcane bud scale extract absorbance peak at 1940 nm

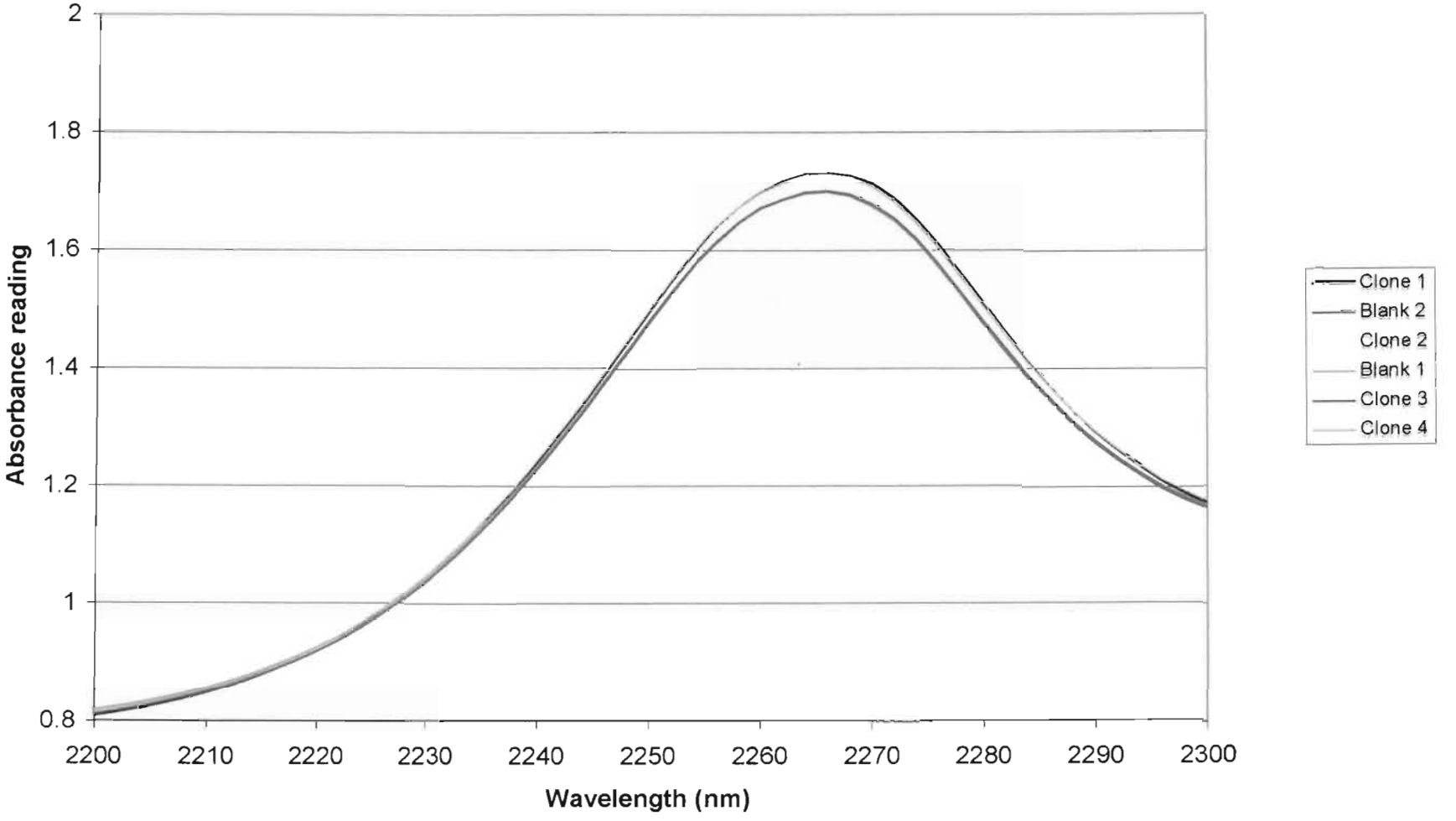

Figure 3.5: Enlargement of the graph area of the sugarcane bud scale extract absorbance peak at 2270 nm

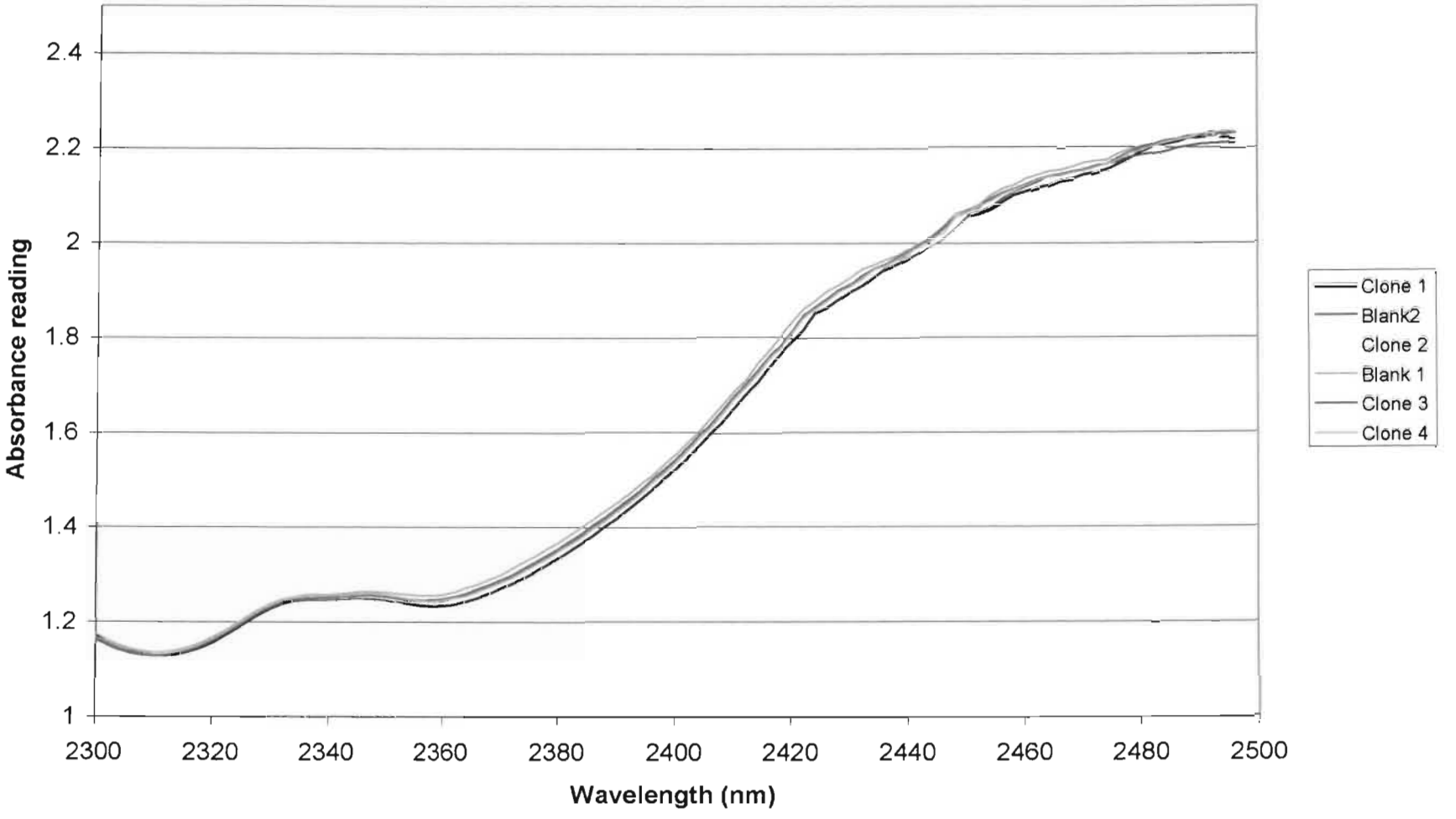

Figure 3.6: Enlargement of the graph area of the sugarcane bud scale extract absorbance peak at 2490 nm

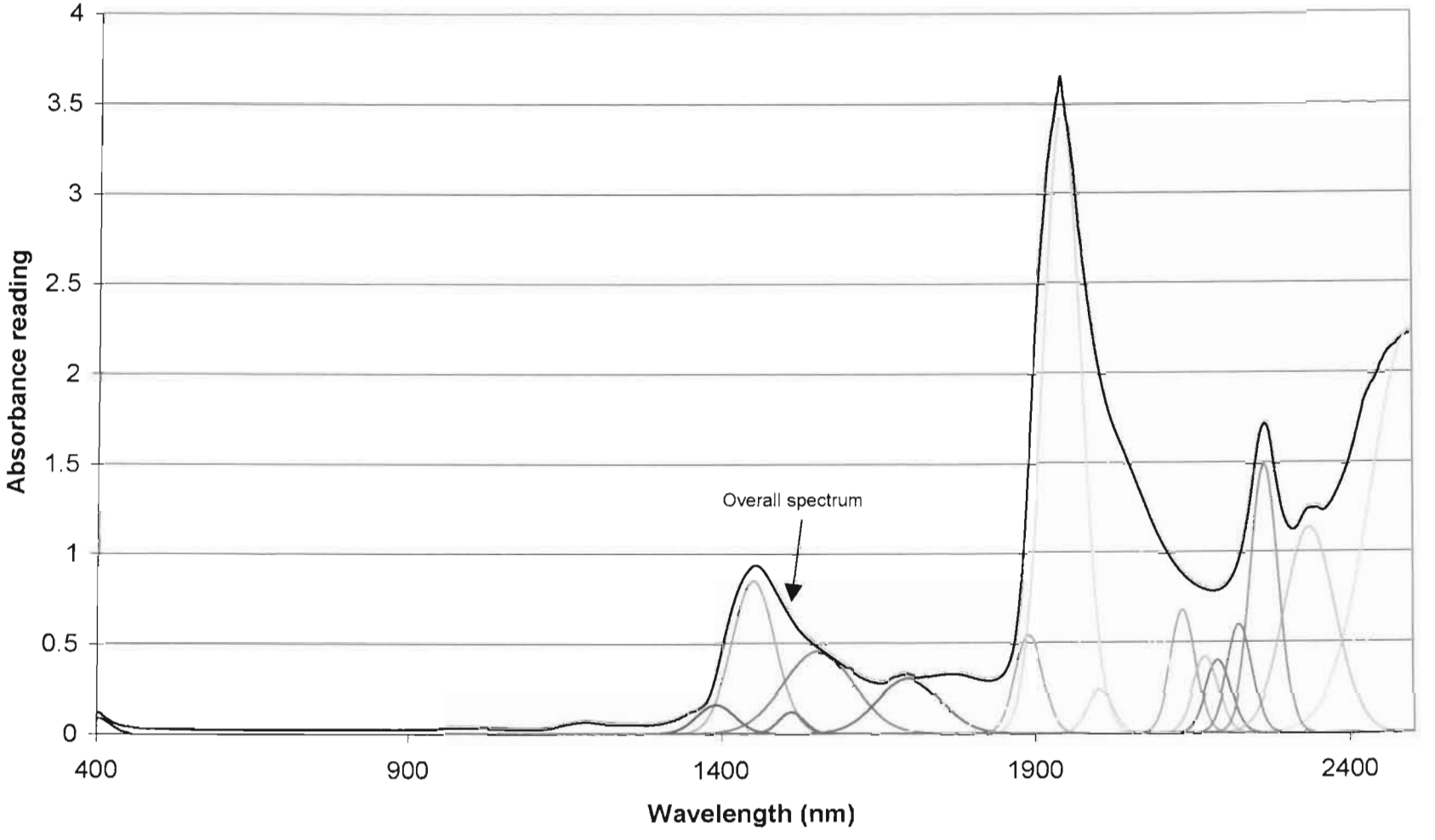

Figure 3.7: Breakdown of overall bud scale spectrum of a single sugarcane genotype into individual peaks, in order to estimate individual peak size and location

differences between chemical profiles of the bud scales. The inability of the calibration equations to predict eldana resistance ratings from one sample set to another were assumed to be due to the environmental influence on the spectral readings. With the subsequent data from the NIRSystems 6500 instrument, it became clear that these differences were due to error. Instrument error was high in the NIRSystems 5000 instrument, creating the false impression that a global library was necessary. Due to the small number of samples that were measured in the original experiment, false positives were obtained in the correlation analysis. With the introduction of the more accurate NIRSystems 6500 instrument, it became evident that incorrect assumptions had been made from the data from the NIRSystems 5000 instrument. Increasing the number of samples also showed that the correlations obtained in the original experiment were due to overfitting, and not to an actual difference between samples. The non-overlap of the samples in the original experiment also added to the erroneous conclusions drawn.

The new NIRS instrument included the wavelengths from the visible light spectrum, from 400 – 1100 nm. An additional peak was noted at the end of the instrument's range, namely at 400 nm. This peak was investigated to determine if it contained any information on eldana resistance. The correlation between samples sets for this peak was very promising, both for the samples taken from the top stalk positions (0.63 – 0.74) and those taken from the lower stalk positions (0.55 – 0.63). This suggested that the bud scale constituent that the peak represented was not random or error, as was the case with the peaks in the NIR region. However, the chemical component associated with this peak, as detected by NIRS, does not appear to have a major influence on eldana resistance. Correlations between peak amplitude and eldana resistance ratings were low (0.18 – 0.30 for the upper stalk position and 0.03 to 0.12 for the lower stalk position).

More importantly, the relationship between the 400 nm peak and eldana resistance is negligible. The plot of predicted eldana resistance ratings against eldana resistance ratings (Figure 3.8), taken from the sample set with the highest correlation, shows that predictions using the 400 nm peak would be inadequate for effective use in the breeding programme. If the prediction of eldana resistance was accurate enough to remove a fair number of susceptible clones without losing too many resistant clones, it would be useful. In the example of the best sample set, choosing to discard those clones with a rating above a

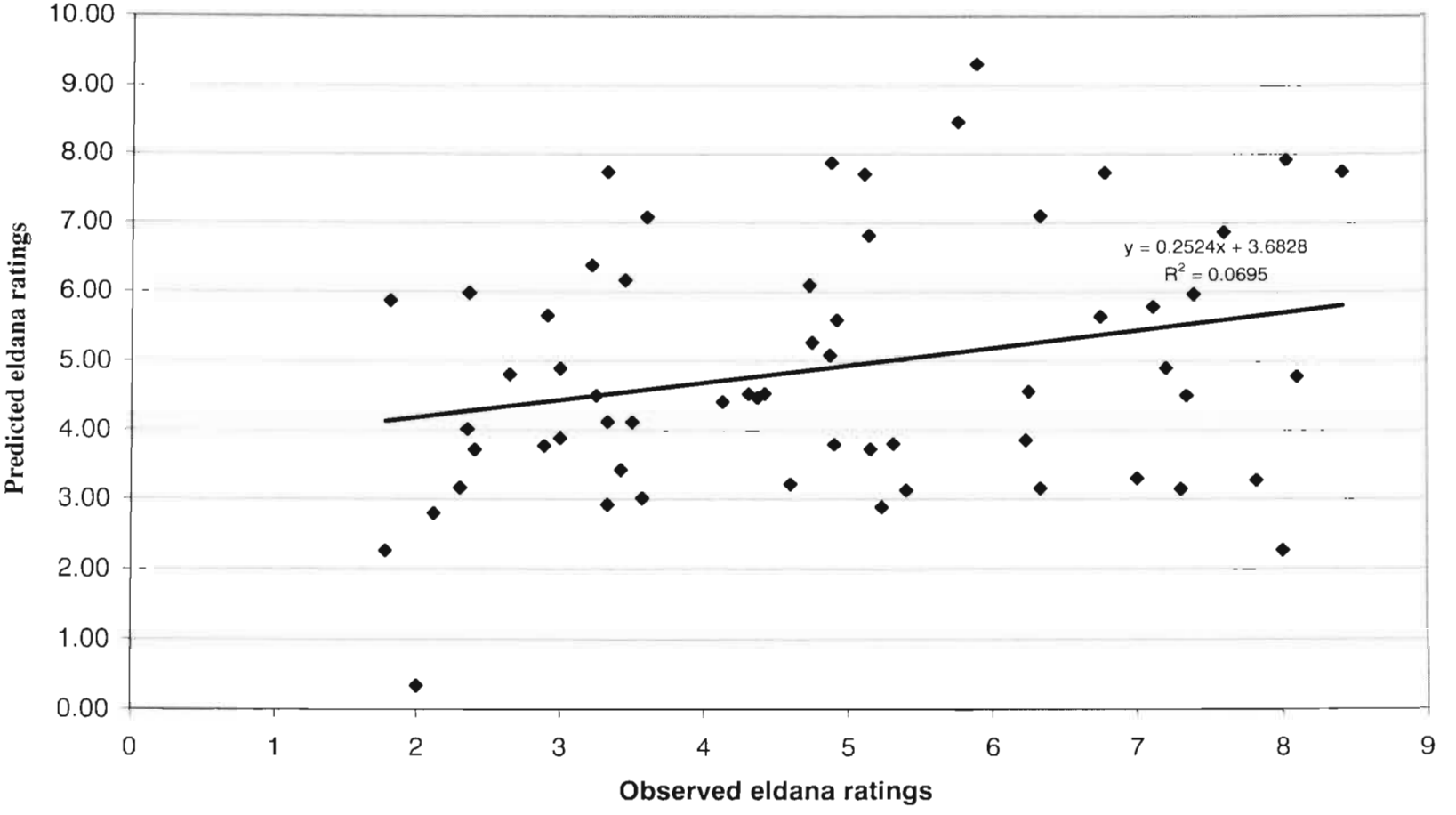

Figure 3.8: Predicted vs actual eldana resistance ratings on sugarcane for the sample set with the highest correlation with eldana ratings (20 month plant crop), using 400 nm absorbance peak values converted to a 1-9 scale for predicted values

certain amount would result in as many resistant clones being removed as susceptible ones, and an even larger number of intermediates (Table 3.3).

In the previous work conducted by Rutherford (Rutherford and van Staden, 1996), the NIRS readings were compared to high performance liquid chromatography (HPLC) results, obtained by running the samples through the column after having been read by the NIRS instrument. It was thought that various peaks were associated with eldana resistance (Figure 3.9), and that these peaks were what the NIRS instrument was detecting. The chemical identity of these peaks was tentatively investigated by Rutherford *et al.* (1993). However the number of sample sets used for this study were small, introducing the possibility of overfitting of correlations.

Samples from the 12 and 16 month sample sets for the plant crop were also run through HPLC, in order to determine whether there was a relationship between the peak areas and eldana resistance. Although HPLC analysis is more time-consuming than NIRS prediction, if the association was good, it could provide an alternative means for predicting eldana resistance. Correlations between samples sets were once again good (0.52 – 0.90), proving that the peaks were measuring components that remained consistent across environments within a specific clone. Correlations were done between each peak and eldana ratings, as well as for the total quantity measured by the column. Most correlation values were small (less than 0.2) and changed from positive to negative between different sample sets. The maximum correlation value obtained was 0.28. The predicted values for eldana ratings were plotted against the actual values to evaluate the possibility of using HPLC peaks for prediction, despite the low correlations (Figure 3.10). As in the predictions using the 400 nm peak, the number of resistant and intermediate clones that would be removed from the population by this prediction was unacceptable (Table 3.4). If the cut-off for removal from the population was rating 5 or 6, then equal numbers of resistant clones would be removed as intermediate and susceptible clones. At higher cut-offs, insufficient susceptible clones would be removed to justify the expense of the technique.

The environmental study was designed not only to develop a calibration equation that would work with any environment or year, but also to give information on the possible alterations that occur in the chemical profiles of bud scales in different environments. It is important to know how the environmental interaction may affect the chemical composition

Table 3.3: Number of sugarcane clones that would be removed from the population using different ratings on the 1-9 scale as the cut-off value, for eldana predictions based on 400 nm absorbance values

Cut-off rating	No of clones removed from category		
	Susceptible	Intermediate	Resistant
9	0	1	0
8	0	2	0
7	3	6	1
6	4	8	4
5	6	12	6

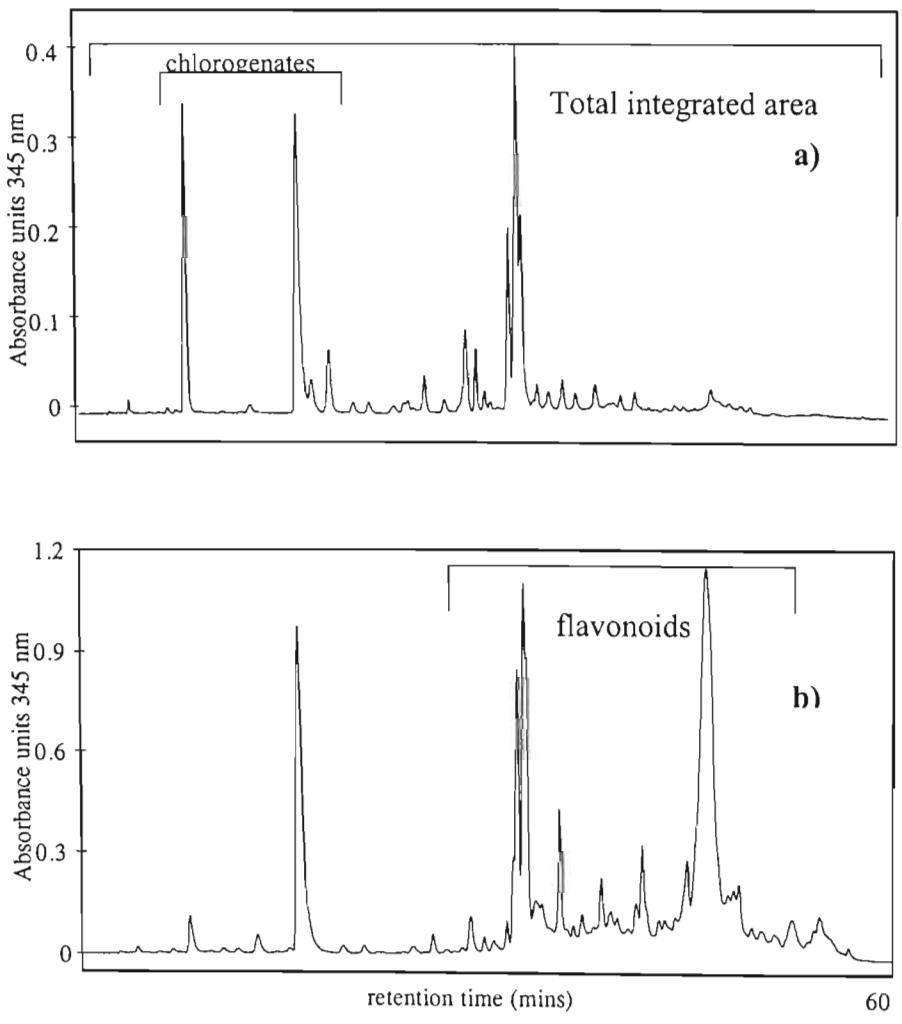

Figure 3.9: Examples of HPLC profiles of bud scale extracts of resistant (a) and susceptible (b) sugarcane clones (Rutherford and van Staden, 1996)

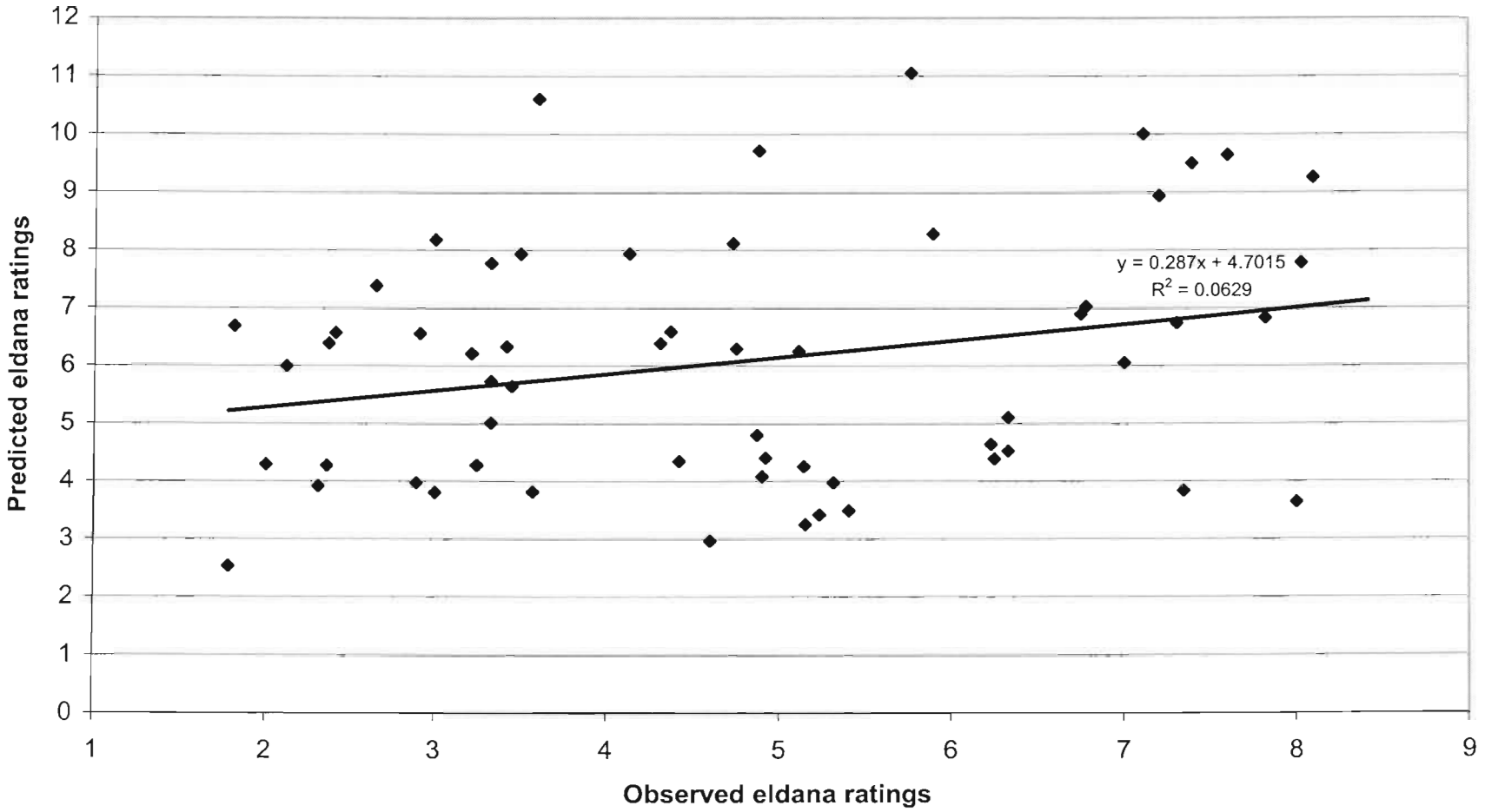

Figure 3.10: Predicted vs actual eldana resistance ratings on sugarcane for the sample set with the highest correlation with eldana ratings (12 month plant crop), using HPLC peak area converted to a 1-9 scale for predicted values

of bud scales if the method is to be useful for breeding and selection purposes. It also needs to be known if sampling has to be limited to a certain age of crop. It is important to know if the chemical components break down on the older lower sections of the stalk, as this will affect the usefulness of that method of resistance. However, because the NIRS technique was unable to predict eldana resistance ratings, the environmental study could not be carried out on the predictions of eldana resistance based on NIRS. Instead G x E interaction analyses were conducted on bud scale mass, in order to test the selected analyses on real data.

Table 3.4: Number of sugarcane clones that would be removed from the population using different ratings on the 1-9 scale as the cut-off value, for eldana predictions based on HPLC peak area

Cut-off rating	No of clones removed from category		
	Susceptible	Intermediate	Resistant
9	4	3	0
8	5	5	1
7	7	6	3
6	10	11	10
5	10	13	12

3.6 Results and Discussion – G x E interaction analyses

The fresh mass of bud scales was measured after removal of the bud scales, in order to calculate the quantities of chemicals to be added for the extraction process. These bud scale masses were investigated for correlation to eldana resistance, and were used to test the G x E interaction analyses that were to be used on the chemical profiles of the bud scales. The average bud scale masses of the 60 cultivars in the calibration set are shown in Appendix 1.

Correlation of bud scale mass (Tables 3.5 and 3.6) to eldana resistance ranged from -0.21 to 0.39 for top of stalk samples, showing inconsistency between different sample sets, possibly due to moisture content. Negative correlations seemed to occur more in the Coastal site for all ages in the plant crop, and more in the Midlands region for all ages in the ratoon crop. This suggests that there is a seasonal and site influence on the relationship between bud scale mass and eldana resistance. However, since none of the correlations

were very strong, interpretation was ineffectual. A maximum correlation of 0.39 was obtained from a 16-month Coastal sample, and this was the only value out of 30 that exceeded 0.2.

Table 3.5: Correlation of eldana ratings to bud scale mass of sugarcane clones for plant crop of calibration study

Site	Age of crop	Replication	Top of stalk	Base of stalk
Coastal	16	1	-0.13	0.11
		2	-0.11	0.06
		3	-0.18	-0.14
	20	1	-0.21	0.22
		2	-0.05	-0.09
		3	-0.10	0.08
Midlands	16	1	0.13	0.17
		2	0.14	0.15
		3	0.15	0.31
	20	1	0.07	0.19
		2	0.02	0.09
		3	0.13	0.26

Table 3.6: Correlation of eldana ratings to bud scale mass of sugarcane clones for ratoon crop of calibration study

Site	Age of crop	Replication	Top of stalk	Base of stalk
Coastal	12	1	-0.10	
		2	-0.21	
		3	-0.05	
	16	1	0.39	0.26
		2	0.10	0.08
		3	0.14	0.27
	20	1	0.05	0.15
		2	-0.01	0.24
		3	0.10	0.17
Midlands	12	1	-0.17	
		2	-0.02	
		3	0.07	
	16	1	0.06	0.25
		2	-0.03	0.15
		3	0.19	0.28
	20	1	0.06	0.11
		2	-0.03	0.09
		3	0.08	0.29

The correlation values for the bud scale mass from the lower section of the stalk to eldana resistance were much better (Tables 3.5 and 3.6), having only two negative values (-0.14 and –0.08), with a maximum of 0.31. However, nine of the 24 values exceeded 0.2, suggesting that there was a closer correlation between bud scale mass at the lower stalk positions than at the top of the stalk. Eldana prefers the lower section of the stalk for entry, and the buds here tend to decay or suffer damage over time. Since the mass, and therefore size, of the bud scale is positively correlated with eldana resistance ratings, the implication is that the less the buds degenerate with age the higher the susceptibility (a higher rating value is given for susceptible clones). However, the relationship is not strong, and would not be useful for predicting eldana resistance.

Correlation between replications within a site ranged from 0.28 to 0.70 (Table 3.7), suggesting reasonable consistency of bud scale mass within one environment. The average bud scale mass was lower for the base of the stalk at all sites and ages, except one. There was no trend in average bud scale mass as age increased for the top of stalk buds, but older bud scales at the base of the stalk tended to be lighter. Differences in average bud scale mass between sites were not consistent across the different ages.

Table 3.7: Correlation of bud scale mass of sugarcane clones in calibration study, between replications within sites and ages

Crop	Site	Age of crop	Top of stalk		Base of stalk	
			Correlation	Average bud scale mass	Correlation	Average bud scale mass
Plant	Coastal	16	0.68	0.228	0.48	0.172
		20	0.52	0.303	0.41	0.163
	Midlands	16	0.70	0.195	0.64	0.238
		20	0.54	0.251	0.48	0.203
Ratoon	Coastal	12	0.51	0.260		
		16	0.36	0.232	0.33	0.166
		20	0.35	0.236	0.35	0.198
	Midlands	12	0.59	0.260		
		16	0.61	0.324	0.60	0.202
		20	0.53	0.260	0.28	0.146

Bud scale mass was averaged over replications, within sites and ages, before correlating to eldana ratings. The results were similar to the correlations obtained on a per replication basis (Table 3.8). None of the correlations exceeded 0.3.

Table 3.8: Correlation of eldana ratings to bud scale mass of sugarcane clones in calibration study, averaged over replications

Crop	Site	Age of crop	Top of stalk	Base of stalk
Plant	Coastal	16	-0.16	0.02
		20	-0.16	0.08
	Midlands	16	0.16	0.24
		20	0.08	0.20
Ratoon	Coastal	12	-0.14	
		16	0.28	0.29
		20	0.08	0.23
	Midlands	12	-0.05	
		16	0.07	0.26
		20	0.04	0.23

Despite the low correlations between eldana resistance and bud scale mass, it was decided to continue analysis of G x E interactions on bud scale mass, in order to study the techniques available.

3.6.1 Analysis of variance

An analysis of variance was carried out for all possible interactions of treatment effects. The results showed that all main effects were significant at the 1% level (Table 3.9). All interactions were significant at the 1% level, except for the year.replication interaction which was significant at the 5% level, and the site.age.replication and age.year.replication interaction which were not significant. The standard error was 0.065 and the CV was 25.7%. This indicates the bud scale mass differs across sites, ages and years. More importantly, there is an interaction at all levels between sites, ages and years, suggesting that the effect of each of these is not consistent. Because the higher order interactions are difficult to interpret, a further ANOVA was carried out for only the first order interactions (Table 3.10). In this analysis, all main effects and interactions were significant at the 1% level. The standard error was 0.0716 and the CV was 28.4%. This ANOVA was then used to estimate V_G, using subtraction in the formulae shown in Table 3.1. Adding together V_G,

Table 3.9: ANOVA for bud scale mass of 60 sugarcane clones evaluated for stability at two sites, three ages and in two crops, including all interactions

Source	df	MS
Genotypes (G)	59	0.1285**
Sites (S)	1	0.0740**
Years (Y)	1	0.1629**
Ages (A)	2	0.0641**
Rep (R)	2	0.0339**
G x S	59	0.0192**
G x A	118	0.0170**
S x A	2	0.3103**
G x Y	59	0.0227**
S x Y	1	1.4049**
A x Y	1 (1)	0.7973**
G x R	118	0.0065**
S x R	2	0.0306**
A x R	4	0.0169**
Y x R	2	0.0123*
G x S x A	118	0.0123**
G x S x Y	59	0.0168**
G x A x Y	59 (59)	0.0064**
S x A x Y	1 (1)	0.0712**
G x S x R	118	0.0073**
G x A x R	236	0.0066**
S x A x R	4	0.0021
G x Y x R	118	0.0092**
S x Y x R	2	0.0276**
A x Y x R	2 (2)	0.0090
Residual	629 (319)	0.0042
Total	1777 (382)	

CV 25.7%

* Significant at 5% level

** Significant at 1% level

Table 3.10: ANOVA for bud scale mass of 60 sugarcane clones evaluated for stability at two sites, three ages and in two crops, including only first order interactions for ease of interpretation

Source	df	MS
Genotypes (G)	59	0.1281**
Sites (S)	1	0.0726**
Years (Y)	1	0.1677**
Ages (A)	2	0.0625**
G x S	59	0.0181**
G x Y	59	0.0216**
G x A	118	0.0162**
S x Y	1	1.4035**
S x A	2	0.3055**
Y x A	1 (1)	0.8039**
Residual	1474 (381)	0.0051
Total	1777 (382)	
CV 28.4%		

** Significant at 1% level

V_E (square of the standard error) and $V_{G \times E}$ (the sum of the estimates of the first order interactions) as the estimate of V_P, the broad-sense heritability for bud scale mass was then calculated as 0.15. Since the interactions were all significant, further methods of G x E interaction evaluation were employed.

3.6.2 Regression analysis and variance

A regression analysis was carried out on the bud scale mass data, using the mean of all genotypes at a site as the environmental index. The definition that was used for a stable genotype was one that had a slope parallel to the mean of all genotypes, i.e. a slope that did not differ significantly from one. The variance of each genotype was also calculated, as another measure of stability is low variance. The results are summarised in Table 3.11.

Examination of residual plots showed no non-linear trends or other problems (results not shown).

Table 3.11: Regression and variance data for sugarcane genotypes evaluated for stability in two environments at 12, 16 and 20 months at plant and ratoon stages

Clone	Slope	Var	Clone	Slope	Var	Clone	Slope	Var
1	1.199	0.02103	21	0.416	0.00458	41	1.168	0.00554
2	1.772*	0.00893	22	0.674	0.00664	42	1.704	0.01119
3	1.333	0.00817	23	0.509*	0.00266	43	1.043	0.00490
4	1.228	0.00836	24	0.466*	0.00282	44	0.726	0.00456
5	0.999	0.00399	25	2.125*	0.01837	45	2.053*	0.01124
6	0.653	0.00494	26	0.738	0.00474	46	1.365	0.00552
7	0.783	0.00238	27	0.092*	0.00316	47	0.683	0.00270
8	1.455*	0.00478	28	1.947*	0.01238	48	0.255*	0.00327
9	1.855	0.01289	29	1.282	0.00528	49	0.540	0.00735
10	1.183	0.01371	30	0.308*	0.00357	50	1.242	0.00707
11	0.327*	0.00335	31	1.437	0.00762	51	0.308*	0.00225
12	1.371	0.00615	32	2.195	0.02151	52	-0.019*	0.00278
13	0.734	0.01160	33	0.432	0.00498	53	0.771	0.00313
14	0.383*	0.00274	34	1.271	0.00895	54	0.621	0.00339
15	0.806	0.00791	35	0.935	0.00564	55	2.322*	0.01571
16	1.226	0.01162	36	1.786*	0.00844	56	0.525	0.00269
17	0.907	0.00447	37	1.413	0.00974	57	1.046	0.01457
18	1.439	0.00689	38	0.761	0.00367	58	0.850	0.00629
19	0.950	0.01153	39	0.553	0.00678	59	0.503*	0.00174
20	0.682	0.00322	40	1.058	0.00690	60	0.553	0.00445

* significant at 5% level

Results between the two methods are inconsistent. There are a number of genotypes that are stable based on variance, but are unstable based on regression as they have a regression slope significantly different from one. This is not unexpected as the two methods measure different aspects of stability. These results do highlight the problem that stability depends

on the method used to define it. Those clones that rank well for both methods might be classified as stable. One observation to note is that those clones with low variance and significant slope tend to have a slope less than one, and that those clones with high variance and significant slope tend to have a slope greater than one. This suggests that clones with low variance across sites have low variability because of small bud sizes across all sites, whilst those clones with high variance values have high variability because of a greater than normal mass increase at sites that exhibit higher bud scale mass on average. As eldana resistance is slightly associated with small buds, the low variance clones with slopes less than one may be preferential to the stable clones.

3.6.3 Qualitative or rank order analysis

An evaluation of the stability of clones on a qualitative basis is important even in disease evaluation. A change from resistant to susceptible under different environmental conditions makes a particular clone undesirable, as the resistance is not stable. Therefore the tests for measuring rank-order changes of genotypes between environments were carried out on bud scale mass using the methods detailed in section 3.4.3 (Table 3.12). To remove the effect of genotype on the stability analysis, corrected values were also calculated (using Equation 4) and analysed for the stability parameter estimates (Table 3.13). Correlations between the different types of stability parameters are listed in Table 3.14.

There are a high number of unstable clones in the uncorrected data (78% for $S^{(1)}$ parameter and 73% for $S^{(2)}$ parameter). This number drops drastically in the corrected data to 27% for both parameters. The significances for the corrected data are considered more reliable as they are independent of bud scale mass effects. Because of the drop in number of significantly unstable clones from uncorrected to corrected data, there is a strong possibility that genotype yield level has a major effect on perceived stability. The correlation between $S^{(1)}$ and $S^{(2)}$ in the uncorrected data is good, but $S^{(3)}$ does not agree as well with the other two parameters. Correlations between parameters of uncorrected and corrected data are poor, and negative in the case of $S^{(3)}$. However, agreement between the more reliable corrected parameters is high. This suggests that on corrected data the calculation of only one stability parameter should be sufficient. Agreement with stability estimators based on regression and variance are poor. However, these measure stability

Table 3.12: Stability parameters of bud scale mass of 60 sugarcane clones over two environments, three ages and two crops, based on qualitative or rank order analysis for uncorrected sugarcane bud scale mass

Clone	$S^{(1)}$	$S^{(2)}$	$S^{(3)}$	Clone	$S^{(1)}$	$S^{(2)}$	$S^{(3)}$
1	23.90*	420.7*	14.63	31	11.24**	94.3**	5.55
2	15.18**	179.6*	8.80	32	7.46**	50.4**	3.14
3	17.98	246.0	13.38	33	15.60*	194.7*	8.07
4	20.72	319.8	18.03	34	16.91	216.3	9.58
5	14.22**	156.5**	15.29	35	16.49*	201.1	17.13
6	15.51*	181.6*	11.23	36	18.03	244.5	12.16
7	9.92**	84.0**	21.19	37	20.11	297.3	14.46
8	14.59**	161.2**	12.40	38	13.02**	155.9**	16.88
9	11.23**	102.7**	4.92	39	16.57*	217.9	8.77
10	12.65**	160.0**	5.85	40	16.04*	194.0*	8.99
11	16.59	203.3	12.97	41	15.67*	183.8*	13.16
12	11.92**	109.6**	6.35	42	17.72	232.7	11.42
13	14.85**	170.8*	8.83	43	14.65**	165.6**	11.13
14	15.75*	190.2*	16.21	44	14.92**	171.2*	13.69
15	16.90	209.1	10.71	45	17.41	223.4	11.06
16	13.55**	176.9*	6.24	46	12.56**	125.2**	7.17
17	14.25**	151.6**	14.44	47	11.79**	105.5**	12.76
18	14.90**	167.2**	8.76	48	15.96*	189.1*	15.14
19	20.34	307.5	15.38	49	17.10	215.4	11.21
20	14.31**	155.0**	11.20	50	18.18	245.0	14.81
21	18.36	255.1	16.60	51	15.92*	186.1*	13.26
22	16.25*	202.0	10.98	52	11.49**	157.4**	31.67
23	11.23**	97.0**	19.08	53	10.16**	102.0**	24.71
24	12.33**	130.2**	16.96	54	14.52**	156.3**	13.35
25	5.42**	34.2**	2.09	55	12.08**	140.8**	5.40
26	14.08**	154.2**	8.84	56	11.68**	117.8**	18.99
27	15.86*	190.8*	8.77	57	23.55*	411.4*	15.30
28	6.68**	38.3**	2.66	58	13.82**	149.3**	7.40
29	12.11**	119.3**	7.58	59	10.65**	84.3**	12.70
30	15.33**	184.0*	16.59	60	15.92*	192.9*	19.20

* significant at 5% level

** significant at 1% level

For definitions of $S^{(1)}$, $S^{(2)}$ and $S^{(3)}$ see Section 3.4.3, equations 1-3

Table 3.13: Stability parameters of bud scale mass of 60 sugarcane clones over two environments, three ages and two crops, based on qualitative or rank order analysis for corrected bud scale mass

Clone	$S^{(1)}$	$S^{(2)}$	$S^{(3)}$	Clone	$S^{(1)}$	$S^{(2)}$	$S^{(3)}$
1	26.94**	563.3**	22.06	31	19.56	283.2	14.77
2	18.71	262.2	13.72	32	25.22**	482.9**	21.66
3	22.06	378.8	18.99	33	20.48	307.7	13.56
4	23.56*	416.9*	18.88	34	21.44	335.9	15.06
5	16.61	204.1	11.06	35	19.94	292.1	13.91
6	18.70	263.4	11.86	36	21.07	326.4	15.39
7	14.03**	143.9**	9.42	37	22.57	376.4	17.95
8	15.63*	183.8*	11.64	38	15.67*	188.6*	10.67
9	22.03	358.2	16.97	39	22.15	359.1	15.53
10	24.67**	456.9**	18.55	40	20.19	309.3	14.77
11	19.67	287.9	13.24	41	17.48	228.1	13.00
12	18.03	240.2	12.83	42	22.24	376.1	18.89
13	18.97	265.6	15.63	43	18.56	257.3	11.90
14	18.49	253.4	12.55	44	18.77	265.2	12.93
15	21.14	329.3	16.28	45	21.70	352.7	18.09
16	22.56	376.8	16.05	46	18.34	246.9	12.96
17	18.13	245.3	11.52	47	15.21**	173.9*	9.84
18	20.64	313.9	15.48	48	19.83	291.4	13.96
19	23.48*	429.1*	20.91	49	22.78	383.3	17.37
20	16.99	214.0	12.00	50	20.86	319.7	16.08
21	20.60	317.7	15.89	51	18.40	248.6	12.02
22	19.40	279.6	14.86	52	18.40	250.9	13.83
23	17.07	215.4	11.21	53	16.20*	194.4*	11.21
24	16.69	203.6	10.76	54	18.25	243.2	12.35
25	25.01**	473.1**	22.04	55	25.02**	474.9**	21.39
26	17.78	235.1	12.30	56	17.10	215.3	11.34
27	21.28	334.0	14.49	57	26.05**	512.2**	16.75
28	23.41*	407.3*	19.44	58	19.97	303.4	14.61
29	14.71**	167.8**	9.59	59	14.03**	147.5**	8.89
30	18.85	262.9	13.19	60	19.05	266.4	14.05

* significant at 5% level

** significant at 1% level

For definitions of $S^{(1)}$, $S^{(2)}$ and $S^{(3)}$ see Section 3.4.3, equations 1-3

Table 3.14: Correlations between stability parameters of bud scale mass of 60 sugarcane clones evaluated across two environments, three ages and two crops, based on qualitative or rank order analysis

	$S^{(1)}$	$S^{(2)}$	$S^{(3)}$	$S^{(1)}$ adj	$S^{(2)}$ adj	$S^{(3)}$ adj
$S^{(1)}$	1					
$S^{(2)}$	0.97	1				
$S^{(3)}$	0.24	0.26	1			
$S^{(1)}$ adj	0.34	0.47	-0.38	1		
$S^{(2)}$ adj	0.33	0.47	-0.37	0.99	1	
$S^{(3)}$ adj	0.23	0.36	-0.37	0.94	0.94	1

adj – data corrected for genotype

defined in a different way, and in terms of desirability of clones, may not reflect those that are best for disease resistance. Environmental interactions that cause qualitative changes are an important aspect to evaluate and the stability parameters calculated in this section should be taken into account when determining the best clones for disease resistance.

3.6.4 Stability variance and ecovalence

The stability variance and ecovalence techniques (Equations 7 and 8) were applied to the bud scale mass data (Table 3.15). These techniques allowed the partitioning of G x E interaction variance into components for each genotype, thereby allowing individual clonal determinations of stability. Approximate F distribution tests were applied to determine whether the interaction variance attributable to each genotype was significant. Values for stability variance and ecovalence were similar. Only two clones showed a variance significantly different from the experimental error. This differs from previous techniques, which showed a number of clones exhibiting unstable behaviour across environments.

3.6.5 Deviation of plot mean from maximum plot

An analysis of variance based on the deviation from maximum plot (Equations 9 to 11 and Table 3.2) was carried out on the data. The deviation from maximum plot analysis allowed a stability test for the trial, as well as for individual clones. Minimum bud scale mass was used as the criteria rather than maximum bud scale mass, as minimum bud scale mass is associated with eldana resistance. There was no significant effect for G x E interaction,

Table 3.15: Stability variances and ecovalence parameters for bud scale mass of 60 sugarcane clones evaluated across two environments, three ages and two crops

Clone	Stability variance	Ecovalence	Clone	Stability variance	Ecovalence
1	0.01836**	0.01809**	31	0.02514	0.00241
2	0.00263	0.00260	32	0.00472	0.00436
3	0.00340	0.00338	33	0.00223	0.00219
4	0.00429	0.00430	34	0.00435	0.00429
5	0.00028	0.00041	35	0.00300	0.00310
6	0.00130	0.00137	36	0.00178	0.00180
7	0.00058	0.00079	37	0.00212	0.00213
8	0.00085	0.00094	38	0.00109	0.00123
9	0.00442	0.04220	39	0.00290	0.00282
10	0.00507	0.00481	40	0.00311	0.03080
11	0.00220	0.00225	41	0.00137	0.00143
12	0.00213	0.00207	42	0.00519	0.00512
13	0.00597	0.00587	43	0.00202	0.00210
14	0.00200	0.02105	44	0.00223	0.00233
15	0.00228	0.00227	45	0.00501	0.00493
16	0.03735	0.00357	46	0.00067	0.00067
17	0.00195	0.02058	47	0.00127	0.00140
18	0.00177	0.00175	48	0.00177	0.00187
19	0.00395	0.00392	49	0.00277	0.00276
20	0.00164	0.00172	50	0.00264	0.00270
21	0.00178	0.00185	51	0.00163	0.00171
22	0.00434	0.00431	52	0.00328	0.00348
23	0.00101	0.00120	53	0.00078	0.00100
24	0.00806	0.00762	54	0.00126	0.00136
25	0.00117	0.00133	55	0.00487	0.00464
26	0.00087	0.00090	56	0.00108	0.00126
27	0.00287	0.00282	57	0.01558**	0.01542**
28	0.00408	0.00381	58	0.00232	0.00225
29	0.00112	0.00115	59	0.00080	0.00094
30	0.00266	0.00276	60	0.00356	0.00365

** significant at 1% level

E(V), or genotype, V (Table 3.16). The non-significant E(V) indicated that variances of clonal deviations across environments were the same for all clones. A non-significant V term indicated that none of the genotypes differed in terms of bud scale mass. These results were similar to those obtained for the stability variance and ecovalence parameters. Because of the non-significance of the E(V) term, individual stability parameters were not presented here, as they were all non-significant.

Table 3.16: Deviation from maximum plot stability analysis for bud scale mass of 60 sugarcane clones evaluated across two environments, three ages and two crops

Source	df	SS	MS	F ratio	F prob
E	20	0.61	0.0304	0.758	0.77
V	59	2.00	0.0339	0.847	0.79
E(V)	540	19.85	0.0368	0.918	0.88
Pooled error	1180	47.26	0.0401		
Total	1799	70.32			

3.6.5 Concluding remarks

The analysis of variance provides an overall representation of the G x E interaction effects present in the trial. If the interactions are significant, further analyses can be undertaken. The variance of clones provides a simple measure of differences in performance between environments, but shows nothing of the pattern of interactions. Regression shows how clones tend to perform across good and poor environments, but the interpretation is complicated by the definition of stability that is used. The results are also dependent on the genotypes and environments used in the trial, rather than representing the general scenario.

Qualitative or rank order analysis proved useful in determining those clones that are consistently better performing in terms of eldana resistance, in this case those with small bud scale mass. The uncorrected data was subject to interference by the genotype effect and should be regarded with suspicion. The corrected data with removed genotypic effect provides more accurate information with greater agreement between the stability parameters. It probably gave the best results for this trial. The correlations between regression and the uncorrected stability parameters were negative (-0.17, -0.12 and -0.57

for $S^{(1)}$, $S^{(2)}$ and $S^{(3)}$ respectively) and between regression and the corrected stability parameters were positive (0.45, 0.47 and 0.57 respectively). Interestingly, the correlations for $S^{(3)}$ were both high, although the signs were opposite, while corrected $S^{(1)}$ and $S^{(2)}$ showed fair correlations.

The stability variance and ecovalence parameters showed only two unstable clones. Based on the data from the other techniques, this is unlikely and it is evident that the technique is not performing well. Replications were not considered in these techniques and this may contribute to the lower differences noted between environments. The two parameters gave very similar results.

The deviation of plot mean from maximum plot analysis showed no significance for clones or G x E interactions. Once again, this does not agree with the majority of the other techniques. This technique uses a very different classification of stability, which may explain why it shows different results. For example, the regression analysis uses the average as the stability comparison and this technique uses the maximum plot value.

The contrasting results from the various stability analyses demonstrate the problem with stability analysis, in that there are many definitions of stability. In determining the best option for use in a trial, other aspects should be considered. In disease resistance, the main criteria is not to have a clone close to the average performance of the trial, but rather to have a clone that performs well in all environments. Therefore regression is probably not the best choice. The rank order analysis is perhaps a good choice as it provides an idea of the relationship between the clones. Deviation from maximum plot may also be a good way to find the most stable and best performing clone, but in this trial no differentiation between clones was evident.

CHAPTER 4
HERITABILITY TRIAL

4.1 Introduction

In a breeding programme, it is important that parents chosen for disease resistance do actually carry mechanisms that confer resistance to that disease. If the level of resistance is difficult to determine, less progress will be made as some parent selections will be more susceptible than desired. The heritability of the trait is also useful information as it will give an indication of the gain that can be made in breeding for the trait. The mechanism of inheritance needs to be known as well, in order to plan the breeding strategy.

Bud scale chemical composition may form one part of an intricate system that confers resistance to eldana. Very little is known about the heritability of resistance to eldana as a whole, and bud scale profiles have only recently been evaluated as a mechanism of eldana resistance. Therefore it is important to determine the heritability and behaviour of bud scale profiles in sugarcane. Because of the polyploid nature of sugarcane, simple mechanisms of inheritance are unlikely, but if the mechanism of inheritance of bud scale profiles can be approximated, the information will provide breeders with a strategy for increasing eldana resistance in the population.

In this study 24 crosses were planted in a trial together with their parents in order to determine the heritability of bud scale chemical profiles. The trial was sampled in both the plant and first ratoon crops, in order to provide more information. The set of calibration clones used in the calibration study were included in order to provide local calibration. The inheritance study was to be based on the NIRS predictions of eldana resistance ratings.; however, NIRS spectra and HPLC profiles on bud scale components showed no relationship between peaks and eldana resistance. Therefore an inheritance study of the chemical components related to eldana resistance could not be carried out. Instead the methods of analysis of cross information were applied to bud scale mass, as taken on fresh samples during preparation of bud scale extraction.

4.2 Materials and Methods

A biparental mating design of 24 pairs of parents was used, with no genotype being a parent more than once. The crosses were chosen randomly from all available crosses, excluding those with multiple possibilities of male parents in one female flower, or those that could have undergone self-pollination. Eight crosses that were planted were lost due to poor germination and assorted damage to young plants. The trial design was randomised block. The list of 24 crosses and their parents are shown in Appendix 2, along with the maximum number of offspring in each cross for those that were sampled.

The offspring were planted in three replications, with unique seedlings in each replication. In other words, the seedlings were not replicated, but were divided into three groups. Each offspring was represented by one stool. The parents were planted in an adjacent plot, separate from the offspring, in order to prevent environmental correlation between parents and offspring from affecting the heritability estimate. Each parent was planted in a 3 m row. The calibration set was planted in three replications, between the parent rows and the offspring plots. This was to provide a standard with which to compare the parents and offspring. Each cultivar was planted in a 3 m row (Appendix 5).

The trial was planted in March 1996, cut back in February 1997, and sampled at 12 months. Each replication of the offspring was sampled at the same time as the adjoining replication of the calibration set, in order to minimise time differences. The trial was then cut back and allowed to ratoon. The ratoon crop was then sampled again at 12 months, in the same way as the plant crop.

4.3 Data analysis

An analysis of variance can be used to determine heritability estimates if information on individual offspring in each cross is available, where expected values of mean squares for each type of variance are shown (Table 4.1).

Table 4.1: Sample ANOVA table for cross analysis to determine heritability

(Falconer and Mackay, 1996)

Source	df	Expected mean squares
Between crosses	(n/2)-1	$V_w + kV_c$
Within crosses	(n/2)(k-1)	V_w
Total	(nk/2)-1	

where n = number of parents, k = number of offspring within each cross, V_w = within cross variance and V_c = between cross variance.

The following formulae can then be used:

$V_w = ½ V_A + V_E = V_P - V_c$

$V_c = ½ V_A$

where V_A is an estimate of additive variance, V_P phenotypic variance and V_E error variance. These formulae assume that dominance effects are zero. They allow heritability to be calculated as V_A/V_P.

An intra-class correlation, r_I, can also be calculated from these values, using the formulae (Falconer and Mackay, 1996):

$r_I = V_c / (V_c + V_w)$

This correlation provides an estimate of repeatability. It represents the correlation between repeated measures of the same cross, and therefore expresses the amount of variance in a cross which is due to permanent differences between individuals in that cross.

A regression analysis can also be used to determine the heritability of a trait (Table 4.2), using offspring as the dependent variable and parents as the independent variable. An F-test will show whether the regression is significant or not.

Table 4.2: Sample regression analysis of crosses for determination of heritability

Source	df
SS (due to regression)	1
SS(deviations from regression)	n-2
Total SS	n-1

The regression coefficient, b, can be calculated from the sum of squares and sum of products (Falconer and Mackay, 1996):

$$b = \frac{SP(xy)}{SS(x)}$$

where $SP(xy) = \Sigma xy - (\Sigma x \Sigma y / n)$ and $SS(x) = \Sigma x^2 - ((\Sigma x)^2 / n)$

Because this is a biparental layout and the mid-parent value is being used in the regression calculations, the regression coefficient is equal to the heritability estimate, h^2.

Variance and regression methods will only agree if all gene effects are additive. Analyses can also be carried out using only the male or female parent, with appropriate changes made in the heritability calculations, in order to determine whether there are any parental effects.

An important step is to examine the graph of the residuals of all analyses, in order to determine whether there are any trends not accounted for by the linear analysis. Residual graphs are plotted using (observed minus predicted values) as the y-axis values and the predicted values as the x-axis values.

4.4 Results and Discussion

The heritability experiment contained both parents and offspring of a number of biparental crosses. These were chosen on a random basis from the seed storage unit, therefore representing a random sample of the breeding population. The field layout was designed so that the parents were in a separate area from the offspring, therefore removing any environmental covariance between parents and offspring that may come with field effects. The calibration set was included in the same field to provide a sample set grown in the same environment. Because of the small sample size of only seven stalks, individuals could be sampled rather than a family mean. Parents had single seven-stalk samples taken from them.

Not all the offspring that were planted survived in the field, due to poor growth and other factors. The maximum number of offspring that were available for all of the 24 crosses was

21. For those crosses where additional offspring survived, samples were taken from all available offspring, up to a total of 30. Analyses were carried out using different combinations of numbers of crosses and offspring, in order to determine whether more crosses with less offspring gave better information than less crosses with more offspring. Appendix 2 gives the average bud scale mass for both parents and offspring from the 24 crosses.

The results for the heritability calculations are given in Table 4.3. The cross/offspring column indicates the number of crosses and the number of offspring in each cross. The 24/24 and 24/30 options had missing values, due to some crosses having insufficient offspring, but were included in the analysis for additional information. The analysis tables are listed in Tables 4.4 and 4.5.

Table 4.3: Heritability estimates of bud scale mass of sugarcane crosses calculated by variance and regression analysis, including repeatabilty estimates

	Cross/offspring	Type of analysis		Repeatability (r_I)
		Variance	Regression	
Plant crop	24/21	0.29**	0.28**	0.15
	24/24	0.28**	0.28**	0.14
	24/30	0.26**	0.27**	0.13
	18/24	0.18**	0.10	0.09
	12/30	0.27**	0.17	0.14
Ratoon crop	24/21	0.29**	0.30**	0.15
	24/24	0.25**	0.30**	0.13
	24/30	0.22**	0.29**	0.11
	18/24	0.27**	0.29**	0.14
	12/30	0.31**	0.28*	0.16

** significant at 1% level

* significant at 5% level

The heritability estimates determined by the two methods were similar, ranging from 0.18 to 0.31 in the cross analysis estimates, to 0.10 to 0.30 in the regression analysis estimates. Narrow sense heritability seems to be low for the characteristic of bud scale mass, while broad sense heritability, determined in the calibration analysis, seems to be even lower. The crosses with higher number of crosses seemed to perform better than the crosses with

lower number of crosses and higher number of offspring. However, these results may be misleading due to the relatively small differences between cross sizes, and the overall low number of crosses that were available. In the analyses of the 24/24 and 24/30 cross options, even with the large number of missing values, the results were significant.

Table 4.4: Summary of regression analyses for heritability study of bud scale mass of sugarcane crosses

Crop	Crosses	Offspring	MS Regression	MS Residual	F Probability
Plant	24	21	0.01033	0.00085	0.00210
	24	24	0.01010	0.00090	0.00290
	24	30	0.00983	0.00092	0.00350
	18	24	0.00073	0.00080	0.35287
	12	30	0.00133	0.00100	0.27579
Ratoon	24	21	0.01479	0.00102	0.00090
	24	24	0.01417	0.00086	0.00051
	24	30	0.01380	0.00076	0.00033
	18	24	0.00989	0.00094	0.00502
	12	30	0.00714	0.00103	0.02479

Table 4.5: Summary of variance analyses for heritability study of bud scale mass of sugarcane crosses

Crop	Crosses	Offspring	MS Between	MS Within	F Probability
Plant	24	21	0.02647	0.00577	3.91E -11
	24	24	0.02899	0.00592	2.58E -12
	24	30	0.03214	0.00589	2.03E -14
	18	24	0.01903	0.00575	1.11E -05
	12	30	0.03083	0.00540	1.8E -08
Ratoon	24	21	0.03386	0.00736	3.63E -11
	24	24	0.03325	0.00753	1.13E -10
	24	30	0.03519	0.00754	1.17E -11
	18	24	0.03490	0.00731	2.46E -09
	12	30	0.04720	0.00717	5.41E -10

Repeatabilty of the heritability estimate, r_I, was low, as expected, ranging from 0.09 to 0.16. No obvious pattern emerged in terms of which type of cross produced more repeatable results, either those with higher numbers of crosses, or those with higher numbers of offspring. The options with 24 crosses did, however, show a decrease in repeatability with increase in number of offspring, i.e., with an increase in missing values.

This suggests that it may be better to use analyses that contain less crosses, if there are less missing values in these crosses.

Examination of the residual plots is an important aspect of an analysis, in order to verify if there are non-linear trends in the data, as well as other problems such as unequal variances. The residual plots were drawn for the 24/21 (Figures 4.1 and 4.4), 24/24 (Figures 4.2 and 4.5) and 24/30 (Figures 4.3 and 4.6) options. They did not indicate any non-linear trends or problems with unequal variances across the data.

Bud scale mass is correlated slightly with eldana, suggesting that bud size may be one aspect of eldana resistance. Bud scale mass heritability estimates are significant, but fairly low, suggesting that breeding for smaller bud size may be feasible, but difficult. Changes in bud scale mass in the population would be slow, and, given the low correlation with eldana resistance, would probably not improve eldana resistance to any great extent. It would therefore not be feasible to introduce the screening of bud scale mass of clones to the breeding programme and to attempt to breed for smaller bud size.

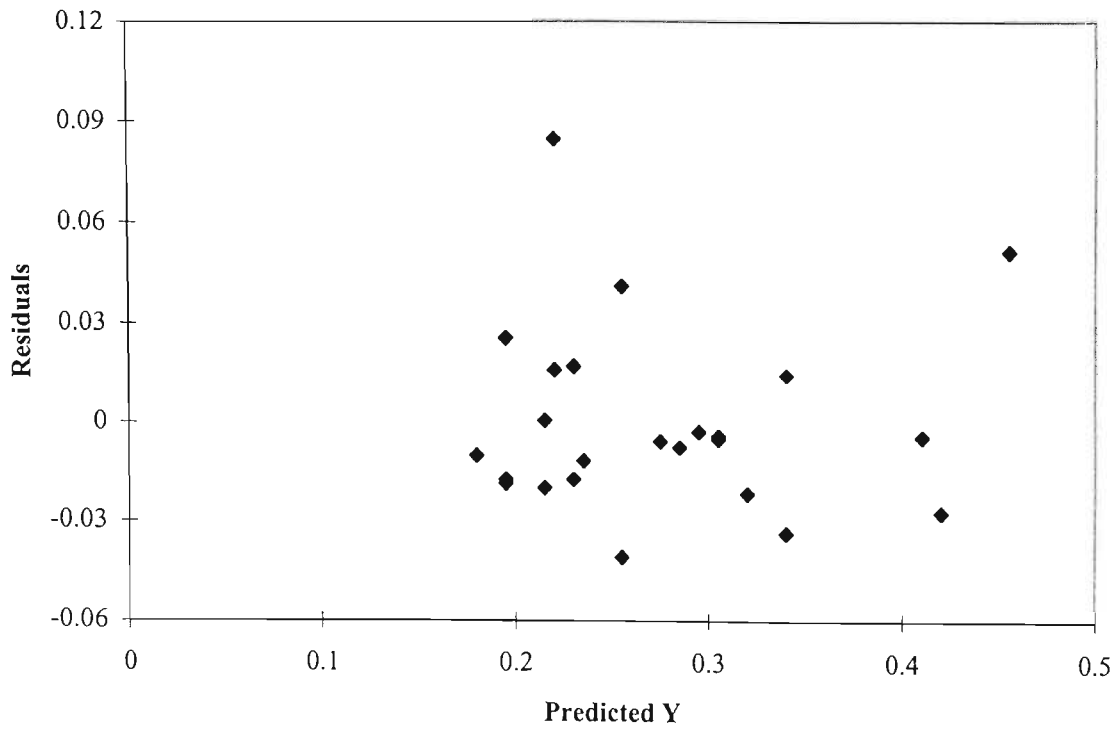

Figure 4.1: Residual plot of 24 crosses/21 offspring plant crop in sugarcane heritability study of bud scale mass

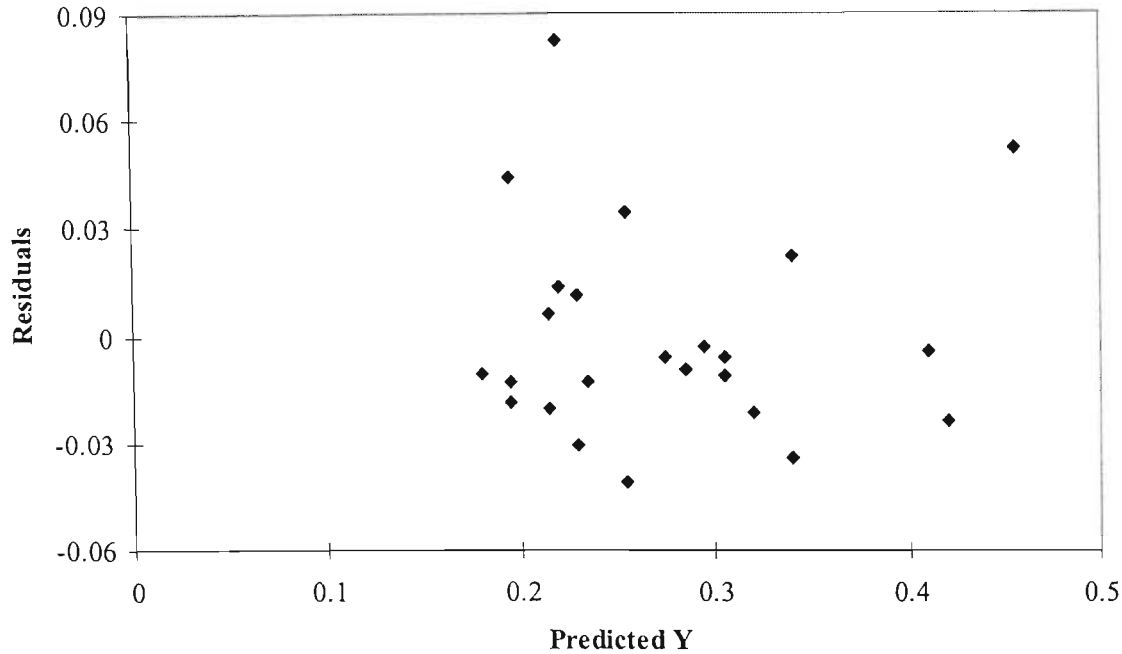

Figure 4.2: Residual plot of 24 crosses/24 offspring plant crop in sugarcane heritability study of bud scale mass

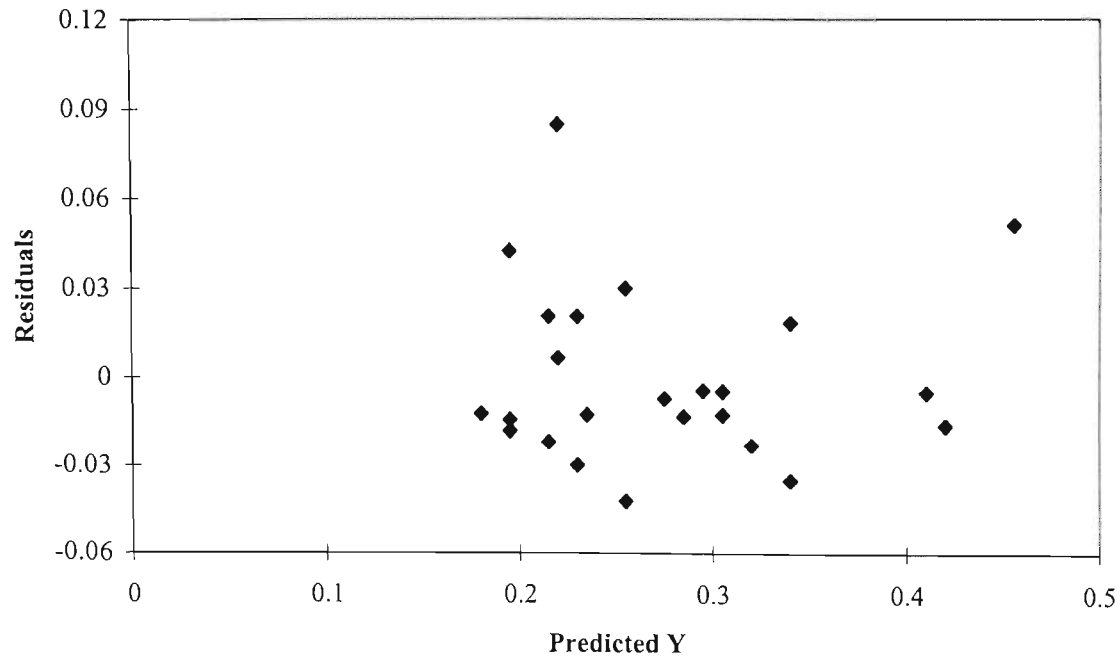

Figure 4.3: Residual plot of 24 crosses/30 offspring plant crop in sugarcane heritability study of bud scale mass

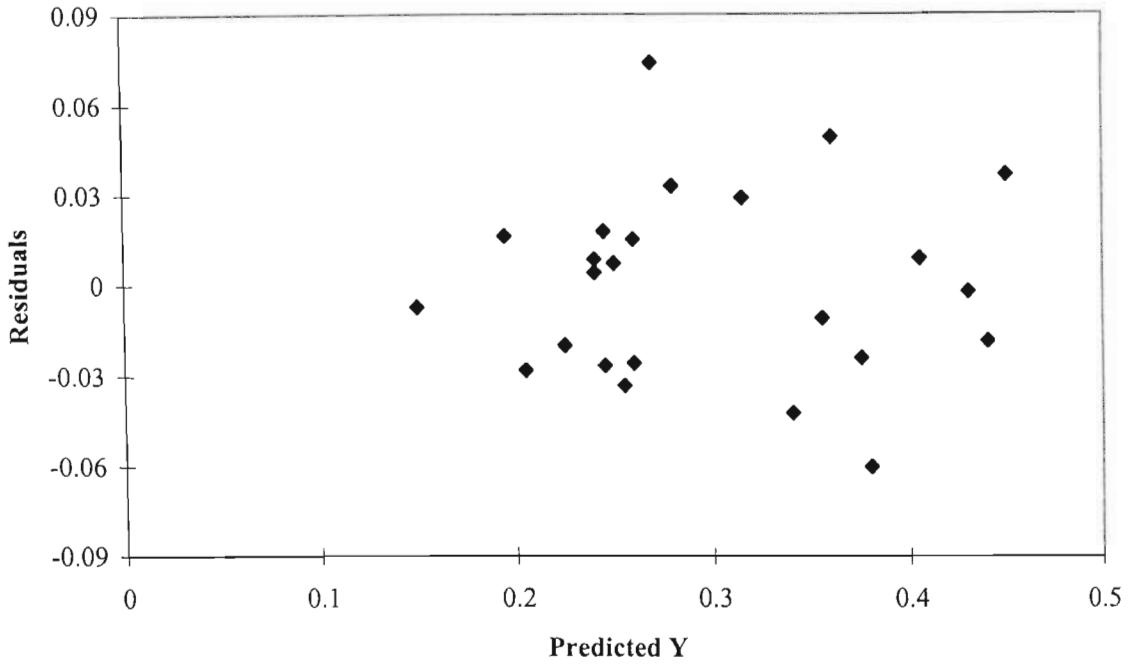

Figure 4.4: Residual plot of 24 crosses/21 offspring ratoon crop in sugarcane heritability study of bud scale mass

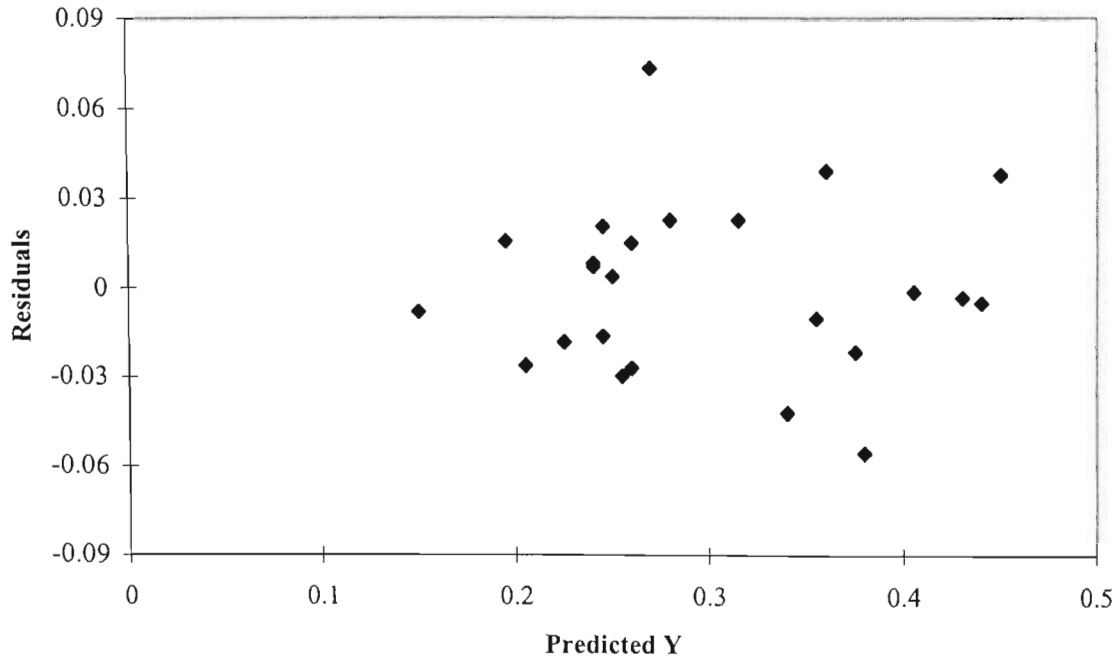

Figure 4.5: Residual plot of 24 crosses/24 offspring ratoon crop in sugarcane heritability study of bud scale mass

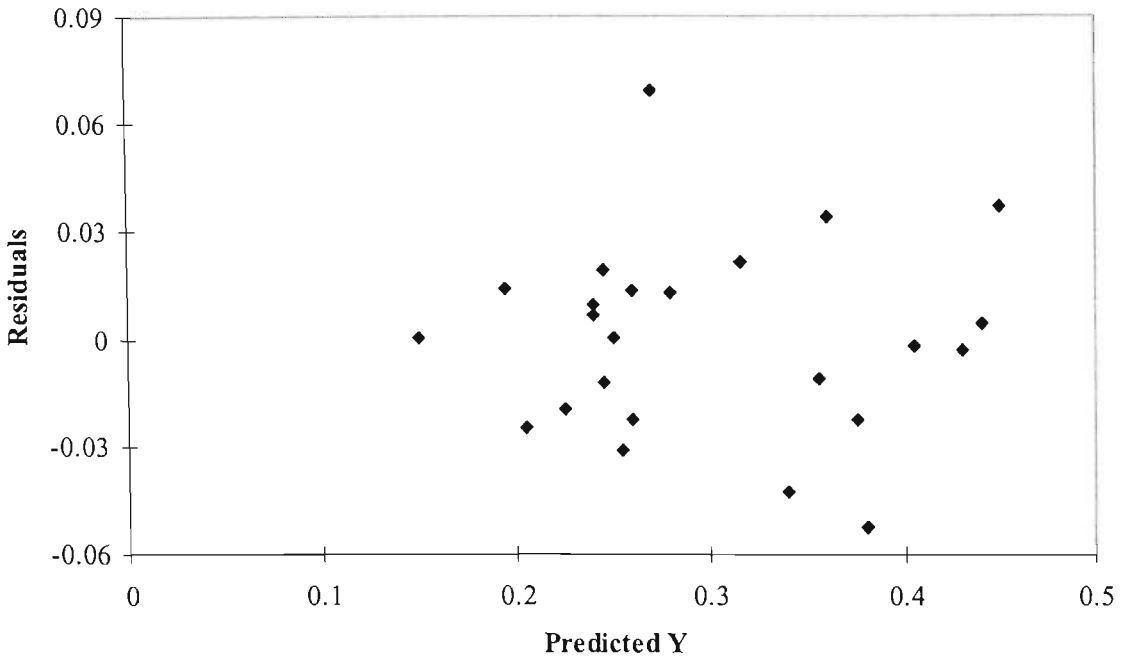

Figure 4.6: Residual plot of 24 crosses/30 offspring ratoon crop in sugarcane heritability study of bud scale mass

CHAPTER 5
CONCLUSION

The original work carried out by Rutherford (Rutherford *et al.*, 1993; Rutherford and van Staden, 1996) suggested that there was a possibility of predicting eldana resistance in sugarcane using the near infrared analysis of bud scale chemical profiles. However, this work was carried out on a limited number of sample sets on an NIRS instrument that exhibited baseshifts within sample sets and inconsistencies between sample sets. The variation in NIRS readings between sample sets were thought to be due to environmental effects, and that this could be overcome with the development of sufficient samples to cover the range of conditions that influence the sampling of bud scale profiles.

The current study had two aims: to develop a global set so that accurate calibration equations could be developed, and to study the environmental and genetic influences on bud scale chemical profiles once the calibration was available. On the new NIRSystems 6500 instrument acquired during the study, various points came to note. Firstly, the baseshifts between samples in the same sample set were no longer observed, suggesting that the old NIRSystems 5000 instrument had a considerable error component even within one session of sample scanning. Secondly, there were no longer differences between sample sets, as the H distances indicated that all samples belonged to the same set. However, the ability to calibrate from one sample set to another did not improve, remaining negligible. The inability to predict samples from one sample set using the calibration developed from another sample set on the old NIRS instrument was thought to be due to the non-overlap of sample sets in multidimensional space, requiring a global library to cover all types of sampling conditions in order to overcome the problem. However, these differences were obviously caused by instrument error on the old NIRS instrument as they were no longer evident on the new NIRS instrument.

Examination of spectra obtained on the new NIRS instrument revealed important details that were not observable with the old instrument. The differences between spectra were small, compared to the strength of the background signal caused by the solvents. These differences were of the same order of magnitude as differences between spectra read repeatedly on the same sample. Furthermore, spectra read on samples containing only the solvents varied as widely as samples from different genotypes, and sometimes had

absorbance readings higher than those containing bud scale extracts. This suggested that if there were differences between spectra from different genotypes they were being masked by other factors, such as small differences between batches of solvents used, evaporation rate of solvents from the solution during scanning, or temperature of the laboratory during scanning. The NIRS technique was therefore not sensitive enough to detect valid differences between samples.

Further study of the samples using HPLC revealed that the chemical profiles between genotypes were different. Rutherford (Rutherford *et al.*, 1993; Rutherford and van Staden, 1996) suggested that these chemical profiles were related to eldana resistance, but correlations between eldana resistance ratings and chemical profiles in this study, as determined by NIRS readings and HPLC, did not show this. It is possible that if the chemicals represented by these peaks are associated with eldana resistance, that they are not in the functional form needed to reflect this. Perhaps the preparation method destroys the functional form of the chemical compounds, or some additional processing of the chemical compounds takes place when damage by eldana larvae occurs. Nevertheless, the results indicate that the method used here is insufficient to reveal differences in eldana resistance between genotypes.

Bud scale mass has a relatively low correlation with eldana resistance, but G x E interaction analyses and a heritability study were carried out on the bud scale fresh mass, in order to evaluate the application of these techniques. The broad sense heritability estimate from the G x E interaction analysis was 0.15, and the narrow sense heritability estimate from the parent-offspring analysis was approximately 0.27, suggesting a slight relationship between parent bud scale mass and offspring bud scale mass. The G x E interaction analyses showed varying results depending on the method used. The ANOVA analysis suggested that ages, sites and years had an effect on bud scale mass, while deviation from maximum plot showed no significance for G x E interactions. The genotypes that were selected as unstable were different depending on the method used to measure stability in individual genotypes. The number of unstable genotypes also varied, with regression analysis and rank order analysis revealing a number of unstable genotypes, while stability variance and ecovalence, which produced similar results to one another, showed only two unstable genotypes. In the rank order analysis, correction of data to remove genotype effect reduced the number of unstable genotypes, suggesting that some of the G x E interaction

effect being measured came from the level of the character being studied. This was a more reliable method than the uncorrected rank order analysis, and would be the preferred analysis type of all those tried.

A number of problems were experienced in this study. Spectra from the old NIRS instrument were misleading, suggesting legitimate differences between samples sets as the reason for non-compatibility between sample sets. It is well-documented that calibration development frequently needs a large number of samples to form a global set, to incorporate all possible types of sampling conditions that may be encountered. These conditions differ from the measurement of environmental effects on the characteristic being studied. An example would be the differing moisture content of a sample affecting the spectral detection of a chemical profile, even though moisture content is not of interest. Samples containing all possible moisture contents in conjunction with the full range of the characteristic being studied would have to be included in the calibration in order to overcome the problem. The assumption was made that an effect of this type was involved in making bud scale sample sets non-comparable. This assumption was proved incorrect with the acquisition of the new NIRS instrument, as there were no such differences between sample sets evident on the new instrument.

The baseshifts experienced on the old NIRS instrument were also cause for concern. The effects were partially mitigated by the use of data transformations such as first and second order derivatives, but the effects could not be removed completely. Baseshifts were not merely a movement up or down on the y-axis, but also had a tilt effect where the baseshift increased or decreased as wavelength increased. The new NIRS instrument fortunately did not show any baseshifts, and the spectra showed great consistency of position between sample sets as well as within sample sets. This suggested that the size of the error on the old instrument was considerable.

Part of the reason for this study was to expand the number of samples available. The original study consisted of a limited number of sample sets, which led to the false impression that the chemicals being detected in the procedure were correlated with eldana resistance. Based on knowledge of chemical interactions with insect pests, Rutherford (Rutherford *et al.*, 1993; Rutherford and van Staden, 1996) determined that the chemical components he identified in the bud scale extracts could be connected to eldana resistance.

However, the chemical components, as detected by HPLC, showed no connection to eldana resistance statistically. It is still possible that these chemicals do play a role in eldana resistance, but they are not in the functional form for the important differences to be detected. As bioassays seem to suggest a link with eldana feeding behaviour, this is a good possibility (Rutherford, 1993).

The results obtained in this study, while not positive, are still important. It would have meant an improvement to the breeding and selection programmes if a rapid screening method for eldana resistance, unaffected by environment, could have been developed. However, various results from this study suggest that eldana resistance is affected by the environment, suggesting that whichever method is used to determine level of resistance, trials would have to be repeated over a number of years to obtain an "average" eldana resistance value. This conclusion is based on the HPLC results and the bud scale mass differences between environments. Even though the HPLC profiles are not correlated with eldana resistance, it is a possibility that the chemicals are associated with resistance in other forms. Correlations between samples sets ranged from 0.52 to 0.90, suggesting that some environments were quite similar with regard to HPLC profiles, and others could be quite different. Similarly, the bud scale mass had a low correlation with eldana resistance, but possibly has an effect on it. Bud scale mass correlations from different sites can be almost zero, suggesting that results between sites differ widely. There is therefore a possibility that eldana resistance itself is influenced by environment. This needs to be investigated further as it would have a great impact on the determination of eldana resistance in the future. Should eldana resistance vary with environment, it would have disturbing implications for the performance of cultivars in the South African sugar industry. Stability of resistance would then become as important a trait to breed and select for as eldana resistance itself. At present, eldana resistance is evaluated by looking at the average performance over a number of environments. This would have to be altered in order to determine whether a genotype is consistently resistant over a number of environments.

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APPENDICES

Table of Contents

Appendix 1

List of the 60 sugarcane cultivars used for the standard sample set for calibration of the NIRS instrument, including their eldana ratings (1- resistant … 9 – susceptible) and average bud scale mass

Clone	Eldana rating	Average bud scale mass (g)
74M0659	3.3	0.289
75E0247	3.2	0.272
75L1157	3.0	0.260
75L1463	2.3	0.237
76F0879	3.0	0.206
76H0333	7.8	0.232
76M1101	1.8	0.163
76M1566	2.0	0.230
77F0637	3.5	0.346
77L1143	7.3	0.380
77L1720	3.6	0.232
77W1241	4.9	0.297
78F0909	6.3	0.280
78L0960	2.9	0.209
79H0181	6.8	0.268
79L0181	5.1	0.336
79M0955	5.2	0.203
80F2147	7.4	0.277
80L0432	7.3	0.262
80W1459	2.4	0.228
81L1308	6.3	0.227
81W0133	3.3	0.259
81W0447	4.4	0.171
82F0675	6.3	0.182
82F2907	4.9	0.443
83F0448	7.0	0.255
84F2753	7.2	0.281
84F3078	4.9	0.392

Clone	Eldana rating	Average bud scale mass (g)
85F2805	5.3	0.254
87W1198	4.1	0.205
88L1077	2.9	0.314
B42231	2.1	0.435
CB38/22	3.3	0.288
CB40/35	8.1	0.274
Co281	1.8	0.207
Co285	5.2	0.256
Co301	6.2	0.260
Co331	3.5	0.194
Co421	4.4	0.299
Co62175	3.4	0.272
J59/3	8.0	0.234
NCo293	8.4	0.295
NCo376	5.1	0.197
NM214	7.6	0.198
N52/219	8.0	0.176
N8	2.4	0.244
N11	7.1	0.277
N12	3.3	0.223
N13	5.8	0.210
N14	4.6	0.280
N16	5.4	0.276
N17	3.6	0.201
N18	5.9	0.215
N19	4.3	0.266
N20	2.7	0.230
N21	2.4	0.224
N22	4.9	0.144
N23	4.7	0.156
N24	6.8	0.214
N25	4.8	0.364

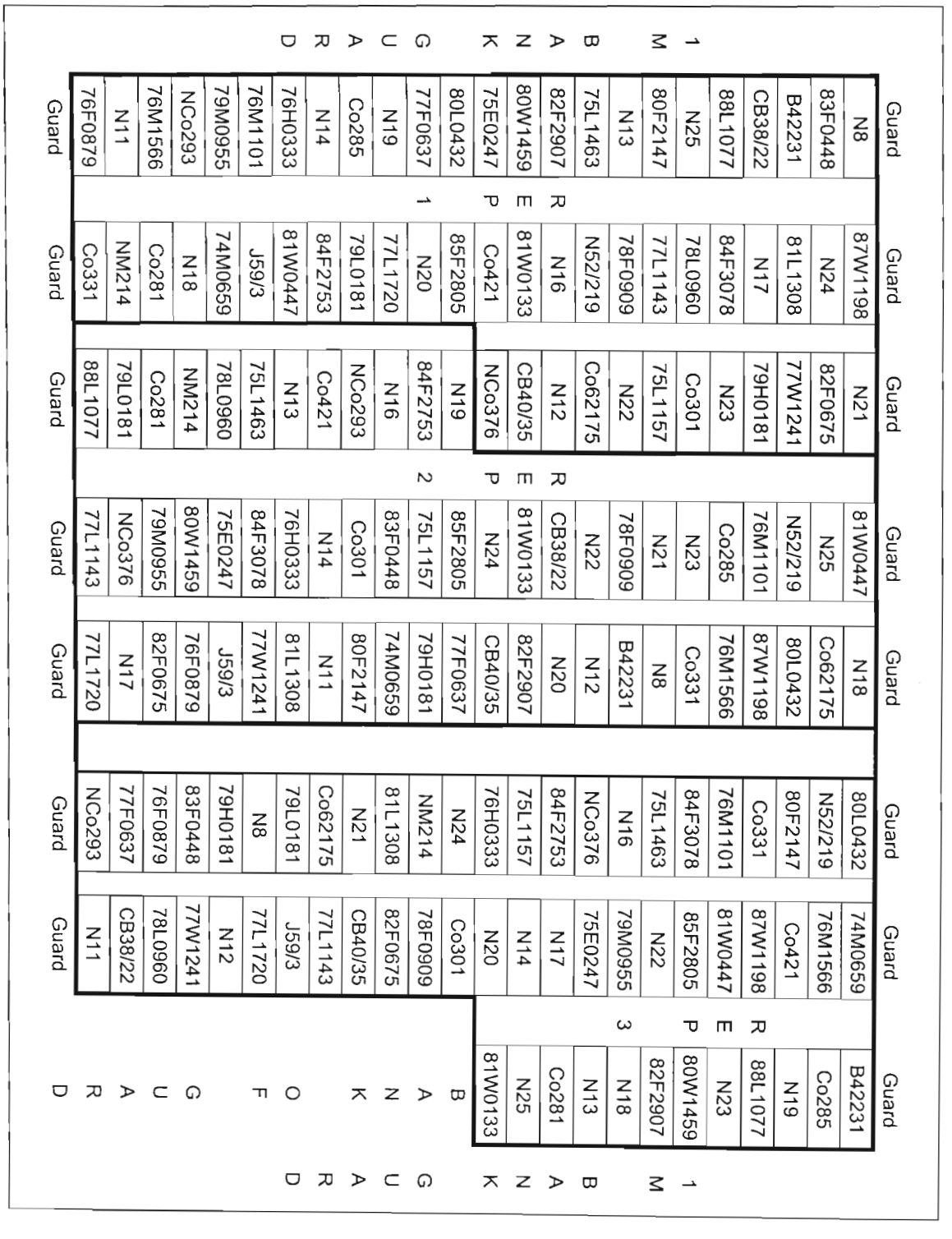
Appendix 2

List of 24 sugarcane crosses and parents selected for heritability study of NIRS-based eldana resistance, the total number of offspring available in each cross and the average bud scale mass of both parents and offspring

Cross	Offspring		Female parent		Male Parent	
	No	Average bud scale mass (g)		Average bud scale mass (g)		Average bud scale mass (g)
AA1213	21	0.34	N6	0.50	75L1463	0.41
BB0307	21	0.21	US82-42	0.24	Co312	0.36
BB0574	21	0.27	81W0133	0.31	83E0266	0.46
BB0890	24	0.26	79M0955	0.16	70E0517	0.29
BB1715	30	0.20	76M1101	0.22	76F0879	0.19
CC0271	21	0.23	NCo376	0.24	Co285	0.22
DD0084	30	0.29	N16	0.31	84F3078	0.55
DD0451	30	0.26	75L1157	0.27	CP70/1133	0.24
DD0926	30	0.24	84F2753	0.33	81E0892	0.13
DD1084	24	0.26	81W0050	0.35	77L1143	0.37
DD1273	30	0.24	N17	0.22	N25	0.33
EE0015	30	0.24	Co281	0.33	US1694	0.21
EE0085	30	0.22	N20	0.27	Kloet	0.12
EE0351	24	0.21	84H0192	0.25	78L0960	0.20
FF0546	24	0.24	N21	0.28	CP57/614	0.42
FF0879	21	0.22	77W0635	0.36	85F3710	0.21
V1175	21	0.25	76M1566	0.24	N18	0.30
V147	30	0.21	NCo310	0.39	IK76-86	0.11
V224	21	0.22	CP63/588	0.23	NiN2	0.15
V506	24	0.26	70E0457	0.32	NCo293	0.30
X55	30	0.23	N8	0.40	NM214	0.21
X84	30	0.32	NCo334	0.25	80E1178	
Y1475	30	0.28	70L1025	0.40	MZC74/275	0.29
Y163	30	0.29	80M0214	0.40	70L1569	0.31

Appendix 3

Field plan for Coastal (Shakaskraal) site calibration and G x E interaction study, consisting of standard sample set of 60 sugarcane cultivars in a randomised block design with three replications

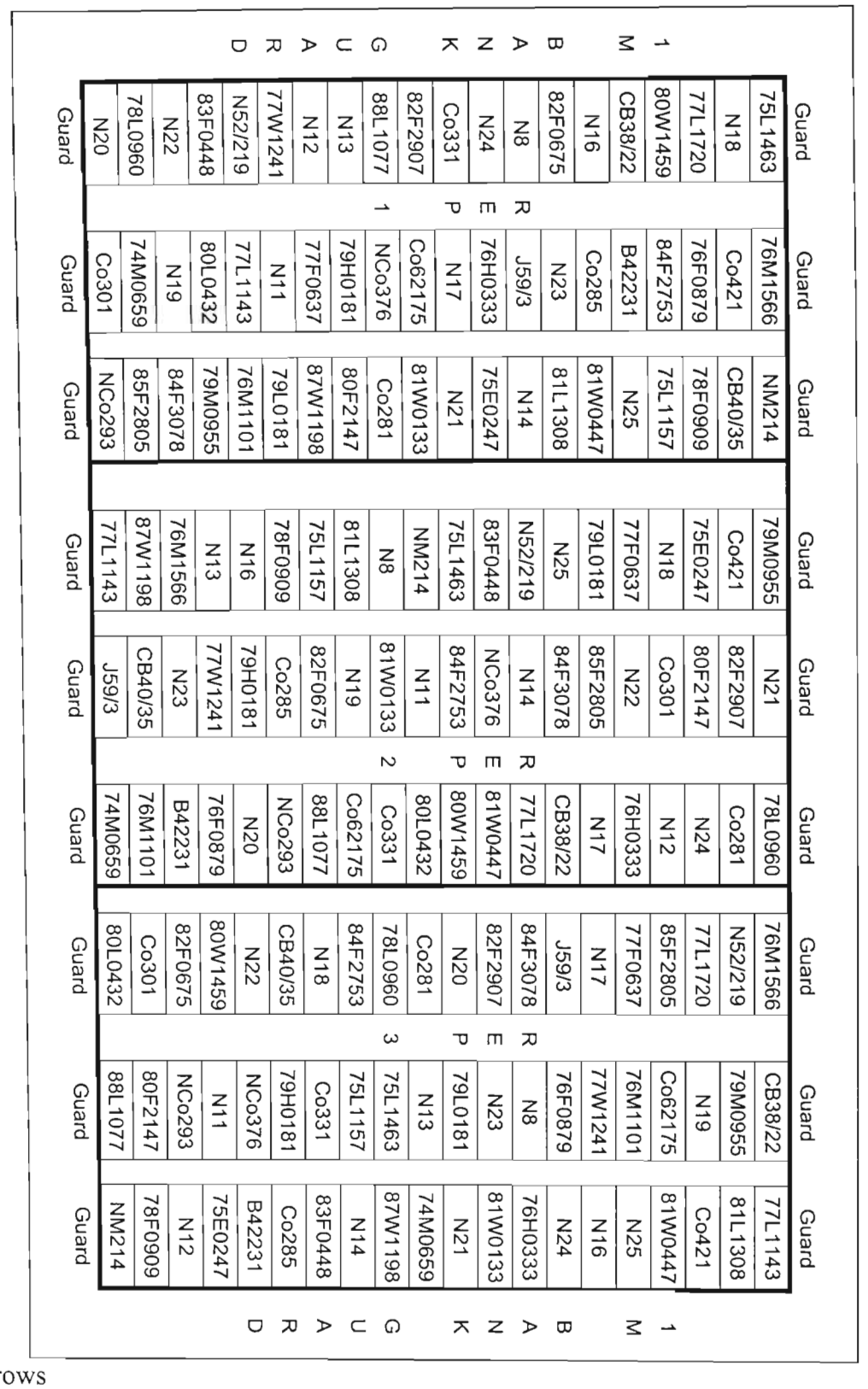


5 m rows

Alternating 2 m and 1m breaks

Appendix 4

Field plan for Midlands (Bryuns Hill) site calibration and G x E interaction study, consisting of standard sample set of 60 sugarcane cultivars in a randomised block design with three replications

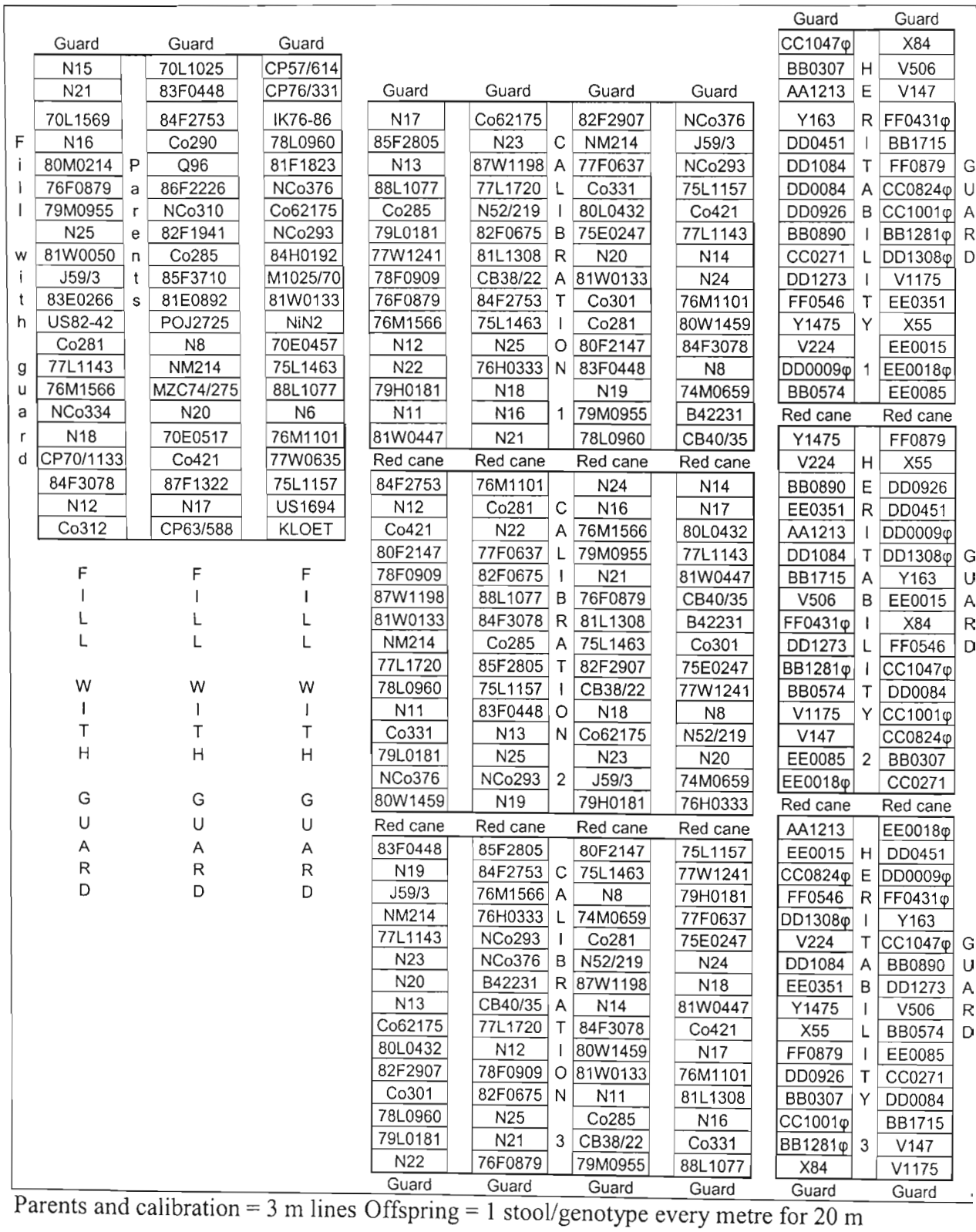


5 m rows

Alternating 2 m and 1m breaks

Appendix 5

Field plan for heritability trial of NIRS-based eldana resistance, planted at Mount Edgecombe in a randomised block design with three replications, consisting of 24 sugarcane crosses and their parents in a separate block, with three randomised replications of the calibration set planted for use as a reference



Parents and calibration = 3 m lines Offspring = 1 stool/genotype every metre for 20 m

φ cross did not survive to harvest, excluded from analysis