EVALUATION OF INOCULATION TECHNIQUES AND SCREENING MARKERS FOR SMUT (Ustilago scitaminea syd) RESISTANCE IN SUGARCANE

By

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ABSTRACT

Sugarcane smut in Kenya cause severe losses in susceptible varieties. Seedling smut screening may select for sugarcane genotypes with physiological resistance, and may therefore be of great use to the industry because genotypes with physiological smut resistance are sought for commercialization.This study was conducted in the green house first to assess the feasibility of inoculating sugarcane (Saccharum spp) seedlings with smut (*Ustilago scitaminea* syd), to evaluate different inoculation methods and secondly to screen DNA markers for smut resistance. Crosses involving two populations emanating from resistant and susceptible varieties was undertaken at Sugarcane Breeding Center-Mtwapa(Mombasa).To investigate the reaction of seedlings to smut, three different inoculation methods were employed. The first method involved soaking seedlings in smut spore suspension at a concentration of 4 x 10^6 spores/ml for 30 minutes. The second method involved wounding the seedlings at the bud with a scalpel then applying a paste of smut made at a concentration of 2 grammes of spore for 2 ml of sterile water. The third method was a paste method that involved a paste of smut at the seedling buds. Each treatment had 30 entries (seedlings) planted in plastic bags in the glass house. Two controls of the un-inoculated progenies were included. The experiment was randomized complete block design replicated three times. There was no significant difference in whip production between the two populations. Population Co 331 X Co 945 had near significant results with wound paste method leading on whip production followed closely by paste method. Inoculation had significant effect on seedlings survival across four months under observation. There was significant difference in tiller production. There was significant difference in whip production between the two families at three months. The study recommends screening for smut resistance at first stage of selection to assess seedlings reaction to smut and to avoid carrying large numbers of clones that are eventually discarded at the advanced stage of selection. To screen molecular markers for smut resistance, tissues of the seedlings were harvested and DNA extracted, quantified and electrophoresis performed. DNA extraction method using lyophilized leaves was better than sap extraction method in terms of DNA quantity and quality. Sugarcane tissue gave a lot of DNA. The primers used in the experiment were obtained from SUCEST database with expected homology of protein like Kinase. Another primer XLRR was

designed from conserved motifs of LRR. NBS and kinase domain. The primers were synthesized by the oligo sequencing unit at the International Livestock Research Institute (ILRI). The concentration of primers used in this study was between 200-300ng. DNA templates of 30ng gave fairly good PCR products with clear bands. Only one hot- start PCR temperature profile with a ramped decrease in annealing temperature was necessary to display amplicons of high quality for the primer pairs tested. The amplicons in the figures presented in this study represent the presence of segment that contains resistance gene analog. Empty wells without any band indicate there was no amplification and therefore the gene analog was absent. The bands were scored as present or absent depending on their intensity and clarity. DNA extracted from plants that exhibited smut whips phenotypically did not produce any band when PCR was done. Every primer pair produced scorable loci in the progeny analyzed. The size of the product from primer SCB 07 and SCC09 was around 800bp. However the size of amplification products from primer XLRR was near the expected size 900bp. Primer SCC09 produced very clear bands.

DECLARATION

This thesis is my original work and has not been presented for a degree award in any other university.

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision and has been submitted for examination with our approval.

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DEDICATION

I dedicate this thesis to my late brother Martin, whose death gave me the purpose to live.

LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
<	Less than
>	Greater than
ANOVA	Analysis of variance
AFLP	Amplified fragment length polymorphism
AP- PCR	Arbitrary primed PCR
BIOSYS	A computer programme for analysis of allelic variation in population genetics and biochemical systematics
CAPS	Cleaved amplified polymorphic sequences
CTAB	Mixed alkyl-trimethyl-ammonium bromide
C. V	Coefficient of variation
DAF	DNA amplification fingerprinting
dATP	deoxyadenosine 5' – triphosphate
dCTP	deoxycytidine 5' – triphosphate
ddH_2O	double-distilled water
dNTPs	deoxynuleoside 5' –triphosphate
DNA	Deoxyribonucleic acid
F – STAT	Computer Programme to analyze F- statistics
GDA	Genetic Data Analysis: Computer programme to analyze discrete genetic data from P.O Lewis and D. Zaytn 1996
GENEPOP	Population genetics software from M. Raymond and F. Ronsset 1995
KARI	Kenya Agricultural Research Institute
KESREF	Kenya Sugar Research Foundation
LSD	Least significant difference
LRR	Leucin –rich repeat
MAS	Marker assisted selection
NBS	Nucleotide-binding sites
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistance gene analog
SAS	General software package for statistical analysis
SASRI	South Africa Sugar Research Institute
SSR	Simple sequence repeat
STS	Sequence –tagged site
UPGMA	Unweighted pair group method with arithmetic averages
WINAMOVA	Program for analysis of molecular data by L. Excoffier
g	gram
kb	kilo bases
min	minute
ng	nanogram = 10^{-9} gram
RNA	ribonucleic acid
RT	room temperature
sec	second
SGB	sample gel buffer
TAE	Tris –acetate EDTA (buffer)
U	unit of enzyme
UV	ultra violet
V	volts
^{0}C	degree Celsius
ug	microgram = 10^{-6} gram
ul	micro litre = 10^{-6} litre
cM	Centimorgan

CHAPTER ONE

1.0 INTRODUCTION

In Africa Sugarcane smut was first noted in South Africa in 1877. In Kenya, the disease was reported in 1958, although it was suspected to have been present since 1956. The fungus *Ustilago scitaminea* causes smut. A description of the disease has been given by Antoine (1961). Smut is transmitted in two ways; one way is by wind borne spores gaining entry into the standing cane through the bud; and the second way is by the spores in the soil, or in irrigation water, entering planted setts.

Many methods of control have been suggested; one of which is hot –water treatment, the use of fungicides and rouging diseased cane has been applied as control measures under Zimbabwe conditions (James, 1974) MC Martin (1948) found that canes derived from crosses between S. officinarum and S. spontaneum were most resistant than those from S. officinarum and S. barberi. Some of the characteristics, which might cause such differences, were examined by Muthusamy (1974) at Tamilnadu, India. The methods used to determine the resistance of varieties to smut was described by Leu and Teng (1974).

Sugarcane resistance to *U. scitaminea* appears to be the result of several characteristics and is probably determined by a number of genes (Hector et al., 1995; Lloyd and Naidoo, 1983). In addition, commercial sugarcane varieties are polyploidy hybrids of several saccharum species. Genetic resistance in these hybrids does not follow the strict gene for gene pattern as seen in some fungal pathogen-host interaction. However differences in variety susceptibility to different smut isolates have been reported (Com stock & Heinz, 1977; Gillaspie et al. 1983; Grisham, 2001*). Ustilago scitaminea* as well as other *Ustilago* species readily hybridize between races and even between species (Bakkeren & Kronstad. 1996). Determination of the identity of sugarcane smut races and maintenance of resistance commercial varieties has thus proven to be difficult. Resistance to sugarcane smut is thought to be determined by combinations of preformed bud structural characteristics (Waller, 1970) and pre-formed bud phenyl-propanoids and glycosy

flavonoids (Lloyd and Naidoo, 1983) Dean, (1982) proposed that resistance is occasioned by physiological barriers, which are partially reduced by wounding the bud prior to inoculation.

1.1 Justification

Smut disease of sugarcane, caused by the fungus *Ustilago scitaminea* Sydow, can cause considerable yield losses and reduction in cane quality (Ferraira and Comstock, 1989). Many observations of the disease were made in Africa and Asia (reviewed by Antoine, 1961; Persley, 1978). Smut remained confined to the Eastern hemisphere until it was found in Argentina in 1940. It has since been recorded in most sugarcane growing countries of the world (Persley, 1978). In July 1998, sugarcane smut was observed in Australia for the first time in the Ord river irrigation area of Western Australia. The most likely source of this infection was thought to be wind blown spores from Indonesia (Riley et al., 1999.In Tanzania smut was first observed at Arusha Chini near Moshi in 1960 on plant material from Morogoro (Anon. 1962). Smut is considered to be the most important pathogen of sugarcane in Tanzania. Twenty three years ago it was estimated that the Tanzania sugar industry was losing approximately US$ 1.23 million per annum to this disease (Keswani and Msechu, 1981). A survey of major sugar-cane growing estates, namely Kilombero Sugar Company, Mtibwa sugar Estate, Tanganyika planting company and Kagera sugar Estate, showed that the incident of this disease had increased (Msechu, 1979). Sugarcane smut is known to reduce the quality and yield of sugarcane (James, 1973; Bachchhau et al. 1979; and Makerjee et al, 1979). The disease has been reported to be one of the most important diseases of sugarcane in Sudan (Ibrahim & Ahmed, 1974), in Zimbabwe (James, 1973) and in India (Mohan Rao & Prakasam, 1956). A past report indicated that the sugar industry in South Africa incurs a loss of 1.4 million Rands per annum from this disease (Anon. 1979). Antoine (1961) in reviewing the losses caused by this disease has referred to the tendency to report disease incidence rather than yield loss.

Research done in Kenya showed that sugarcane smut occurred in all the areas surveyed in cane growing zones (Osoro et al, 1980). The popular commercial variety Co. 421 was graded susceptible. Trials have been conducted to evaluate the effectiveness of fungicide to control sugarcane smut (Kariaga, 1980). Studies have also been conducted on the

relationship between hot water treatment and the sugarcane smut pathogen infection. (Osoro, 1984) suggested that smut pathogen infection is more enhanced in the heat-treated and inoculated plant tissue. Hot water treatment appeared to reduce the plant tissue resistance mechanism against smut. A yield difference between inoculated and non-inoculated varieties to smut was 20.3 tones (Ongoma, 1984). Smut inoculation also reduced tillering capacity in all varieties except for variety Co. 331 (Ongoma 1988). Smut screening trial of KEN 82 series varieties and commercial varieties showed that 78% of the varieties were susceptible (Ongoma, 1988). Screening KEN 82 and 89 series of varieties against smut showed that KEN 82-62 was resistant to smut. Seventeen varieties were intermediate resistant. Some varieties were rated susceptible to highly susceptible (Osoro et al, 1995).

Sugarcane smut in Kenya cause severe losses in susceptible varieties. Other economic losses occur in the expenses incurred during rouging. Preliminary plant cane results indicated yield loss of 38% on susceptible varieties (Nzioki and Jamoza, 2007 unpublished). Muhoroni sugarcane farms, about Ksh. 300 per hectare are spent in rouging smut whips (Osoro et al., 2000 unpublished). Other expenses incurred include cost of fungicide used in seed treatment. The Kenya Sugar Research Foundation (KESREF) has produced some varieties with field resistance to smut. Never the less it has taken many years to develop resistant varieties. It takes up to fourteen years to develop a variety from the initial stage of seedling to a selected commercial variety. In these fourteen years program resistant varieties are selected only at a very late stage.

Breeding for resistance is the best alternative to overcoming smut in Kenya. However the process takes 14 years from germplasm characterization to producing a commercial variety. This process is further compounded by the inability to phenotypically select for smut resistance in the field. Therefore it is necessary to identify resistant plants in early generations.

One way of aiding early generation selection is through molecular markers closely linked to smut resistance. Once markers have been identified breeders would then embark on molecular assisted selection for smut resistance.

In this study the objectives were:

I) To evaluate different inoculation methods for smut in sugarcane seedlings

11) To screen markers associated with smut resistance for use in marker assisted selection

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biology, origin, distribution and genetics of sugarcane

Sugarcane is a tall monocotyledonous crop that is cultivated in the tropical and subtropical regions of the world primarily for its ability to store high concentrations of sucrose, or sugar, in the internodes of the stem. Modern sugarcane varieties that are cultivated for sugar production are complex interspecific hybrids (*Saccharum spp.*) that have risen through intensive selective breeding of species within *Saccharum* genus primarily involving crosses between species *Saccharum officinarum* L. and *S. spontaneum* L. (Cox et al. 2000)

S. officinarum or the 'noble canes' accumulate very high levels of sucrose in the stem and generally have poor disease resistance. *S. officinarum* is presumed to be the products of complex introgressions among *S. spontaneum, Erianthius arundinaceus* and *Miscanthus sinensis* (Daniels and Roach 1987). The center of origin of *S. officinarum* is presumed to be in Indonesia. The species was probably transported throughout south East Asia by humans, leading to a modern center of diversity in Papua New Guinea and Iranian Java (Indonesia) where the majority of the species were collected in the late 1800s (Daniels and Roach 1987).

Suggestion for the origin of *S.officinarum* involve selection of sweet forms of *S. robustum* for use as food, possibly with the aid of animals such as pigs or rats that were attracted to sweeter individual plants (Daniels and Roach 1987).

S. Spontaneum is a much more adaptable species and grows in a wide range of habitats and at various altitudes in the tropics through to temperate regions from latitude 8° S to 40° N extending across the three geographical zones.a) The East zone comprises South Pacific islands, Philippines, Taiwan, Japan, China, Vietnam, Thailand, Malaysia and Burma.b) The central zone, which comprises India, Nepal, Bangladesh, Sri Lanka, Pakistan, Afghanistan, Iran and Middle east whereas the c.) The West zone which

includes, Egypt, Sudan, Kenya, Tanzania and other countries in the Mediterranean (Tai and Miller 2001; Daniels and Roach 1987; Pursglove 1972).

Saccharum is a complex genus characterized by and composed of at least six distinct species – *S. officinarum, S.barberi, S. sinensi, S. spontaneum, S. robustum and S. edule* (Daniels and Roach, 1987; Naidu and Sreenivasan, 1987). Described as allopolyploids, modern cultivated sugarcane has approximately 80-140 chromosomes with 8-18 copies of a basic set (i.e. x = 8 or x = 10 haploid chromosome number) (D'hont et al., 1995; Ha et al., 1999; Ming et al., 2001). Sugarcane *S. officinarum* was vegetatively cultivated as noble canes (i.e. *S. officinarum*) until the end of the 20th century when it succumbed to the devastating sereh disease, which prompted plant breeders to hybridize it with its wild relative, *S.spontaneum*. In a process termed as nobilization, the resulting hybrid progeny was repeatedly backcrossed to *S. officinarum* to restore the high sucrose producing plant types. *S. officinarum*, the noble cane from Asia, is thought to comprise a large part of cultivated sugarcane genome and confers the genes for high sucrose content, low fiber, thick stalks, sparse pubescence, rare flowering and limited tillering (Ming et al., 2001). The wild relative, *S. spontaneum*, comprise about 10% of the cultivated sugarcane as evident from the in situ hybridization (D'hont et al., 1996) and is believed to confer genes for pest and disease resistance and a biotic stress tolerance (Screnivasan et al., 1987).

The nobilization endeavors proved successful in averting the threat of diseases. For the most part of the last century, sugarcane-breeding activity thrived essentially by intercrossing the original nobilized clones and their derived progeny. It is well known that only a few clones were involved in the original nobilization event (Arcenueaux, 1965). There is growing concern among sugarcane breeders regarding the narrowness of genetic diversity in the existing sugarcane germplasm worldwide. Data from various studies that looked at chloroplast (cpDNA) and mitochondrial (mtDNA) DNA in the clones of *Saccharum spp.* and hybrids indicated a narrow genetic base (Al- Janabi et al., 1994; Dereen, 1995; D'hont et al., 1994; Mangelsdorf, 1983). There have been no active bases broadening programs alongside cultivar development programs in most sugarcane breeding stations.

This is compounded further by the proven cross method of choosing parents. The proven cross method has the bias of repeatedly using, in high frequency, parents from good performing crosses. Such concentrated use of a few parental clones each season seems to lessen the genetic base broadening efforts. (Heinz and Tew, 1987; Kimbeng et al., 2004).

2.2 Biology and epidemiology of smut

Owing to its vegetative mode of propagation, sugarcane (Saccharum *spp.*) is prone to infection by systemic pathogens. In the smut life cycle, airborne diploid teliospores are produced from long, unbranched sori (whips) and serve to transmit the fungus between plants (Ferreria and Comstock, 1989). Teliospores germinate on wet plant surfaces to produce promycelia that produce haploid sporidia following meiosis. Of the four sporadia produced, two are of plus mating types and two are of minus mating types (Alexander and Scrinivasan, 1966). After compatible sporadia fuse, a dikaryon is formed and becomes the infectious hypha of the organism (Ferreria and Comstock, 1989).

The most effective method to control sugarcane diseases is the use of resistant cultivars (Schenck, 1998). A traditional method of screening sugarcane cultivars for resistance to smut usually takes 6-18 months and requires a large acreage. The most widely used technique to evaluate for resistance to smut involves immersing sugarcane setts (seed pieces containing 1-3 nodal buds) in a teliospore suspension before planting, and counting the number of sori (whips) that develop (Ferreria et al.1980). Resistance is rated on a scale of 1(highly resistant) to 9 (highly susceptible) (Hutchinson, 1969) based on the percentage sori produced. If sori are not produced within growing season, the plants are grown for a second season and scored again (Lloyd and Pillay, 1980). The 20th century saw the steady spread of sugarcane smut to almost all sugar industry of the world (reviewed by Presley, 1978). A widely stable smut genotype may have been involved in this spread.

Pathogenic races of sugarcane smut have been observed in several countries including two races (A and B) from Hawaii (Comstock and Heinz, 1977) and three races (1,2,3)

reported in Taiwan (Leu and Teng, 1972; Lee et al., 1999). However, Ferreira and Comstock (1989) consider the true prevalence of races to be controversial. Many claims are based on the reaction of the same cultivar in different countries but the interpretation of these claims is confused by test- to- test variation and the use of different inoculation methods in different countries. Two international collaborative efforts have attempted to standardize race typing. Gillaspie et al. (1983) performed race typing under glass house conditions to standardize the environment and six races were identified. Grisham (2001) co-ordinated a race type study in nine countries using local isolates tested against a standardized set of 11 differential cultivars.

U.scitaminea is phenotypically variable with regard to morphology, cultural characteristics and pathogenicity (e.g. Muhammad and Kausar, 1962; Abo and Okusanya, 1996; Alexander, 1981; Perez and Mauri, 1983).

A single breeding cycle for smut resistance may take as long as fifteen years. Considerable time and expense are spent on clones that are found to be susceptible to smut. Therefore detection at early stages of selection would be beneficial (Bailey and Bechet, 1982). Cultivar reaction to smut differs from country to country, and from region to region because of existence of different smut races (James, 1969; Ferreira et al.1980; Perez and Mauri, 1983). Ricaud (1981) recommended that a broad classification system should be used for cultivars tested against a given disease for the first time. Thereafter, the present nine level category rating system (Hutchinson, 1969) may be applied when greater confidence can be given to the results because selected genotypes would have already shown resistance to smut during preliminary selection. In pursuit of a suitable and rapid smut – screening method to eliminate sugarcane genotypes rated as susceptible and highly susceptible, staining techniques (Sinha et al., 1982) and chemical assay (Lloyd and Naidoo, 1983) have been used. Neither efficiency nor speed of evaluation has been improved by either method. Albert and Schenk (1969) introduced a PCR procedure for detecting smut DNA in sugarcane tissue that may be useful in early screening programs. This assay does not directly have any indications of the susceptibility levels of the sugarcane cutivars.

In vitro culture has been used to screen for eye spot disease, Fiji disease and downy mildew in sugarcane, but was unsuccessful for smut (Heinz et al., 1977). Peros and Chagvardieff (1983) Demonstrated that callus initiated from both susceptible and resistant sugarcane plants reacted similarly when challenged with *U. scitaminea* under *in vitro* conditions. It was later shown that fungal metabolites of *U.scitaminea* caused complete necrosis of sugarcane callus tissue during co-culture (Peros and Chagvardieff, 1987). Hence, it appears that varietal resistance is not always expressed in callus tissue, and that in vitro test with whole plants would be more appropriate.

2.3 Smut disease assessment

The early and accurate diagnosis of plant disease is a crucial component of any crop-management system. Plant diseases can be managed most effectively if control measures are introduced at early stages of disease development. Reliance on symptoms often is not adequate since the disease may be well established before symptoms first appear, and symptoms expression can be highly variable. Recent advances in molecular biotechnology are being applied to develop rapid, specific and sensitive tools for the detection and evaluation of many plant diseases.

The genus *Ustilago* (Smut fungi), belonging to the basidiomycetes, includes a group of phytopathogens that cause disease on a variety of plants, including cereal crops. Sugarcane smut was one of the first diseases of sugarcane to be recognized in 1877 because of the conspicuous whip-like sorus produced by fungus in infected plants. Sori arise either from the terminal meristem or from side shoots of infected stalks (culms) and contain a central core of parenchyma and vascular host tissue around which a thin cylinder of teliospores is produced. The life cycles of smut fungi are similar for all species and involve transitions between three cell types. Diploid teliospores are the resting cell type and are disseminated mainly by wind and rain splashes. They germinate by forming a probasidium on which, following meiosis, four sporidia emerge. The haploid sporidia represent the second cell type. They grow by budding, and compatible (opposite-mating types/plus and minus) sporidia fuse to give rise to the dikaryotic pathogenic third cell type, which exhibits mycelial growth (Alexopoulos, 1962).

Karyogamy takes place in the dikayotic mycelium and diploid teliospores are formed within the host tissues. (Bakkeren and Kronstad, 1993).The life cycle is regulated by the a and b mating-type loci within the sporidia. a has two alleles which encode a pheromone and a receptor whilst b is multiallelic and appears to control pathogenicity and sexual development (Bakkeren et al., 1992). With the use of the primers based on U. maydis bE mating type, gene, Albert and Schenck (1996) sequenced the corresponding gene in *U.scitaminea*. Molecular detection of the smut pathogen in sugarcane has since become possible by using PCR to amplify the bE mating –type gene of *U.scitaminea*.Histopathology on the stalk and meristem colonization may be used to detect *U.scitaminea* within infected sugarcane (Alexander et al., 1980). Mycological establishment and colonization during the prolonged period between the penetration and soral (whip) formation phases are vague. Information does not extend to the differences in colonization of resistant and susceptible cultivars. It is reported that susceptible sugarcane cultivars produce great number of sori, which develop early than in resistant cultivars (Waller, 1970). Lloyd and Pillay (Lloyd et al., 1981) found that the rate of colonization of *U.scitaminea* in sugarcane tissues correlated with the ratings for resistance. An ultramicroscopic study of resistance and susceptible buds also revealed that buds of resistant sugarcane cultivars were not subjected to intracellular penetration by *U.scitaminea* (Solas et al., 1999). This indicates that the rates and patterns of colonization differ in susceptible and resistant varieties.

Any approach of evaluating sugarcane for smut resistance will need to be based on better and more complete knowledge about the etiology of smut and the nature of resistance. The tissue culture smut-evaluation assays (Singh, 2003), conducted in a controlled physical and chemical environment without the interaction of other microorganisms, and therefore facilitates the study of host- parasite relationships.

Varietal reaction to smut can be evaluated using several inoculation techniques. The most widely used is the dip inoculation method (Byther & Steiner 1974). Another is the wound - paste technique (Leu et al., 1976) and the brushing technique (Luthra et al., 1938).

2.4 Testing sugarcane for reaction to smut

Smut resistance in sugarcane is influenced by nodal bud morphology (Waller, 1970), chemical inhibitors present in bud scales (Lloyd & Pillay 1980), and host physiology (Dean1982; Waller 1970). To assess smut reaction, researchers typically use a dip inoculation assay in which nodal buds are immersed briefly in a suspension of teliospores, planted in a green house, and evaluated in a green house or field (Alexander et al., 1991; Chao et al., 1990; Comstock et al., 1983; Grishan&Breaux 1988, Wu et al., 1983). Chen and Lo (1989) used a pinkprick method to inject teliospores into buds of a clone of *Misanthus Anderss*, which generally produces insignificant stalks with no root primodia at stalk nodes (Daniels& Roach 1987). Teliospore injection circumvents the protection afforded by intact bud scales and provides an estimate of the physiological resistance to fungal development in the plant. Injection inoculation may induce greater smut infection than dip inoculation, and cultivars can respond differently to the two methods of inoculation (Dean 1982; Waller 1970).

Since varietal reaction to smut can be influenced by smut race or isolate, the environment, the experimental design and procedure used, and interactions among these factors (Chao et al., 1990; Comstock et al., 1983), empirical evaluation of smut resistance under local conditions appears to be necessary. *S. Spontaneum* and *S.sinensi* tend to have higher frequency of resistant (Sreenivasan & Alexander 1971) plus moderately resistant (Alexander et al., 1991) clones than do *S.baberi* Jesw, *S.officinarum*, and *S. robustum*. However Chona (1957) considered *S. officinarum* to be immune or highly resistant compared to *S. spontaneum and S.baberi*. Results of this study were based on dip inoculation.

The protocol presently used to screen for resistance to covered kernel smut in sorghum is inconsistent and escapes are common (Claflin and Ramundo, 1996). Escapes can be reduced by improving existing screening methods or by devising new inoculation methods. In sugarcane little has been done on screening seedlings with smut and evaluating the inoculation methods.

2.5 DNA-Based Molecular Markers

DNA markers techniques have been widely applied in the improvement of plant traits. There are many journal articles and numerous reviews, books and technical bulletins that have been written in various aspects of DNA markers system and their various applications to plant genetics and crop improvement. For useful texts and figures, which describe basic principles and procedures of molecular markers, Paterson et al (1991) have documented comparison of techniques and their numerous applications (Stuber (1993); Lee (1995); Mohan et al., (1997); Powell et al., (1997); Weising et al., (1998), Karp et al., (1997), Joshi et al., (1999) and Koebner et al., (2001).

Ever since their development, DNA marker systems are being modified continuously to enhance their utility, discriminating power and ease of use. Since the development of the first DNA marker technique, restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), subsequent exponential increase in DNA marker system has resulted in virtually unlimited source of genetic polymorphisms for plant genome analysis and crop improvement.

2.5.1 Microsatellites, also called 'simple sequence repeats' (SSRs)

SSRs marker system is a second –generation PCR-based marker technique based primarily on short tandem sequence motifs, such as di- e.g. (CA) n, tri- and tetra-nucleotides that occur everywhere in the eukaryotic genome. This marker system exploits the extensive level of polymorphism offered by the highly repetitive DNA fraction. Microsatellite marker is analyzed by PCR using a pair of specific primers designed from sequence data flanking a specific motif. Polymorphism is detected because of differences in the number of tandem repeats in a specific repeat motif. A multitude of techniques that exploit microsatellite variability as molecular markers were described in the reviews of Weising et al., (1998) and Joshi et al., (1999)

2.5.2 Resistance gene analog (RGA) marker system

RGA is a genome region targeted molecular marker system generated from degenerate PCR primers based on conserved regions of cloned plant resistance genes (Kanazin et al., 1996; Yu et al., 1996; Shen et al., 1998).This type of molecular marker system is

appropriate for genetic mapping studies that attempt to identify plant resistance genes. However not all amplified products may correspond to a functional disease resistance gene. RGA primers have been shown to amplify the conserved sequences of leucin-rich repeats (LRR), kinase and/ or nucleotide-binding sites (NBS), thereby targeting genes for disease resistance or other important signal-transduction processes in plants (Bent, 1996). The mapping of RGAs on linkage maps has also been used as a candidate-gene approach to identify genes for resistance to various pathogens (Kanazin et al., 1996; Leister et al., 1996; Yu et al., 1996; Feuillet et al., 1997). RGA screening has also been successfully applied in various quantitative trait loci (QTL) analyses. For example, Byrne et al., (1996) was able to link the candidate genes involved in the flavone synthesis pathway of maize with the host defense response phenotype associated with a QTL for resistance to corn earworm.

The establishment of a non-radioactive silver staining protocol for polyacrylamide gel electrophoresis(PAGE) under local laboratory condition in Philippines (Hautea et al., 2001) has paved the way for successful adoption and maximized utilization of more powerful DNA marker systems in genome mapping and genetic diversity studies of Philippines crops. These included foremost, RGA fragment analysis, of which the candidate RGA fragment had to be resolved on PAGE (Chen et al., 1998) and detected for silver staining for high resolution of DNA polymorphism.

For ultimate tagging of candidate –resistance gene sequence, RGA markers have been successfully used in QTL mapping studies in corn (Ruswandi, 2001) and tomato (Balatero, 2000), for downy mildew and bacterial wilt resistances, respectively. In corn, four RGA markers were linked to QTLs for resistance evaluated using various disease reaction parameters. In the study rga-1 marker was associated with the major QTL – resistance 'block' in chromosome 8.

2.5.3 Marker assisted selection

Marker-assisted selection (MAS) offer promise for i) indirect selection of desirable traits free of environmental, pleiotrophic or epistatic effects ii) the ability to discriminate between homo and heterozygote, iii) pyramiding of genes, iv) monitoring and

retrogression and v) identification of recombinants possessing least amount of linkage drag donor DNA flanking the gene of interest. However, reports on actual application of marker assisted selection (MAS) are still limited. Among them, successful application includes identification of homozygous and heterozygous resistant plants against barley mild mosaic virus (BaMMV) (Tuvesson et al., 1998).

Marker- assisted breeding has been used to develop a super broccoli variety that contains 100 times the concentration of cancer fighting sulphoraphanes as a normal broccoli do. This was achieved through a number of biochemical and DNA fingerprinting techniques to identify Brassica species that could be effectively used in breeding programs with cultivated broccoli. The species belonging to Sicilian *B.villosa-rupestris* increased sulphoraphane levels without significantly increasing the levels of more volatile unpalatable compounds (Fulkner et al., 1998). The development of supper broccoli took only 4 years to complete with marker-assisted breeding compared 10-15 years that may take through conventional breeding. This variety is already being tested for human consumption.

Most of the agricultural important characters are quantitative, strongly influenced by the environment and expensive to evaluate directly. Quantitatively inherited traits have strong genetic components but, under normal conditions of measurements, cannot be measured by individually recognizable loci. Such quantitative traits can best be selected phenotypically provided they have high heritability (Falconer and Mackay, 1996). However, with the help of DNA markers, such complex traits have been resolved into discrete QTL, which can then be modified through marker-assisted selection. If marker assisted selection for QTL is to be applied in the long-term breeding programs, it would be imperative to understand that MAS combined with phenotypic selection will be superior initially but will become inferior when QTL approaches fixation (Hospital et al., 1997).

In breeding for disease and pest resistance, in general, the segregating populations are derived from crosses between the resistance and susceptible sources. Genotypes are selected either at natural disease or pest 'hot spots' or under artificially created disease

and pest nurseries or by infecting individual plants under controlled environments. Although these procedures have given excellent results, they are time consuming and expensive. Besides, there are always susceptible plants that escape attack. Furthermore, the pests or the pathogens have to be maintained either on the host or alternate hosts if they are obligate parasites. Screening of plants with several pathogens and their pathotypes or pests and their biotypes simultaneously or even sequentially is difficult if not impossible. Availability of tightly linked genetic makers for resistance genes will help in identifying plants carrying these genes without subjecting them to pathogen or pest attack in early generations. The breeder need little amount of DNA from each of the individual plants to be tested without destroying the plant. Using a known set of primers for PCR, the products of reaction are run on agarose gels and the genotypes of the individual plant for resistance or susceptibility could be directly ascertained by presence or absence of the marker band on the gel. Only the materials in the advanced generations would be required to be tested in disease and insect nurseries. Thus, with MAS, it is now possible for the breeder to conduct many rounds of selection in a year without depending on the natural occurrence of the pest or pathogens as well. However, the presence of different races or biotypes complicates the development and application of molecular marker assisted selection. Markers developed for one pathotype or biotype may not have application to other locations in which different pathotypes or biotypes occur unless the same gene controls resistance.

Marker assisted selection (MAS) for resistance genes (R) can be useful in all these approaches. Based on host – pathogen or host – insect interaction alone it is often not possible to discriminate the presence of additional (R) gene (s). With MAS, new R gene segregation can be followed in the presence of the existing R gene(s) and hence R genes from diverse sources can be incorporated in a single genotype for durable resistance. Pyramiding of bacterial blight resistance genes Xa1, Xa3, Xa4, Xa5 and Xa10 in different combinations using molecular markers has been reported (Yoshimura et al., 1996).

Timmerman et al., (1994) allele specific associate primers (ASAPs) for the gene, er-1, which confers resistance to powdery mildew in pea. One of the major advantages of developing allele specific PCR is its potential for MAS. Sequence characterized amplified regions (SCARs) were developed for downy mildew resistance in lettuce and

nematode resistance gene in tomato. Nair et al., (1995&1996) converted the RAPD markers to SCARs for greater reliability as selection tool. Alternatively, two allele specific associate primers(ASAP) markers, one for each allele could be developed and two amplifications performed to identify both alleles or they can be multiplexed in a single PCR reaction as successfully shown by Nair et al. (1995) to discriminate between the susceptible and resistant biotype of gall midge in rice.

2.5.4 Molecular markers in sugarcane

Molecular genetic markers are valuable in the studies of complex genomes such as that of sugarcane. (Daugrois et al., 1996). Their incorporation in the selection of economic traits during the early stages of a breeding program, as well as in the choice of the best parents in a cross, may significantly reduce the time of development of new varieties. These goals can be successfully achieved with the availability of robust polymorphic markers that co segregate with economically important traits in sugarcane.

Mirosatellite or simple sequence repeats (SSR) have been considered one of the most powerful markers (Jarne and Largoda 1996). In sugarcane, a total of 250 SSRs have been obtained from the analysis of 8679 EST sequences. Despite the low level of polymorphism detected in the sugarcane cultivars evaluated, they were highly transferable to related species or genera (Cordeiro et al. 2001).

The potential to use genetically correlated measures to improve breeding was first recognized by Sax (1923). The marker studies have been underway in sugarcane for more than 15 years (Glaszmann et al. 1989). The polyploid genome means that multiple copies of each chromosome (or locus) are present. Although it is unclear what the copy number will be and it could vary between homeologous groups (Butterfield et al., 2001). Sugarcane genome is large (~ 112 chromosomes and > 17000 Cm, Hoarau et al., 2001). The large size of genome and number of chromosomes present means that large numbers of markers (~ 5000) will be required to give fairly dense map coverage.

Polymorphism assays based on variations in the numbers of short tandemly repeated DNA sequences (microsatellite) have recently been successfully applied to plant breeding

programs (Gupta et al. 1996). Once developed, these markers are easy to apply, although the methodology for their development is complex and costly, which limits their application to important crops, such as sugarcane (*Saccharum_officinarum*) (Scott et al. 2000). Sugarcane genotypes (Cordeiro et al., 1999) make microsatellite the preferred method for use in the construction of reliable frame work genetic map of sugarcane.

2.5.5 Characterizations of microsatellite markers from sugarcane

The principal reason for the increasing success of simple sequence length polymorphisms (SSLPs) as molecular tools is because they provide a higher incidence of detectable polymorphism than other techniques such as RAPDs and RFLPs, (Powell et al., 1996). In addition, studies involving comparisons between different marker systems such s RAPDs, AFLPs, RFLPs and SSLPs shows them to be highly reproducible technique between and within laboratories. The ability of SSLPs to reveal high allelic diversity is useful particularly in distinguishing between genotypes and the success of using these markers in crop species like barley (Sangai Maroof et al., 1994), rice (Wu et al., 1993) and wheat (Roder et al., 1995) has encouraged the testing of SSLPs in sugarcane.

Microsatellites repeats exceeds the capacity of RFLPs (Smith et al., 1997) where characterization of an identification of germplasm for the purposes of research, product development, conservation, measuring and monitoring of genetic diversity in agriculture for support of intellectual property right is concerned.

Much of the earlier characterization of microsatellites has relied on database searches of published sequences or on the construction of genomic libraries. The new development of microsatellite enrichment techniques (Edwards et al., 1996) has however increased the efficiency of microsatellite characterization in species in which little or no previous sequence information is available. The assessment of genetic diversity in sugarcane germplasm is currently based on pedigree records and phenotypic traits. Screening and evaluating the available genetic diversity in sugarcane with microsatellite repeats could both optimize and facilitate the breeding process.

2.6 Genetics of disease resistance

The candidate gene approach has been particularly productive for the investigation of pest and disease resistance because many genes involved in this resistance pathway have been characterized. Candidate genes involved in defense responses can be broadly classified as those involved in the initial recognition of pest or pathogen, the resistance (R) gene and those involved in defense response (DR) triggered by the recognition event (Dixon and Harrison 1990). Candidate gene for R and DR are also potential candidates for insect's resistance genes. The nucleotide binding site leucine rich repeat (NBS-LRR) class of genes has been demonstrated to include resistance to the full taxonomic range of plant pest, including viruses, bacteria, fungi, oomycetes, and nematodes (Hulbert et al. 2001).

The resistance genes identified thus far share common sequence motifs, such as LRRs, NBSs, and kinase domains, reflecting related functions in their roles in pathogen recognition. (Anderson et al.1994; Bent et al.1994; Hulbert et al. 2001). These motifs have been widely used to design degenerate oligonucleotide primers to isolate R gene analogs (RGAs) by polymerase chain reaction (PCR) amplification (Garcia-Mass et al.2001; Kanazin et al. 1996; Mago et al.1999; Yu et al.1996). As a result, a sizeable collection of PCR –derived NBS-LRR sequences from diverse plant species is now available. Many of these sequences have been located to chromosome regions containing major R genes as well as quantitative trait loci (QTL). Collins and associates (1998) demonstrated co- localization of NBS-LRR sequences to rust resistance gene loci (Rp1 and Rp3) in maize.

Although the NBS-LRR sequences govern recognition in plant- pathogen interaction, the DR genes generally are considered downstream from the recognition step of the signal transduction pathway. Thus, the DR genes include a variety of genes that are recognized based on their increased expression during the defense response and whose products are thought to enhance defense in a quantitative manner (McMullen and Simcox 1995; Wang et al. 1994; Young 1996). The proteins encoded by DR genes include structural proteins that are incorporated into the extra cellular matrix and participate in the confinement of

the pathogen; enzymes of secondary metabolism, such as members of the phenylpropanoid pathway important in the synthesis of isoflavonoid and stilben phytoalexins and lignin; enzymes that are implicated to be differently involved in the defense response, including chitinases, peroxidases, catalases, glucanases, sulfotransferases and proteins that inactivate fungal ribosomes or bind chitin ; and regulatory genes controlling the expression of multiple downstream DR genes (Dixon and Harrison 1990).

Resistance (R) genes in plants play a crucial role in preventing disease. Many R genes are dominant, or incompletely dominant, and require specific dominant avirulence (Avr) genes in the pathogen for their function (Flor, 1964). This genetic interaction between plants and pathogen led to the current view that such R genes encode receptors for Avr gene –dependent pathogen molecules (reviewed by Staskawicz et al., 1995). Upon recognition of these molecules, R gene products activate plant defense mechanisms. These defenses include rapid production of an oxidative burst resulting in cell wall cross-linking, localized cell death (the hypersensitive response). Salicylic acid biosynthesis, and induction of genes characteristic of systemic acquired resistance (Levine et al., 1994; Ward et al., 1991; Lamb, 1994).

R genes fall into several distinct classes. The tobacco N gene, the Arabidopsis RPS2 and RPM1 genes, and the flax L6 gene confer resistance, respectively, to viral, bacterial and fungal pathogens, but they all encode proteins that are probably cytoplasmic, contain multiple leucin –rich repeats (LRRs), and nucleotide binding site (Whitham et al., 1994; Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995; Lawrence et al., 1995).
The tomato Cf-9 gene confers resistance to isolates of *cladosporium fulvum*, which express the corresponding Avr9 gene. This has been the sole member of a second class, predicted to be predominantly extracytoplasmic, with c- terminal membrane anchor and no nucleotide- binding site (Jones et al, 1994). The extracytoplasmic domain consists primarily of LRRs, and the predicted cytoplasmic domain of Cf-9 is only 25 amino acids. The tomato Pto gene, conferring resistance to a bacterial pathogen, is a member of a third class and encodes a serine/threonine protein kinase, but lacks LRRs (Martin et al., 1993).

The rice Xa21 gene, also conferring resistance to bacterial pathogen, caries features of both the latter classes, encoding a transmembrane protein kinase with 23 extracelular LRRs (Song et al., 1995).

2.6.1 Resistance in hosts and avirulence in pathogens

Plant pathogen interactions, particularly those involving biotrophic parasites, are governed by specific interactions between pathogen avr (avirulence) gene loci and alleles of the corresponding plant disease resistance(R) locus. When corresponding R and avr genes are present in both host and pathogen, the result is disease resistance. If either is inactive or absent, disease results. (Flor, 1971). The simplest model that accounts for this genetic interaction requires that R products recognize avr- dependent signal and trigger the chain of signal – transduction events that culminate in activation of defense mechanisms and arrest of pathogen growth. R genes specify a polymorphic component of a particular recognition event. Specific R- mediated innate immunity is superimposed onto one or more basal defense pathways. Basal defenses inhibit pathogen spread after successful infection and onset of disease. Genetic overlap between specific and basal resistance responses suggests that one function of R-mediated signaling is to more rapidly and effectively activate defense mechanisms that are shared by both pathways (Glazebrook et al 1997; Dowell & Dangl 2000).

A significant effort by several laboratories in the past 5-10 years has resulted in the identification of many R genes from model and crop species (Bent 1996; Dodds & Pryor 2000). Functional R genes isolated so far encode resistance to bacterial, viral, fungal, oomycete and even nematode and insect pathogens with very different lifestyles, outside or inside the plant cell. Despite this wide range of pathogen taxa and their presumed pathogenicity effector molecules, R genes encode only five classes of proteins.

The largest class of R genes encodes 'nucleotide-binding site plus leucin-rich repeat' (NB-LRR) class of proteins. These function, so far, exclusively as R genes and they are highly evolved for that function. Although computer analyses do not predict localization, at least one NB-LRR is associated with plasma membrane (Boyes and Dangl 1998). Their most striking structural feature is a variable number of carboxyl-terminal LRRs. LRR

domains are found in diverse proteins and functions as sites of protein –protein interaction, peptide-ligand binding and protein- carbohydrate interaction. (Jones & Jones 1996; Kajava 1998). In addition, each R proteins contain a conserved nucleotide –binding (NB) site, which in other proteins is critical for ATP or GTP binding (Saraste et al., 1990) Comparative sequence analyses demonstrated that R specificity resides largely in the LRRs, which are under diversifying selection to increase amino acid variability thought to be solvent exposed (Parniske et al., 1997; McDowell et al., 1998; Botella et al., 1998; Meyers, B.C. et al., 1998). Construction of domains chimaeras has supported these finding for both NB-LRR and extra cellular LRR classes of proteins (Wang et al., 1998; Ellis et al., 1999; Wulff et al., 2001; Van der Hoorn et al.2001). Recent evidence indicate that in the L class of flax rust resistance genes, diversifying selection also acts on residues TIR domain, and that these residues are apparently co-evolving with the corresponding LRR domain to provide specificity. (Luck et al., 2000).

CHAPTER THREE

3.0 MATERIALS AND METHODS

Agro-ecological zone of the study site

The research was conducted at Sugar cane breeding centre within Coast Agricultural Research Station- Mtwapa. The station is located 15km North of Mombasa; Mombasa District; Coast province; center coordinates 03^0 56'S-390 44'E; The altitude is 21km. Dominant soil is well drained, very deep dark yellowish brown, friable sandy loam to sandy clay loam. Annual average rainfall in mm of 1.050 – 1.250mm (MoA – NAL, 1987). It is also referred to as coastal lowland zone (CL) and semi humid (3) (Boxem *et al.*, 1987).

3.1 Developing the progeny for inoculation

Co 421 is a variety native from India. It is widely adapted to various agro- ecological zones in Kenyan sugar industry and yields 80-110t/ha (tonnes of cane per hectare). However the variety has succumbed to smut infection. Co 331 is native to India and is also susceptible to smut. Variety EAK 70-97 was bred during the East Africa Community and selected in Kenya. The variety is smut resistant. Co 945 is native in India. The variety is widely grown in western Kenya for its high sucrose content and resistance to smut.

Two parents involving resistant and susceptible varieties were crossed at Sugarcane Breeding center – Mtwapa. Population one was a cross between Co 421(susceptible) and EAK *70 – 97*(resistant). Population two was a cross between Co 331 (susceptible) and Co 945 (resistant). The seeds were harvested after 21 days following cross pollination, dried under sunny conditions and threshed to recover seed. 2 grams of the seeds were sown in a media containing sandy soil and coconut coir mixed in a ratio of 1: 1 and planted in trays in green house. The seeds germinated after four days and were maintained to ensure fast growth and protection from diseases and pests.

3.2 Inoculating seedlings with smut

Seedlings were inoculated in three different ways. 1) Soaking the seedlings in a smut spore suspension (4 grammes of spores per 1 liter of sterile water at 4×10^6 spores/ ml) for 30 minutes. 2) Wounding the seedlings at the bud with a scalpel then applying a paste of smut made at a concentration of 2 grammes of spore for 2 ml of sterile water. 3) Apply a paste of smut at the seedling buds.

Each inoculation method had 30 progenies planted in plastic bags in the green house. Controls of the un-inoculated progenies from the two populations were included. The experiment was replicated three times. Maximum and minimum temperatures in the green house were recorded daily. Data collection on seedling reaction to infection began two months after inoculation. Data on number of smut whips per stool, seedling survival and mortality, disease incidents per population and number of tillers per plot was recorded. The data collection continued for six months at two months intervals.

3.3 Data analysis

Individual analyses of variance for the green house experiment were performed using the SAS procedure general linear model (GLM) (SAS/STAT, 1994) on plot means of survival, mortality, tillers and disease incidence. Individual ANOVA was done on the inoculation methods and the untreated control. The effect of inoculation on the sugarcane seedlings and the interactions were evaluated. Mean separation and comparisons were tested by Fisher's least significance difference (LSD) test.

3.4 DNA Extraction and Electrophoresis

DNA was extracted from parental lines and F1 generations. Genomic DNA was isolated using the method of saghai-Maroof et al., (1984).The second extraction method to test on sugarcane DNA extraction was done using Sap extractor based on method of Clarke et al., (1989).Using the first method. midribs of fresh leaves were removed and the leaves transferred to the laboratory where they were quick-frozen by placing them in liquid nitrogen held in Styrofoam container at -80^{o}c. The samples were lyophilized and later ground to a fine powder using a mechanical mill. The powder was stored at -20^{o}c in plastic scintillation vials pending analysis. About 300-400 mg of this powder was

weighed into 15 ml polypropylene centrifuge tube and 9.0 ml of warm CTAB extraction buffer added following the method of saghai-Maroof et al., (1984). The samples were incubated in an oven at 65°c for 60-90 minutes and 4.5 ml chloroform/octanol added after 5 minutes. They were mixed gently by spinning them in a tabletop centrifuge at 1300-1500 xg1 at room temperature for 10 minutes. The top aqueous layer was poured off into new 15 ml tubes, 4.5 ml chloroform/octanol added and spinned again for 10 minutes. The top aqueous layer was pipetted into new 15 ml tubes containing 30ul of 10mg/ml Rnase and incubated for 30 minutes. After which, 6.0 ml of isopropanol was added and the mixture shaken gently to allow DNA to precipitate. Precipitated DNA was removed using a glass hook and placed in a 5 ml plastic tubes containing 4ml of DNA wash 1 solution for 20 minutes. It was then removed rinsed in 2 ml of DNA wash 2 solutions for 5 minutes and transferred into sarsted screw tubes containing 0.3% TE solution for storage at 4°c. DNA concentration was determined by comparing the fragment sizes and the DNA ladder . DNA quality was checked on a 0.8% agarose gel.

3.5 Polymerase Chain Reaction (PCR)-based selection

Parental lines and F1 progenies were screened with three EST-microsatellites markers and one RGA marker .The marker sequences constituted the forward and reverse primers in the PCR reactions. PCR samples constituted of 30ng of template DNA, 2pM each of forward and reverse primers, 10mM tris-HCL, and 10mM Mg2+SO4, 0.2mM each of d NTPs and 2.0U/ul of taq DNA polymerase in a reaction volume of 10ul. There were two PCR cycling phases. In the first phase, amplification involved 10 cycles. During this process, DNA was denatured initially at 94°c for 5 minutes, with each amplification cycles lasting 10 seconds; annealing was done at 61°c for 20 seconds (but stepped down at a rate of 1°c) and extension at 74°c for 30 seconds. In the second phase, amplification was for 30 cycles during which reaction components were denatured for 5 minutes at 94°c, with each amplification cycle lasting 10 seconds; reactions were annealed for 20 seconds at 54°c and extended for 30 seconds at 72°c. The last round of elongation was conducted for 4 minutes at 72°c. These amplifications were carried out using a Gene Amp 9700 thermal cycler from Applied Biosystems Ltd. The resultant PCR products were separated first, 0.7% w agarose gels containing 0.1ug/ml ethidium bromide, then in

TBE buffer at 5V/cm for approximately 2 hours. Bands were visualized and photographed using UVI-DOC imaging system.

3.6 Development of primers used in the experiment

Four primers were used for this study of which two were EST-SSRs primers from the SUCEST databases. Primer SCC09 had a forward sequence of 5 (AGGCGGCAGGGGTTGTAGAGAT)3 and a reverse sequence of 5(CATCCTCAGCACCATCACCATT)3. The annealing tempretaure of the primer was at 56 ^{o} c. The expected product size was 251 bp with expected homology of protein like kinase. Primer SCB 07 had a forward sequence of ACGAGAACCACAGCCACCG and reverse sequence of GGAGGTAGTCGGTGAAGTGC and annealing temperature of 51^{o} c . The expected product size was 291 bp and pyrophosphate-dependent phosphofructose1- kinase-like protein. The third primer H201b had a forward sequence of GGTGGGGTTGGGAAGACA and reverse sequence of CAACGCTAGTGGCAATCC. The forth primer XLRR designed from the conserved motifs of LRR, NBS, and kinase domains had a forward of CCGTTGGACAGGAA and reverse sequence of GAGGAAGGACAGGTTGCC. The primers were synthesized by the oligo sequencing unit at the International Livestock Research Institute (ILRI).

CHAPTER FOUR

4.0 RESULTS

Seedling smut screening may select for sugarcane genotypes with physiological resistance, and may therefore be of great use to the industry because genotypes with physiological smut resistance are sought for commercialization (H.Nzioki, personal communication, KESREF). One advantage of the green house assay is that it can be completed in five months and allows for a rapid mass screening of sugarcane genotypes.

The traditional smut evaluation system of using setts has remained unchanged for over 40 years because no other method has proved to be more effective. Approximately 30, 000 sugarcane genotypes (seedlings) are produced annually in Kenya through hybridization, and only a few clones are tested to determine their reaction to smut in controlled experimental plots (J..Jamoza, personal communication, KESREF). In order to evaluate larger numbers there is need for an alternative, simple yet reliable smut-screening procedure for sugarcane.

Table 1: Analysis of variance on tillers at two, three and four months after planting, survival counts at one, two, three and four months after planting and smut whip count at three and five months after inoculation

Dependent variables	Independent variables and model	D.F	F-value	P-Value	R-Sq	Mean
Tiller count at 2 months	Model	9	3.69	0.014	0.70	28.42
	Population	1	0.16	0.6987		
	Inoculation method	3	7.58	<0.0001		
	Interaction	3	2.22	0.1306		
Tiller count at 3 months	Model	9	4.06	0.0097	0.72	28.62
	Population	1	3.40	0.0865		
	Inoculation method	3	7.10	0.0039		
	Interaction	3	2.50	0.1042		
Tiller count at 4 months	Model	9	2.15	0.0960	0.58	19.41
	Population	1	2.36	0.1468		
	Inoculation method	3	3.08	0.0618		
	Interaction	3	0.73	0.5500		
Survival at 1 month	Model	9	9.79	0.0001	0.86	20.17
	Population	1	4.96	0.0428		
	Inoculation method	3	23.70	<0.0001		
	Interaction	3	3.92	0.0467		
Survival at 2 month	Model	9	6.09	0.0015	0.80	19.20
	Population	1	0.72	0.4100		
	Inoculation method	3	14.55	0.0001		
	Interaction	3	3.43	0.0467		
Survival at 3 month	Model	9	6.09	0.0015	0.80	19.20
	Population	1	0.72	0.4100		
	Inoculation method	3	14.55	0.0001		
	Interaction	3	3.43	0.0467		
Survival at 4 month	Model	9	2.44	0.0654	0.61	15.88
	Population	1	0.67	0.427		
	Inoculation method	3	4.94	0.0152		
	Interaction	3	0.98	0.4305		
Smut whip count at 3 months	Model	9	7.78	0.0014	0.83	0.25
	Population	1	19.38	0.0060		
	Inoculation method	3	7.90	0.0025		
	Interaction	3	7.90	0.0020		
Smut whip count at 5 months	Model	9	1.78	0.062	0.74	1.92
	Population	1	2.82	0.1151		
	Inoculation method	3	1.59	0.2352		
	Interaction	3	7.78	0.0010		

Table 2 : Counts on tillers at two, three and four months after planting , mortality at one, two, three and four months after planting and survival at one, two, three and four months after planting

Inoculation method	Counts on various parameter										
	Tillers			Mortality				Survival			
	2	3	4	1	2	3	4	1	2	3	4
Soaking	25	25	17	13	13	13	16	17	17	17	14
Paste	24	25	19	13	15	15	17	17	15	15	13
Wound paste	26	27	16	12	12	12	17	18	18	18	14
Uninoculated	39	38	25	1	3	3	7	29	28	28	23
LSD	7.7^{**}	7.3^{**}	7.9^{ns}	3.6^{***}	4.5^{***}	4.5^{***}	6.5^{*}	3.6^{***}	4.5^{***}	4.5^{***}	6.5^{*}

- Significant at 0.05, ** significant at < 0.01, *** significant at < 0.001, ns not significant

Table 3: Correlation analysis of tillering at two, three and four months after planting and survival counts at one, two, three and four months after planting and smut whip production at three and five months after inoculation

		Til-2	Til-3	Til-4	Surv-1	Surv-2	Surv-3	Surv-4	Smut-3	Smut-5
Til-2	Correlation P-value	1.0000	0.91804 <.0001	0.67597 0.0003	0.81518 <.0001	.085603 <0.0001	.85603 <.0001	0.71559 <.0001	-0.09670 .6531	0.10872 0.6131
Til-3	Correlation P-value	0.91804 <.0001	1.0000	0.77737 <.0001	.75319 <.0001	.82377 <0.0001	0.82377 <.0001	0.77311 <.0001	.049370 0.8188	0.14313 0.5046
Til-4	Correlation P-value	0.67597 0.0003	0.77737 <.0001	1.000	.55370 0.0050	0.65595 .0005	.65595 .0005	0.91338 <.0001	-0.122241 0.5688	0.03488 .8715
Surv-1	Correlation P-value	.81518 <.0001	.75319 <.0001	0.55370 .0050	1.0000	.94672 <0.0001	0.94672 <.0001	0.73498 <.0001	-0.21188 0.3203	0.05751 .7895
Surv-2	Correlation P-value	0.85603 <.0001	.82377 <.0001	0.65595 0.0005	0.94672 <.0001	1.000	1.0000<. <.0001	0.80867 <.0001	-0.17305 0.4187	0.05079 .8137
Surv-3	Correlation P-value	0.85603 <.0001	0.82377 <.0001	.65595 0.0005	0.94672 <.0001	1.0000 <0.0001	1.0000	0.80867 <0.0001	-0.17305 0.4187	0.05079 0.8137
Surv-4	Correlation P-value	0.71559 <.0001	0.77311 <.0001	0.91338 <.0001	0.73498 <0.0001	0.80867 <.0001	0.80867 <.0001	1.00000	-0.10292 0.6323	0.07018 0.7445
Smut-3	Correlation P-value	-0.09670 0.6531	0.04937 0.8188	-0.12241 0.5688	-0.21188 0.3203	-0.17305 0.4187	-0.17305 0.4187	-0.10292 0.6323	1.0000	0.60133 0.0019
Smut-5	Correlation P-value	0.10872 0.6131	0.14313 0.5046	0.03488 .08715	0.05751 0.7895	0.05079 0.8137	0.05079 0.8137	0.07018 0.7445	0.60133 0.0019	1.0000

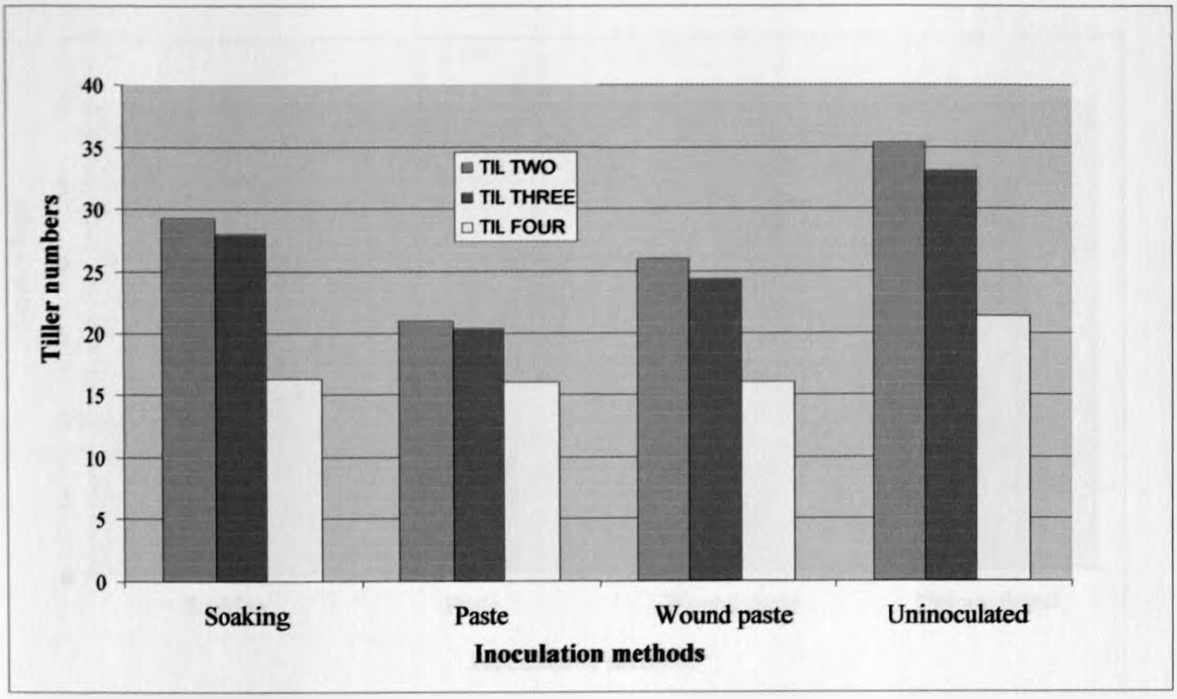

Figure 1: Effect of inoculation methods of soaking, paste, wound paste and uninoculated on tillering across population 1 (Co 421 x EAK 70-97)

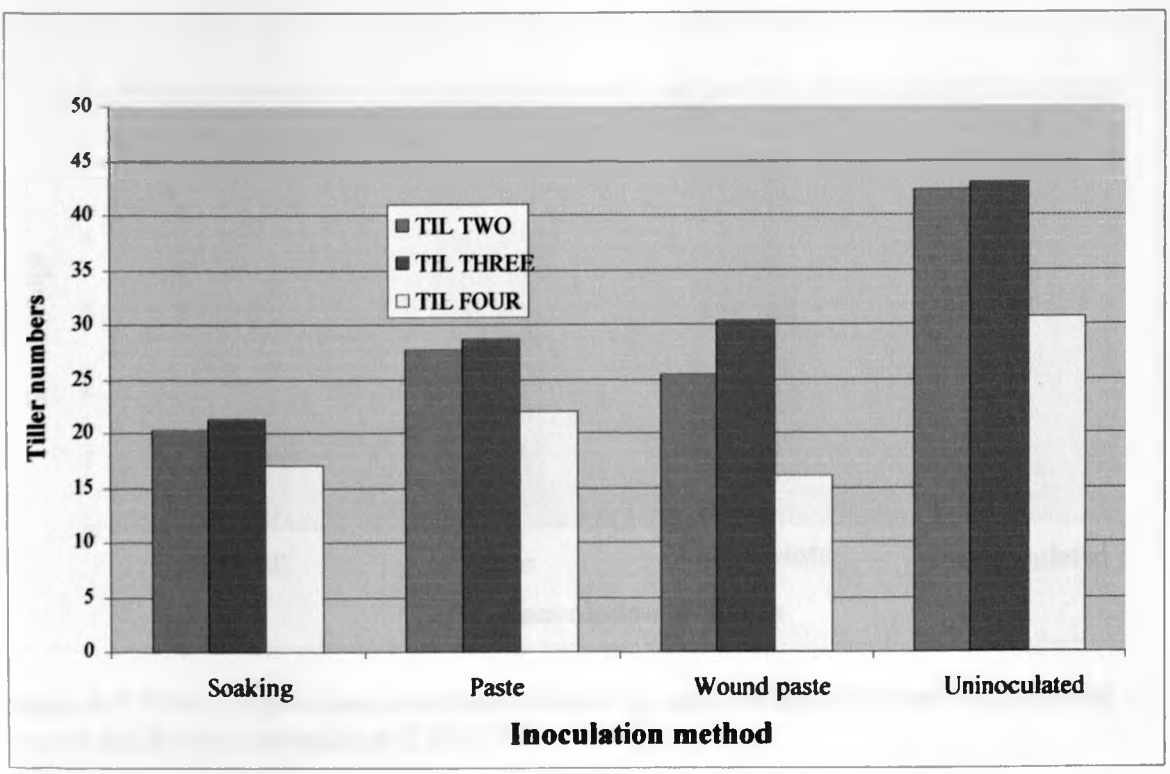

Figure 2: Effect of inoculation methods of soaking, paste, wound paste and uninoculated on tillering for population 2 (Co 331 x Co 945)

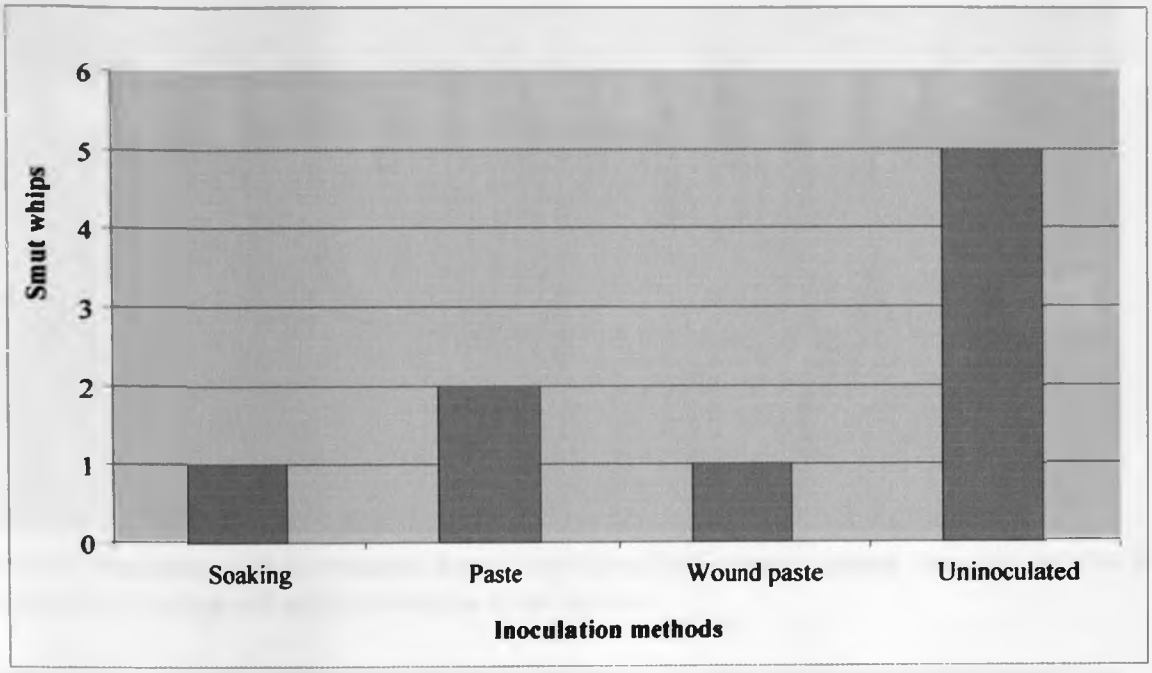

Figure 3: Effects of inoculation methods of soaking, paste wound paste and uninoculated on smut incidence in population 1 (Co 421 x EAK 70-97)

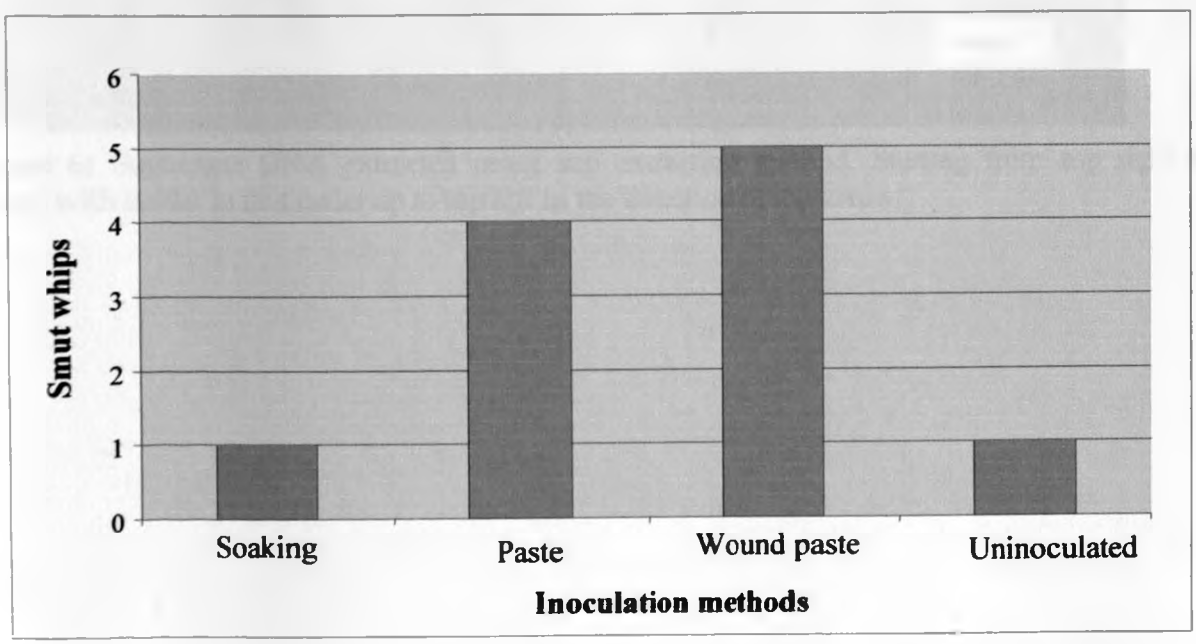

Figure 4: Effects of inoculation methods of soaking, paste wound paste and uninoculated on smut incidence in population 2 (Co 331 x Co 945)

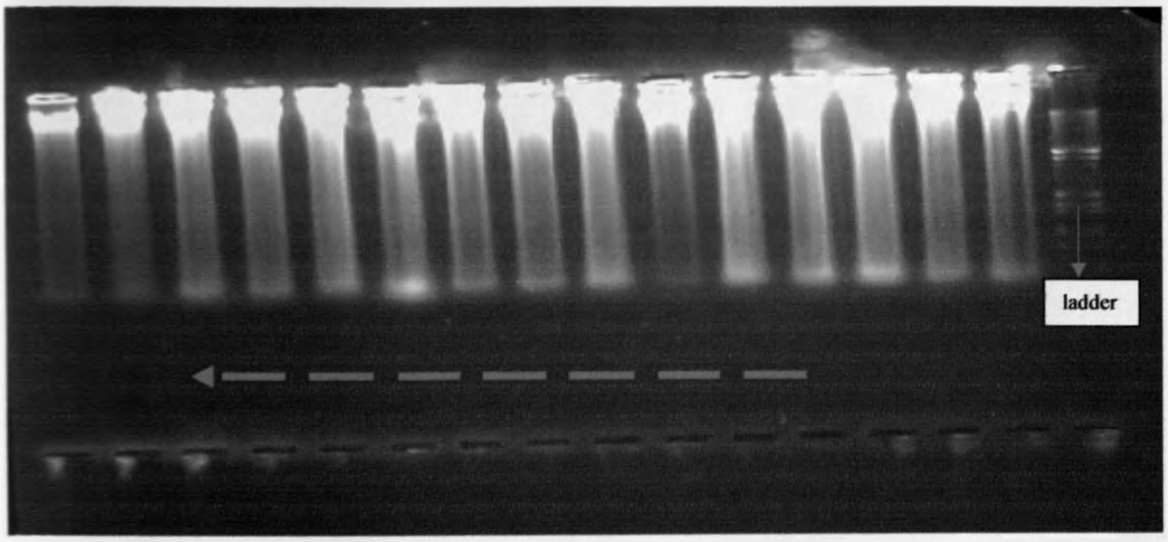

Figure 5: Sugarcane DNA extracted from lyophilized leaf sample starting from top right well stating with ladder up to top left in the direction of the arrow

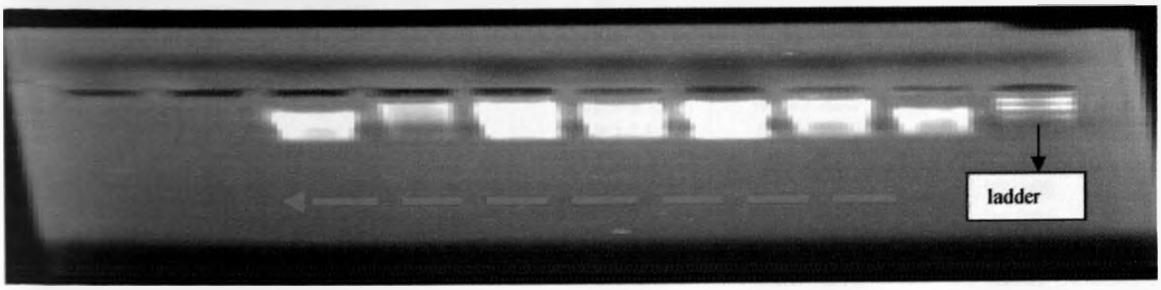

Figure 6: Sugarcane DNA extracted using sap extraction method. Starting from top right well stating with ladder in that order up to top left in the direction of the arrow

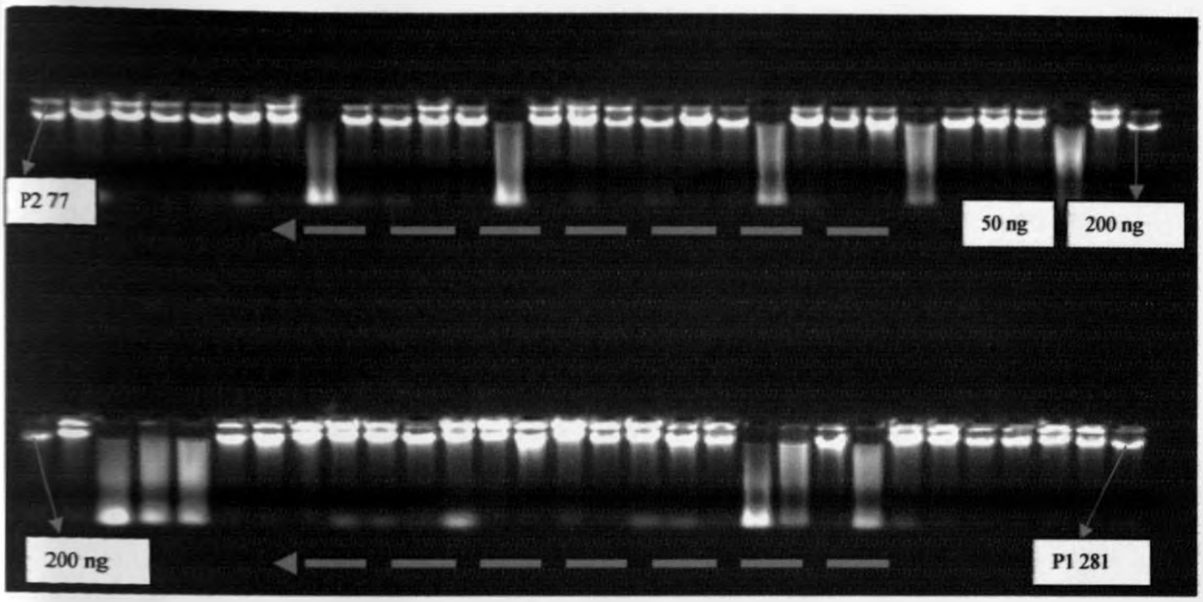

Figure 7 Sugarcane DNA used to check for quality a random sample from DNA extracted from sugarcane progenies as listed below. Top well starting with the ladder 200ng up to p2 77 and the bottom well starting with p1 281 up to ladder of 200ng in the direction of the arrow

1.200ng	11.P2 147	21.P2 191	31.P1 281	41.P1 334	51.P1343
2.100ng	12.P1 101	22.P2 374	32.P1 280	42.P1 287	52.P1 12
3.50ng	13.P1 244	23.P1 237	33.P2 135	43.P1 255	53.P1 34
4.P1 223	14.P1 352	24.P2 123	34.P2 151	44.P2 387	54.P2 304
5.P1 457	15.P2 63	25.P2 48	35.P1 251	45.P1 21	55.P2 145
6.P2 56	16.P2 72	26.P2 85	36.P2 433	46.P2 74	56.P2 189
7.P1 268	17.P2 373	27.P1 22	37.P2 149	47.P1 351	57.P1 231
8.P2 51	18.P1 207	28.P1 369	38.P1 108	48.P2 436	58.50ng
9.CO421	19.P2 59	29.P1 103	39.P2 305	49.P1 379	59.100ng
10.P1 439	20.P2 199	30.P2 77	40.P1 249	50.P2 131	60.200ng

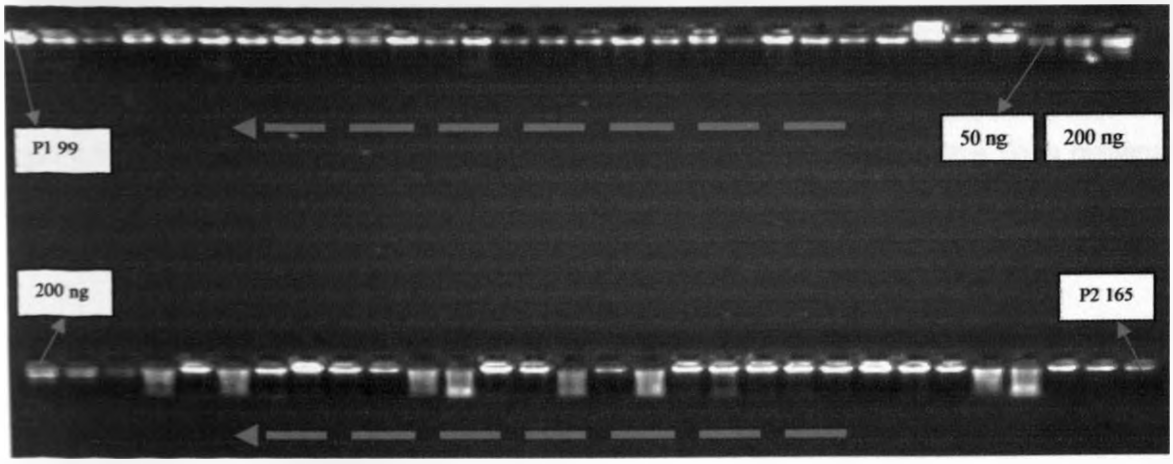

Figure 8: Sugarcane DNA used to check for quality a random sample from DNA extracted from sugarcane progenies as listed below. Top well starting with the ladder 200 ng up to p1 99 and the bottom well starting p2 165 up to ladder of 200ng in the direction of the arrow

1.200ng	11.P2 296	21.P1 110	31.P2 165	41.P1 330	51.P2 133
2.100ng	12.P2 142	22.P2 181	32.P2 294	42.P1 451	52.P2 163
3.50ng	13.P2 427	23.P2 194	33.P2 86	43.P2 316	53.P2 177
4.P2 324	14.P2 122	24.P2 195	34.P2 50	44.P1 44	54.P2 88
5.P1 259	15.P2 146	25.P1 440	35.P1 458	45.P1 437	55.P2 49
6.P1 310	16.P2 420	26.P1 315	36.P2 190	46.P2 55	56.P2 185
7.P2 394	17.P2 90	27.P1 311	37.P2 127	47.P2 178	57.P1 31
8.P1 450	18.P2 89	28.P2 386	38.P2 137	48.P2 193	58.50ng
9.P2 203	19.P2 315	29.P2 325	39.P1 353	49.P1 39	59.100ng
10.P1 275	20.P2 91	30.P1 99	40.P1 213	50.P1 37	60.200ng

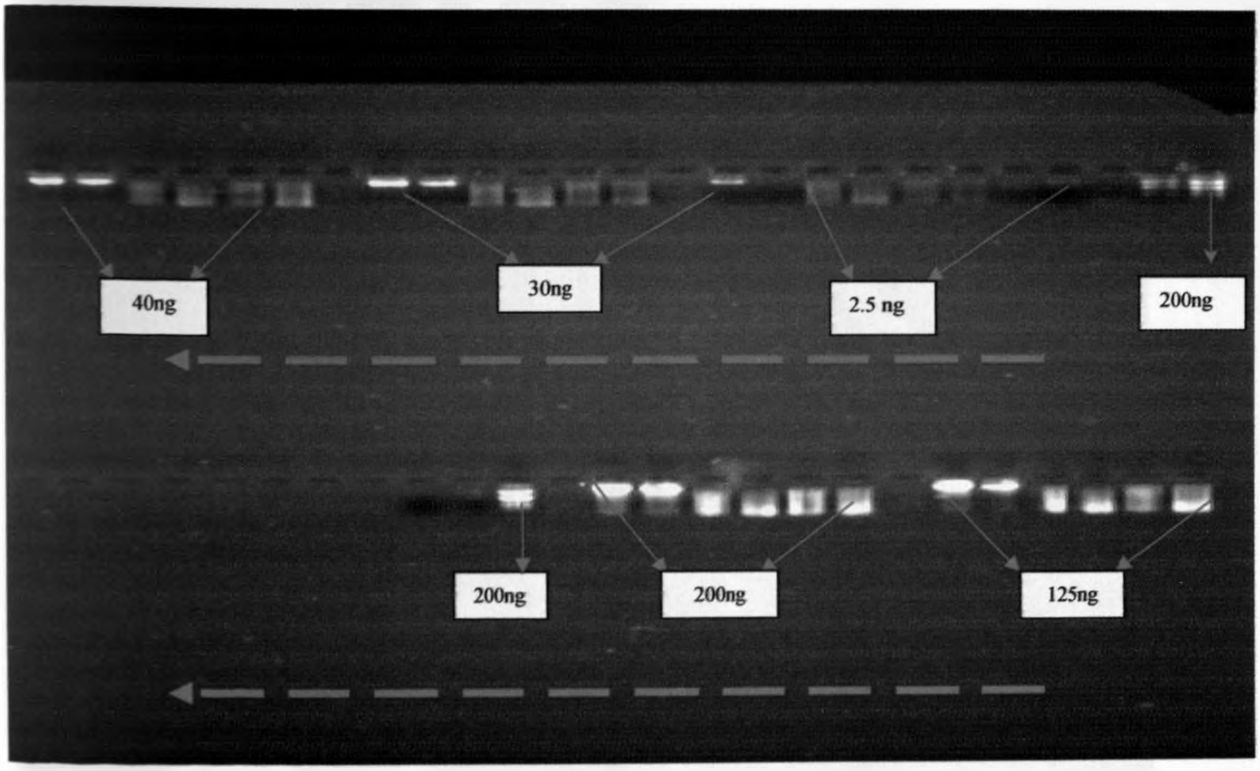

Figure 9: Sugarcane DNA dilution random sample 2.5,30,40,125 and 200ng from progenies listed below in the direction of the arrow for use in PCR.

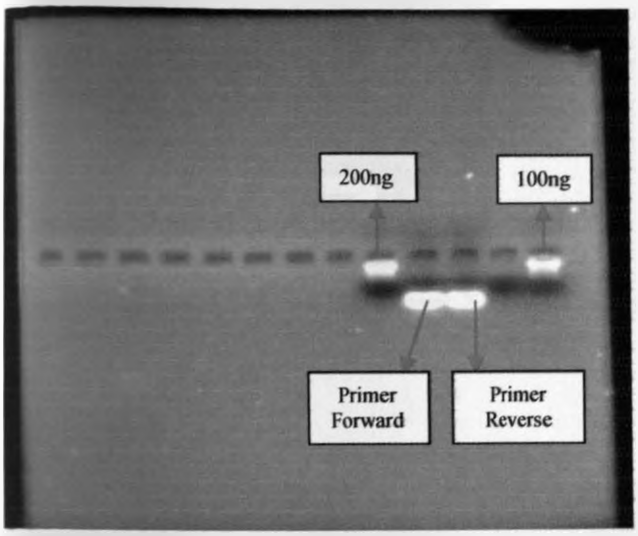

Figure 10: Primer H201 b concentration determination reverse and forward sequence and 100 ng and 200ng ladder

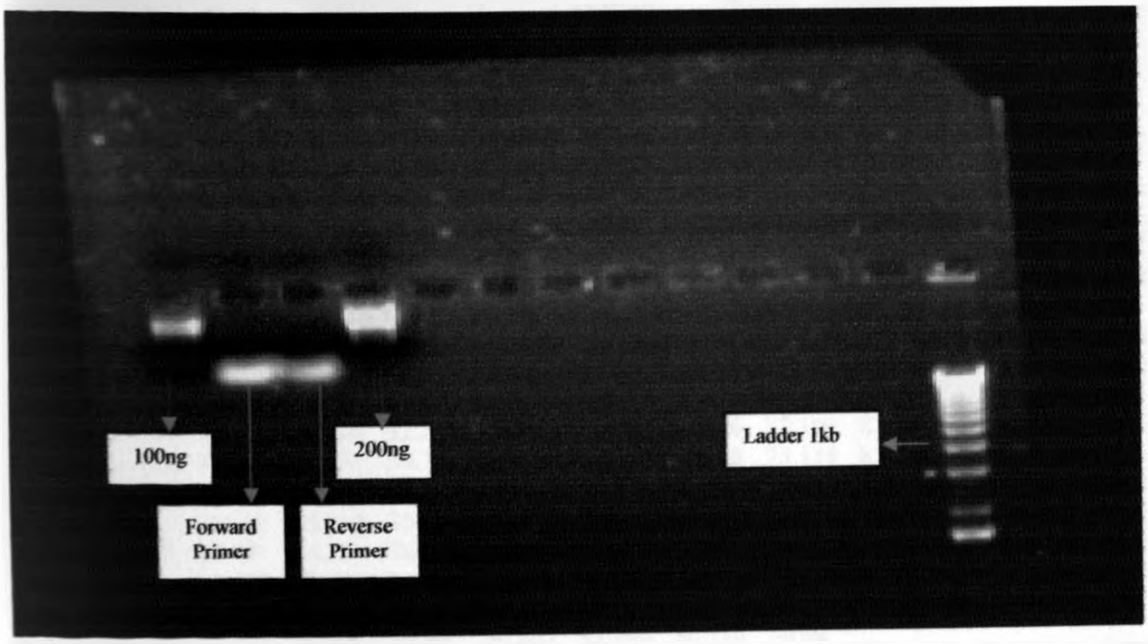

Figure 11: Determination of primer SCB07 concentration, DNA standards of 100 and 200 ng and a ladder of 1kb

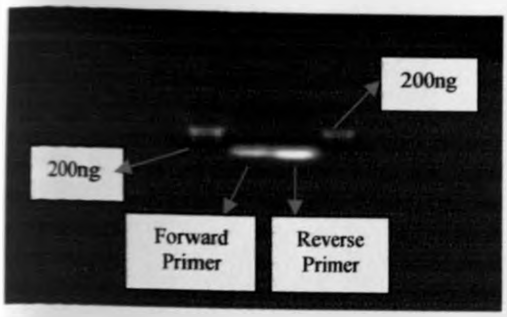

Figure 12: Determination of primer XLRR concentration and DNA standards of 100 ng and 200 ng

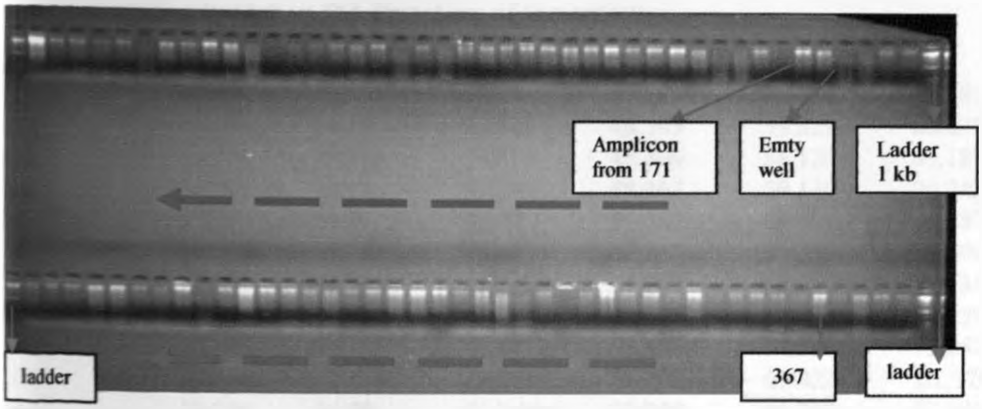

Figure 13: PCR products with primer SCC09 from progenies listed below starting with the ladder on the top right and ending with the ladder. The lower well starting with a ladder at the right bottom and ending with the ladder in the direction of the arrow.

1. ladder	11.47	21.459	31.386	41.152	51.247	61.302	71.287	81.244
2.108	12.306	22.314	32.211	42.207	52.259	62.366	72.109	82. 103
3.133	13.345	23.351	33.70	43.272	53.156	63.165	73.134	83.397
4.5	14.117	24.25	34.298	44.ladd	54.222	64.72	74.157	84.226
5. Co421	15143.	25.310	35.392	45.ladd	55.230	65.422	75.252	85.452
6.443	16.79	26.13	36.214	46.343	56.324	66.339	76.394	86.149
7.141	17.409	27.79	37.317	47.340	57.329	67.281	77.379	87.359
8.328	18257.	28.145	38.350	48.367	58.120	68.271	78.129	88.ladder
9.163	19.438	29.305	39.463	49.102	59.140	69.189	79.68	
10.71	20.419	30.442	40.223	50.367	60.401	70.242	80.1	

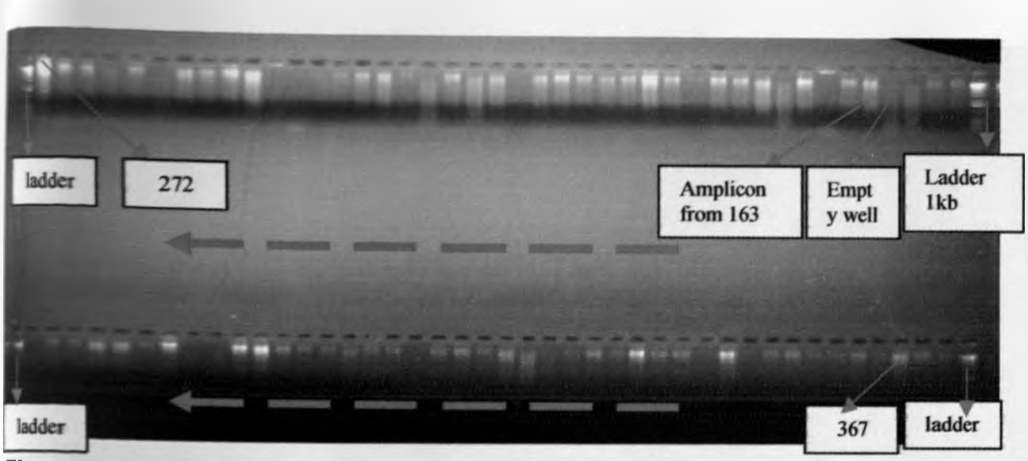

Figure 14: PCR products with primer XLRR for the progenies listed below starting with ladder from top right well to 44th well ending with ladder and the right bottom well starting with ladder and ending with the ladder in the direction of the arrow.

1. ladder	12.306	23.351	34.298	45.ladder	56.324	67.281	78.129
2.108	13.345	24.25	35.392	46.343	57.329	68.271	79.68
3.133	14.117	25.310	36.214	47.340	58.120	69.189	80.135
4.5	15143.	26.13	37.350	48.367	59.140	70.242	81.244
5.Co421	16.79	27.79	38. 317	49.102	60.401	71.287	82.397
6.163	17.409	28.145	39.463	50.367	61.302	72.109	83.103
7.141	18.257	29.305	40.223	51.247	62.366	73.134	84.226
8. 443	19.438	30.442	41.152	52.259	63.165	74.157	85.452
9.328	20.419	31.386	42.207	53.156	64.72	75.252	86.149
10.71	21.459	32.211	43.272	54.222	65.422	76. 379	87.359
11.47	22.314	33.70	44.ladder	55.230	66.339	77.394	88.ladder

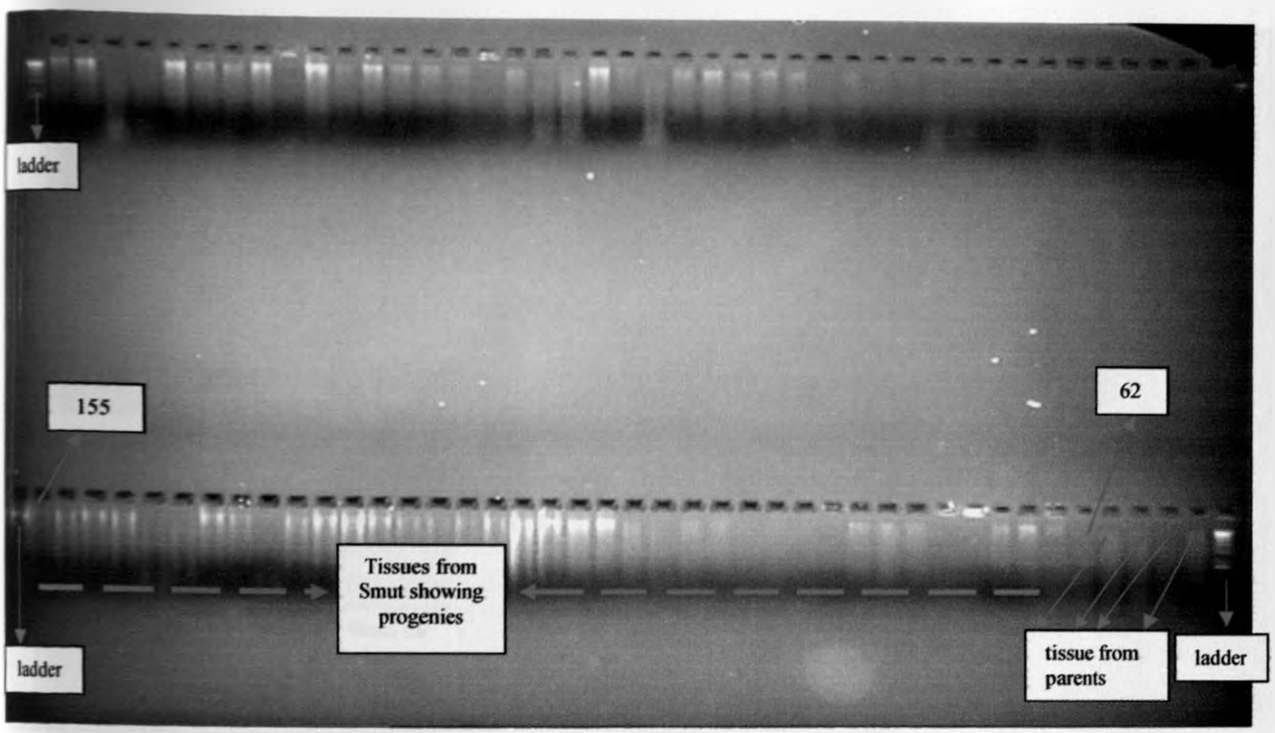

Figure 15: PCR Products with primer XLRR batch 2 starting with ladder on top right well and ending with ladder and the bottom well starting with ladder and ending with a ladder covering progenies that showed smut enclosed within the arrows.

1.ladder	11.32	21. 331	31.402	41. 43	51.114	61. 317	71. 120	81.282
2. water	12 53	22. 12	32. 295	42. 239	52. 94	62. 197	72. 105	82. 423
3.master mix	13. 396	23. 304	33. 273	43. 217	53. 321	63. 425	73. 247	83. 69
4.425	14 87.	24. 136	34. 125	44. ladder	54. 65	64. 443	74. 299	84. 339
5. 236	15. 190	25. 41	35. 9	45. ladder	55. 366	65. 98	75. 197	85. 444
6.58	16. 38	26. 11	36.142	46. co 331	56. 302	66. 88	76. 103	86. 441
7.282	17. 311	27. 232	37.457	47. co 421	57. 96	67. 129	77. 367	87. 155
8.56	18 316	28.429	38. 365	48. 7097	58. 446	68.379	78. 351	88. ladder
9.146	19. 364	29.363	39.408	49.CO945	59. 85	69. 113	79. 196	
10. 373	20. 332	30. 248	40.42	50. 62	60. 386	70. 233	80. 420	

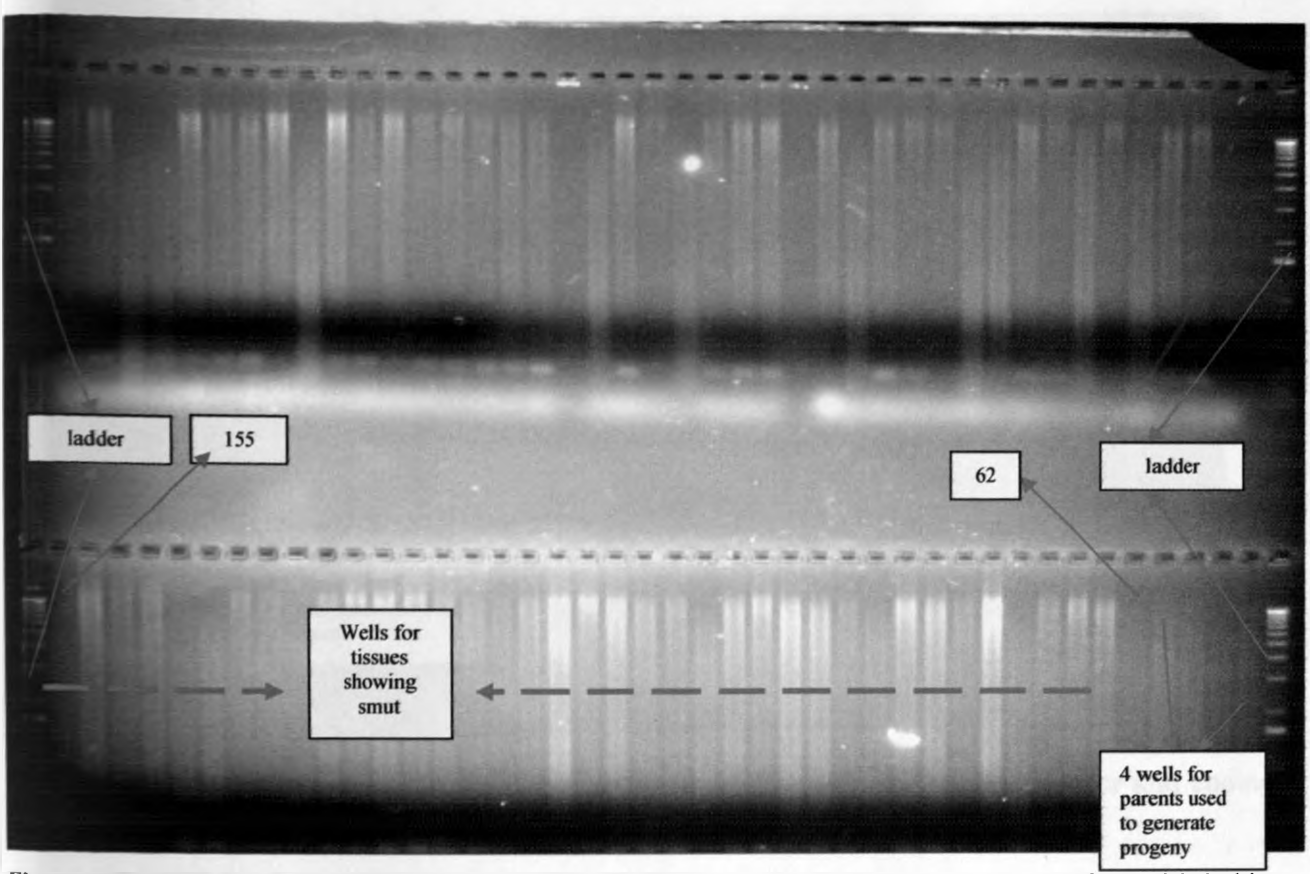

Figure 16: PCR Products with primer SCC09. Wells from top right upper comb starting with ladder and ending with ladder. Lower well from top right starting from ladder to the last well ending with ladder . DNA from progenies that exhibited smut enclosed within the arrow did not have any amplification.

1. ladder	11.32	21. 331	31. 402	41. 43	51..114	61.317	71.120	81.282
2. water	12.53	22. 12	32. 295	42.239	52.94	62.197	72.105	82.423
3.master mix	13. 396	23. 304	33. 273	43 217	53.321	63.425	73.247	83.69
4. 425	14. 87	24. 136	34. 125	44ladder	54.65	64.443	74.299	84.339
5. 236	15. 190	25. 41	35. 9	45.ladder	55.366	65.98	75.197	85444.
6.58	16. 38	26. 11	36. 142	46.Co331	56.302	66.88	76.103	86.441
7.282	17. 311	27. 232	37. 457	47. Co421	57.96	67.129	77.367	87.155
8. 56	18. 316	28. 429	38. 365	48.7097	58.446	68.379	78.351	88.ladder
9. 146	19. 364	29. 363	39. 408	49.Co 945	59.85	69.113	79.196	
10. 373	20. 332	30. 248	40. 42	50.62	60.386	70.233		

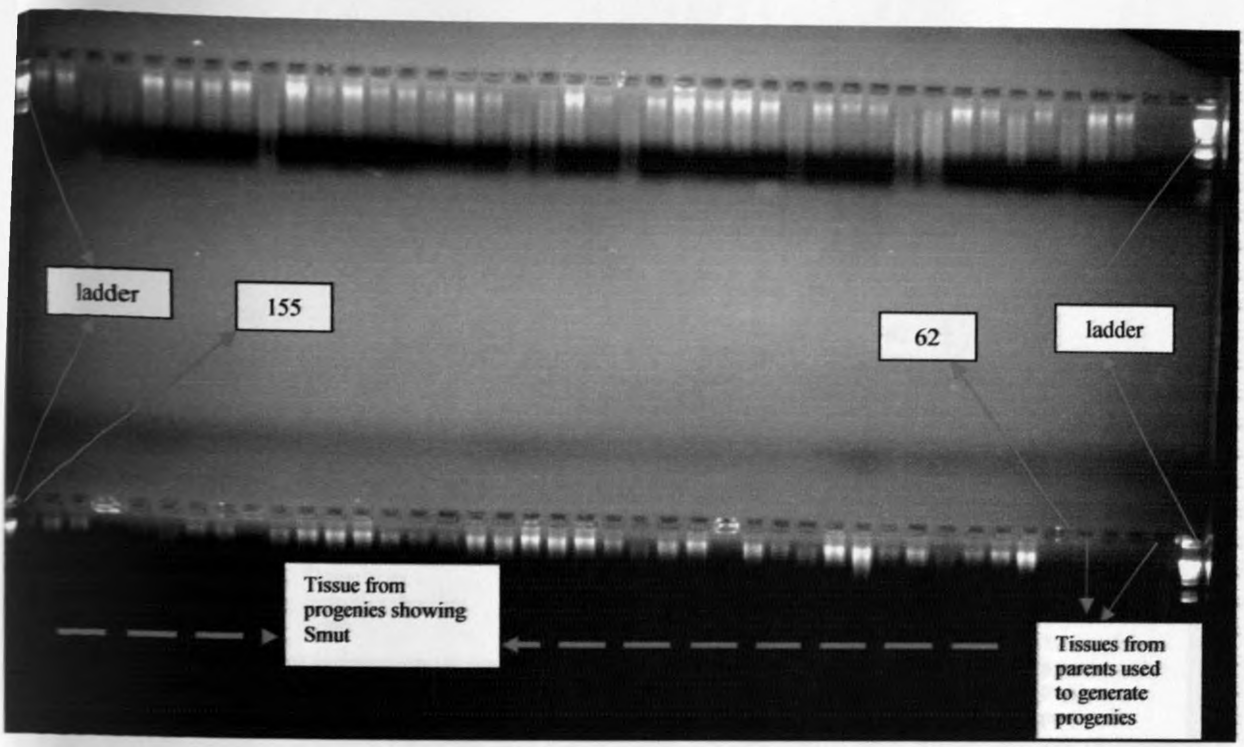

Figure 17: PCR Products with primer H201 .Wells from top right starting with ladder and ending with ladder. Lower well from top right starting from ladder to the last well with ladder

1.ladder	11.32	21. 331	31.402	41. 43	51.114	61. 317	71. 120	81.282
2. water	12 53	22. 12	32. 295	42. 239	52. 94	62. 197	72. 105	82. 423
3.master mix	13. 396	23. 304	33. 273	43. 217	53. 321	63. 425	73. 247	83. 69
4.425	14 87.	24. 136	34. 125	44. ladder	54. 65	64. 443	74. 299	84. 339
5. 236	15. 190	25. 41	35. 9	45. ladder	55. 366	65. 98	75. 197	85. 444
6.58	16. 38	26. 11	36.142	46. Co 331	56. 302	66. 88	76. 103	86. 441
7.282	17. 311	27. 232	37.457	47. Co 421	57. 96	67. 129	77. 367	87. 155
8.56	18 316	28.429	38. 365	48. 7097	58. 446	68.379	78. 351	88. ladder
9.146	19. 364	29.363	39.408	49.Co945	59. 85	69. 113	79. 196	
10. 373	20. 332	30. 248	40.42	50. 62	60. 386	70. 233	80. 420	

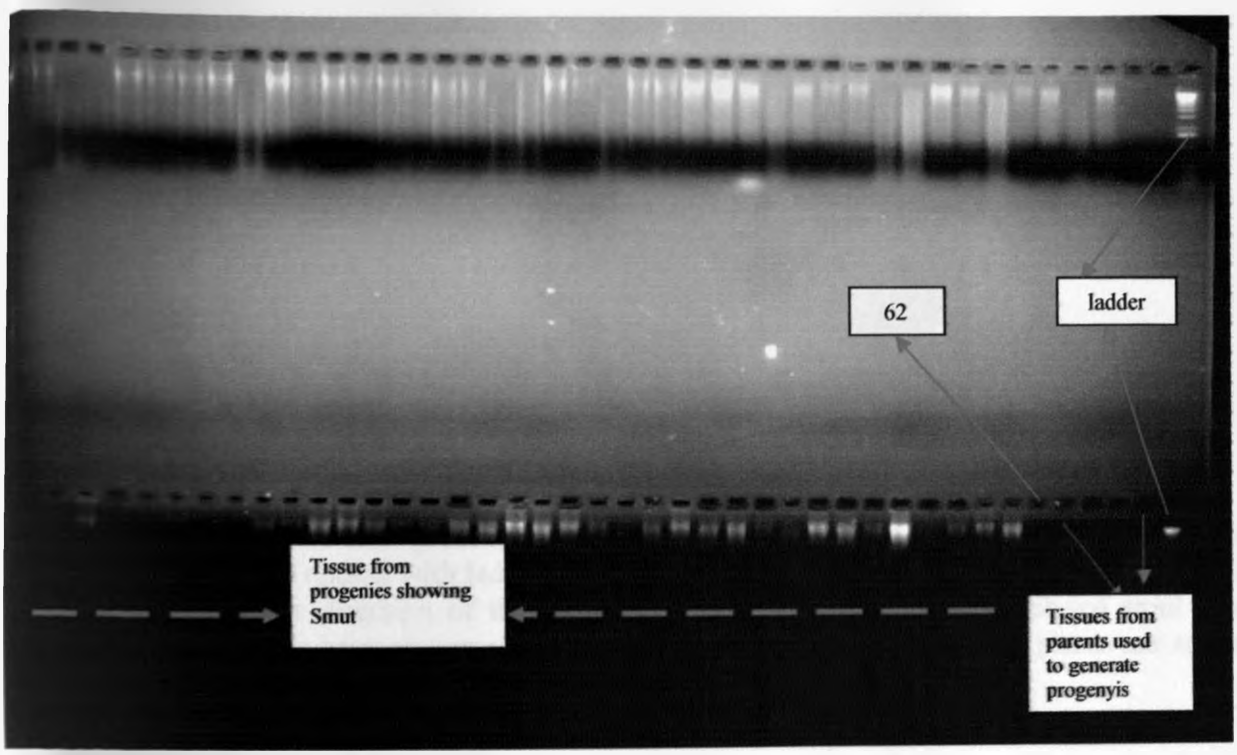

Figure 18: PCR Products Primer SCB07.Wells from top right upper starting with ladder and ending with ladder. Lower well from top right starting from ladder to the last well with ladder.DNA from tissues that exhibited smut shown within the arrow did not have any amplification.

ladder	11.32	21. 331	31.402	41. 43	51.114	61. 317	71. 120	81.282
water	12 53	22. 12	32. 295	42. 239	52. 94	62. 197	72. 105	82. 423
master mix	13. 396	23. 304	33. 273	43. 217	53. 321	63. 425	73. 247	83. 69
425	14 87.	24. 136	34. 125	44. ladder	54. 65	64. 443	74. 299	84. 339
236	15. 190	25. 41	35. 9	45. ladder	55. 366	65. 98	75. 197	85. 444
58	16. 38	26. 11	36.142	46. Co331	56. 302	66. 88	76. 103	86. 441
282	17. 311	27. 232	37.457	47. Co421	57. 96	67. 129	77. 367	87. 155
56	18 316	28.429	38. 365	48. 7097	58. 446	68.379	78. 351	88. ladder
146	19. 364	29.363	39.408	49.Co945	59. 85	69. 113	79. 196	
10.373	20. 332	30. 248	40.42	50. 62	60. 386	70. 233	80. 420	

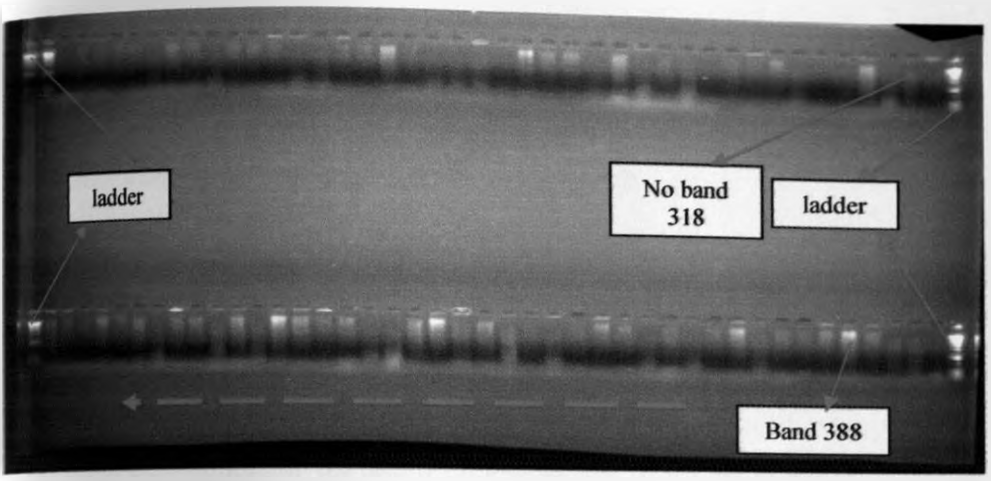

Figure 19: PCR products with primer SCC 09 from progenies listed below. Wells from top right starting with ladder and ending with ladder. Lower well from top right starting from ladder to the last well with ladder in the direction of the arrow. DNA from progenies that exhibited smut shown within the arrow did not have any amplification. Where bands are expressed the plants are assumed to be resistant

1.ladder	9.353	17.65	25.185	33.241	41.299	49.355	57.30	65.23	73.88	81.63
2.354	10.82	18.380	26.441	34.54	42.322	50.338	58.327	66.158	74.424	82.104
3.318	11.234	19.206	27.127	35.237	43.ladd	51.99	59.69	67.Co331	75.330	83.313
4.4	12.231	20.150	28321	36.352	44.ladd	52.325	60.298	68.148	76.337	84.398
5.455	13.52	21.98	29.421	37.215	45.ladd	53.361	61.405	69.106	77.121	85.382
6.192	14.431	22.48	30.Co421	38.292	46.61	54.24	62.256	70.35	78423	86.291
7.356	15.33	23.7097	31.371	39.416	47.Co945	55.193	63.73	71.6	79.131	87.216
8.62	16.461	24.8	32.85	40.411	48.323	56.420	64.439	72.210	80.275	88.ladder

4.1 DISCUSSIONS

4.2 Inoculation methods and traits measured

On the analysis of variance shown in the table 1, inoculation methods of soaking, paste and wound paste was significant in influencing survival and tillering and smut whip count at three months. Seedlings that were inoculated had lower survival rates and tillering ability compared to the seedlings that were not inoculated as shown in table 2. For population 1 (Co 421 x EAK70-97), tillering was highest in uninoculated seedling, then followed by soaking, wound paste and paste in that order as shown in figures 1. For population 2(Co 331 x Co 945) tillering was highest in uninoculated seedlings, and then followed by wound paste, paste and then soaking as shown in figure 2. Tillering rate (the rate at which young shoots appear) has been reported to progressively decrease in the field infected sugarcane cultivars (Waller, 1970). Hector et al., (1995) commented on the lack of in vitro tillering of sugarcane plantlets in the presence of U.scitaminea filtrates as diagnostic feature for smut. In addition to the lack of tillering in smut-inoculated plantlets, susceptible sugarcane cultivars fully express the disease in vitro by producing sori. The high seedling survival registered by seedlings in population two as shown in table 2(Co 331 x Co 945) was probably because seedlings in this population were bigger than those in population 1(Co 421 x EAK 70-97). It appears that resistance is expressed only in fully –grown well developed seedlings, since the very young plantlets and weak ones registers very high mortality rates irrespective of resistance ratings.

Plantlet inoculated with smut further suggests that seedlings need to be fully developed before their resistant traits are expressed. In banana, it was also found that the age of tissue- cultured plantlet influenced resistance to nematodes, and very young plantlets did not express all mechanisms of resistance (Elsen et al., 2002). High seedlings mortality recorded at four months table 2 was due to sooty mould disease that attacked seedlings in the green house then. Most of the smut inoculated plants died after 5 months following inoculation irrespective of whether or not the sori were produced. The inoculation methods of soaking, paste and wound paste however was not significant in influencing smut whip count at the age of five months table 1. Whip production is the most reliable symptom of smut disease in sugarcane, since other features such as the detection of fungal

hyphen and U.scitaminea DNA may not necessarily be a true indication of plant resistance in inoculation experiments.

Population types were significantly different from each other with respect to survival count at one month after planting and smut whip count at 3 months after planting shown in table 1. Population type were not significantly different from each other in terms of survival at two, three and four months after planting and also tillers at two, three and four months after planting. Wound paste was significantly different from control and soaking in terms of Smut whip count at three months after planting as shown in table 1.

Laboratory –based disease evaluation in sugarcane dates back several decades when Bock (1964) showed correlation between laboratory and field inoculation for smut disease formation.

The correlation analysis shown in table 3 above indicates that neither the survival count nor tillers are correlated to smut incidences. The Smut incidences are independent of tillers and survival. This means that the number of smut whips produced is not related to the survival and tillering rates in sugarcane seedlings. Correlation between survival count and tiller is highly significant. This means that the higher the survival, the higher the tillers. This is conventional because if more seedlings survive they eventually produce more tillers. There is a negative correlation between tillering and smut whip production at three months. This means that tillering does not influence smut whip production in the seedlings. The negative correlation between survival and tillering shown in table 4 also means that survival of seedlings does not influence smut whip production.

Based on the scores, population 1 (Co 421 x EAK 70-97) registered poor results in terms of whips counted and soral production per inoculation method figure 3. There was production of whips on un-inoculated seedlings in this population. This could probably be a mixture of low internal resistance of the stalk tissues of individuals in this population or latent infection and inoculation by spores (aero spores) from the nearby-infected seedlings.

For population 2 (Co 331 x Co 945), wound paste method had the highest incidence of smut whip production, followed by paste. Soaking and uninoculated method had the lowest incidence of smut as shown in figure 4 below.

The accuracy of evaluation increased by each crop cycle according to results obtained in a study done in South Africa. Interesting results are therefore expected in the subsequent ratoons. Hence smut levels in the seedlings should be evaluated in the first ratoon as well

4.3 Sugarcane DNA extraction methods

The research was a pioneer of sugarcane DNA extraction in Kenya and there was need to evaluate available protocols and utilize tools available within reach. Two methods of DNA extraction were used. The two methods were lyophilization and sap extaction. These two methods were tested due to difficulty of getting some of the reagents used in extraction of sugarcane. We therefore applied tools and reagents available for DNA extraction method at KARI-Katumani laboratory that specializes mainly in maize and sorghum. Sugarcane DNA extracted using lyophilization shown in figure 5 produced a lot of DNA than sugarcane DNA extracted using sap extraction method figure shown 6. DNA extracted using the two methods were run on agarose gel and compared with the ladder.

4.4 Sugarcane DNA quality

As more efficient methods are developed for gathering DNA sequence, many researchers wish to integrate molecular analyses into their evolutionary studies. Unfortunately, not all specimens contain DNA of suitable quality for molecular analysis. We need a clear understanding of the parameters that influence the degradation of DNA in stored specimens and destructive sampling of specimens that could affect quality. Quality is defined with respect to size of extracted DNA, extraction yield, and ability to amplify from target regions. DNA quality is critical because it influences the quality of PCR products generated and therefore DNA used for any molecular marker study should be of high quality. The DNA extracted was of good quality figure as shown in 7 and 8 below based of their intensity, clarity and yield. However incidences of DNA degradation were observed in a few samples as shown in figure 7.

4.5 Sugarcane DNA quantification

Quantifying DNA is a technique to calculate the quantity (weight) of DNA (deoxyribonucleic acid) in a sample. Using a known volume of sample allows you to calculate the concentration (weight per volume). For enzymatic reactions, efficiency is dependent on the concentration of all components

This includes the DNA template in PCR. Template DNA is the greatest source of potentially deleterious contaminants (more DNA equals more contaminants). Too much DNA binds up available Mg^{++}. Too little DNA means not enough final product. DNA is quantified to ensure the proper amount of template is used in downstream applications. Enzymatic reactions are only successful if all reagents are added in the proper proportions. DNA sequencing requires DNA quantification. Molecular weight markers are DNA fragments of known size. Comparison of sample bands to markers allows visible confirmation of desired product and quantification of sample DNA. Analytical gel densitometry has become a mainstream technique for quantifying DNA. Unlike other methods such as spectrophotometry and the colorimetric diphenylamine assay, gel analysis provides both qualitative and quantitative assessments of a DNA preparation. A gel picture, for example, provides a wealth of information such as quality of the DNA, and, to a certain extent, the contamination by RNA and proteins. The gel can then be digitized and the image used for quantification. With the availability of hardware for gel imaging and software for data acquisition, as well as standard DNA ladders specifically designed for quantification, gel densitometry has become a method of choice by many investigators. DNA extracted was quantified as shown in shown figure 7 and 8 and using a 200ng and 50ng DNA ladder. Most of the DNA extracted was around 200ng in size.

4.6 Sugarcane DNA dilution

For the purpose of PCR, DNA template concentration must be optimized. DNA template concentration determines the legibility of the bands. In this experiment, DNA was diluted to 2.5, 30, 40, 125 and 200ng as shown in figure 9 above to determine the best template for the master mix. Each concentration of the DNA was used in the master mix to establish concentration that gives best PCR products. The bands were clearly visible when the concentration was 30 ng.

4.7 Primer concentration determination

Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products. Once the primers have been constituted, it is important to perform agarose gel electrophoresis to view the presence of oligonucleotides after synthesis and to establish how they should be diluted to be used in the master mix. Enzymatic reactions are only successful if all reagents are added in the proper proportions. Oligonucleotides were compared to 100 and 200ng

DNA ladder. The concentration of the primers used in this study was between 200-300 ng as shown in figures 10, 11 and 12.

4.8 PCR products

PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size. In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants.

The amplicons represented by bands in figures 13, 14, 15, 16, 17, 18 and 19 are assumed to represents the segment that contains resistance gene analog. The bands were scored as either present or absent depending of intensity and clarity. Empty wells without any band indicate that nothing was amplified. The bands were scored as present depending on their intensity and clarity. Consistent with previous reports (Chen et al .1998), PCR- based markers derived using RGA primers were polymorphism and informative, that is, every primer pair produced scorable polymorphic loci in the progeny analyzed. High content of repetitive DNA, which results in the amplification of a smear, constitutes a general problem.

The two primers SCC09 and XLRR used in figure 13 and 14 expressed no bands with DNA progenies that showed smut. The assumption is that they did not have resistant gene analog. Some work done in South Africa by Butterfield et al., (2001) indicated that the candidate gene approach could be efficient way to establish, at least on a coarse scale, the association between candidate genes and functionality. If these genes themselves are involved in resistance, they will be useful for marker –assisted selection breeding programs. The PCR products sizes were not as expected. PCR products may differ from the expected size because of the existence of the introns. As an alternative explanation, the observed size shifts may be the result of indels (insertions/deletions).

DNA extracted from progenies from 62 to 155 that exhibited smut whips shown in figure 15 did not form bands. This might explain their susceptibly on the basis of primer XLRR used to screen them as shown in figure 15. DNA extracted from plants that expressed smut did not show any band of resistance as expected except for primer XRLL that could amplify other resistance components rather than that for disease resistance.

The primer H201 used in figure 17 did not amplify band with DNA extracted from parents used in the development of progeny in this study. It can be assumed that these parents' shows field resistance phenotypically but might not be having resistance in their genotypes. This primer H201 also formed bands with progenies that showed smut and there is possibility that this primer can also amplify other resistance components other than disease resistance.

In contrast the products obtained from two of the primers SCB 07 in figure 18 and SCC09 in figure 19 deviated from the expected size by around 500 bp. The size of the product from primer SCB 07 shown in figure 18 and SCC 09 shown in figure 19 was around 800 bp against the expected size of 290bp and 250 bp respectively. However the size of amplification products from primer XLRR shown in figure 14 was near the expected size 900bp.

The primer SCB07 used in figures 18 did not amplify band with DNA extracted from parents used in the development of progeny in this study. This further confirms that these parents' shows field resistance phenotypically but might not be having resistance in their genotypes. Most of the enzymatic reactions performed with DNA from progenies that had smut did not form any band

Primer SCC09 used in figures 19 formed bands with DNA from progenies that did not show smut and are assumed to be resistant and did not form bands with DNA from progenies that were susceptible to smut like 318.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Summary and conclusions

From the study sugarcane genotypes can and should be screened for smut resistance at first stage of selection to assess seedlings reaction to smut and to avoid carrying large numbers of clones that are eventually discarded at the advanced stage of selection. This will cut on cost and time spent on smut screening.

Wound paste method led to significantly high disease level compared to other methods. It should be adopted as inoculation method due to its ability to identify and discriminate effectively seedlings which are susceptible to smut disease.

Green house sugarcane seedlings smut-evaluation method described in this study is not to be considered as a substitute for field screening. It is a means of pre-screening large numbers of new sugarcane genotypes for resistance to smut disease. Highly resistant and susceptible genotypes by this assay can then be field tested for verification. Susceptible genotypes on the other hand can be detected early and discarded. In this way, it is likely that field tests would be more effective in producing many more smut-resistant sugarcane cultivars.

DNA extracted from lyophilized leave tissue method is better than DNA extracted from sap in terms of DNA quantity and quality. Under good leaf tissue preparation sugarcane tissue give a lot of DNA per a given quantity of tissue.

Primer SCC09 that produced high quality bands (clarity and intensity) was the best for screening seedlings for smut resistance.

5.2 Recommendations

In view of the results of the present study, the following are recommended

1. More populations should be included in the evaluation process to measure the population effect on the smut disease expression in sugarcane seedlings in the plant crops and subsequent ratoons.

2. Further investigations on the resistance levels of the two-selected population (inoculated and non-inoculated) should be done to confirm the usefulness of this method.

3. More primers should be evaluated for smut resistance study in sugarcane seedlings.

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APPENDICES

Appendix 1: LSD mean comparison

t Tests (LSD) for Tiller count at 2 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	38.42262
Critical Value of t	2.14479
Least Significant Difference	5.4275

t Grouping	Mean	N	Family
A	28.917	12	Family2
A	27.917	12	Family1

t Tests (LSD) for Tiller count at 3 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	34.43452
Critical Value of t	2.14479
Least Significant Difference	5.1381

t Grouping	Mean	N	Family
A	30.833	12	Family2
A	26.417	12	Family1

t Tests (LSD) for Tiller count at 4 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	40.69048
Critical Value of t	2.14479
Least Significant Difference	5.5854

t Grouping	Mean	N	Family
A	21.417	12	Family2
A	17.417	12	Family1

t Tests (LSD) for Survival count at 1 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	8.595238
Critical Value of t	2.14479
Least Significant Difference	2.5671

t Grouping	Mean	N	Family
A	21.500	12	Family1
B	18.833	12	Family2

t Tests (LSD) for Survival count at 2 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	13.10119
Critical Value of t	2.14479
Least Significant Difference	3.1693

t Grouping	Mean	N	Family

A	19.833	12	Family1
A	18.583	12	Family2

t Tests (LSD) for Survival count at 3 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	13.10119
Critical Value of t	2.14479
Least Significant Difference	3.1693

t Grouping	Mean	N	Family
A	19.833	12	Family1
A	18.583	12	Family2

t Tests (LSD) for Survival count at 4 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	27.5
Critical Value of t	2.14479
Least Significant Difference	4.5917

t Grouping	Mean	N	Family
A	16.750	12	Family2
A	15.000	12	Family1

t Tests (LSD) for Smut whip count at 3 months after inoculation

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.077381
Critical Value of t	2.14479
Least Significant Difference	0.2436

t Grouping	Mean	N	Family
A	0.5000	12	Family2
B	0.0000	12	Family1

t Tests (LSD) for Smut whip count at 5 months after inoculation

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	2.125
Critical Value of t	2.14479
Least Significant Difference	1.2764

t Grouping	Mean	N	Family
A	2.4167	12	Family2
A	1.4167	12	Family1

t Tests (LSD) for Tiller count at 2 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	38.42262
Critical Value of t	2.14479

Least Significant Difference 7.6757

t Grouping	Mean	N	Inoc_ Method
A	38.833	6	Control
B	25.667	6	W-Paste
B	24.833	6	Soaking
B	24.333	6	Paste

t Tests (LSD) for Tiller count at 3 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	34.43452
Critical Value of t	2.14479
Least Significant Difference	7.2664

t Grouping	Mean	N	Inoc_ Method
A	38.000	6	Control
B	27.333	6	W-Paste
B	24.667	6	Soaking
B	24.500	6	Paste

t Tests (LSD) for Tiller count at 4 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	40.69048
Critical Value of t	2.14479
Least Significant Difference	7.899

t Grouping	Mean	N	Inoc_ Method
A	26.000	6	Control
B A	19.000	6	Paste
B	16.667	6	Soaking
B	16.000	6	W-Paste

t Tests (LSD) for Survival count at 1 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	8.595238
Critical Value of t	2.14479
Least Significant Difference	3.6304

t Grouping	Mean	N	Inoc_ Method
A	28.833	6	Control
B	18.333	6	W-Paste
B	16.833	6	Paste
B	16.667	6	Soaking

t Tests (LSD) for Survival count at 2 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	13.10119
Critical Value of t	2.14479

Least Significant Difference 4.4821

t Grouping	Mean	N	Inoc_ Method
A	27.500	6	Control
B	17.667	6	W-Paste
B	16.667	6	Soaking
B	15.000	6	Paste

t Tests (LSD) for Survival count at 3 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	13.10119
Critical Value of t	2.14479
Least Significant Difference	4.4821

t Grouping	Mean	N	Inoc_ Method
A	27.500	6	Control
B	17.667	6	W-Paste
B	16.667	6	Soaking
B	15.000	6	Paste

t Tests (LSD) for Survival count at 4 months

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	27.5
Critical Value of t	2.14479
Least Significant Difference	6.4937

t Grouping	Mean	N	Inoc_ Method
A	23.000	6	Control
B	13.833	6	Soaking
B	13.500	6	W-Paste
B	13.167	6	Paste

t Tests (LSD) for Smut whip count at 3 months after inoculation

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.077381
Critical Value of t	2.14479
Least Significant Difference	0.3445

t Grouping	Mean	N	Inoc_ Method
A	0.6667	6	W-Paste
B A	0.3333	6	Paste
B	0.0000	6	Control
B	0.0000	6	Soaking

t Tests (LSD) for Smut whip count at 5 months after inoculation

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	2.125

Critical Value of t 2.14479
Least Significant Difference 1.8051

t Grouping	Mean	N	Inoc_ Method
A	2.5000	6	W-Paste
A	2.3333	6	Control
A	2.0000	6	Paste
A	0.8333	6	Soaking

Mean comparison for interactions

Level of Family	Level of Inoc_Method	N	Tiller count at 2 months Mean	Tiller count at 2 months Std Dev	Tiller count at 3 months Mean	Tiller count at 3 months Std Dev
Family1	Control	3	35.3333333	7.5718778	33.0000000	3.0000000
Family1	Paste	3	21.0000000	12.4899960	20.3333333	13.6503968
Family1	Soaking	3	29.3333333	3.0550505	28.0000000	3.6055513
Family1	W-Paste	3	26.0000000	3.6055513	24.3333333	0.5773503
Family2	Control	3	42.3333333	7.5055535	43.0000000	9.0000000
Family2	Paste	3	27.6666667	2.8867513	28.6666667	4.5092498
Family2	Soaking	3	20.3333333	6.0277138	21.3333333	2.0816660
Family2	W-Paste	3	25.3333333	1.5275252	30.3333333	1.1547005

Level of Family	Level of Inoc_Method	N	Tiller count at 4 months Mean	Tiller count at 4 months Std Dev	Survival count at 1 months Mean	Survival count at 1 months Std Dev
Family1	Control	3	21.3333333	3.0550505	28.0000000	2.64575131
Family1	Paste	3	16.0000000	13.0766968	18.0000000	6.55743852
Family1	Soaking	3	16.3333333	8.3266640	21.3333333	1.15470054
Family1	W-Paste	3	16.0000000	9.5393920	18.6666667	1.15470054
Family2	Control	3	30.6666667	3.2145503	29.6666667	0.57735027
Family2	Paste	3	22.0000000	6.2449980	15.6666667	0.57735027
Family2	Soaking	3	17.0000000	2.6457513	12.0000000	2.64575131
Family2	W-Paste	3	16.0000000	1.0000000	18.0000000	1.00000000

Level of Family	Level of Inoc_Method	N	Survival count at 2 months Mean	Survival count at 2 months Std Dev	Survival count at 3 months Mean	Survival count at 3 months Std Dev
Family1	Control	3	25.3333333	2.51661148	25.3333333	2.51661148
Family1	Paste	3	14.6666667	8.14452782	14.6666667	8.14452782
Family1	Soaking	3	21.0000000	2.00000000	21.0000000	2.00000000
Family1	W-Paste	3	18.3333333	1.52752523	18.3333333	1.52752523
Family2	Control	3	29.6666667	0.57735027	29.6666667	0.57735027
Family2	Paste	3	15.3333333	1.15470054	15.3333333	1.15470054
Family2	Soaking	3	12.3333333	2.88675135	12.3333333	2.88675135
Family2	W-Paste	3	17.0000000	2.00000000	17.0000000	2.00000000

Level of Family	Level of Inoc_Method	N	Survival count at 4 months Mean	Survival count at 4 months Std Dev	Smut whip count at 3 months after inoculation Mean	Smut whip count at 3 months after inoculation Std Dev
Family1	Control	3	19.6666667	3.21455025	0.00000000	0.00000000
Family1	Paste	3	12.3333333	9.45163125	0.00000000	0.00000000
Family1	Soaking	3	15.6666667	7.57187779	0.00000000	0.00000000

Family1	W-Paste	3	12.3333333	8.14452782	0.00000000	0.00000000
Family2	Control	3	26.3333333	1.15470054	0.00000000	0.00000000
Family2	Paste	3	14.0000000	2.00000000	0.66666667	0.57735027
Family2	Soaking	3	12.0000000	3.46410162	0.00000000	0.00000000
Family2	W-Paste	3	14.6666667	0.57735027	1.33333333	0.57735027

Level of Level of ----------Smut whip count at 5 months after inoculation---------

Family	Inoc_Method	N	Dead_1 Mean	Dead_1 Std Dev	Mean	Std Dev
Family1	Control	3	4.33333333	2.51661148	2.0000000	2.64575131
Family1	Paste	3	0.00000000	0.00000000	12.0000000	6.55743852
Family1	Soaking	3	0.66666667	1.15470054	8.6666667	1.15470054
Family1	W-Paste	3	0.66666667	1.15470054	11.3333333	1.15470054
Family2	Control	3	0.33333333	0.57735027	0.3333333	0.57735027
Family2	Paste	3	4.00000000	2.00000000	14.3333333	0.57735027
Family2	Soaking	3	1.00000000	1.00000000	18.0000000	2.64575131
Family2	W-Paste	3	4.33333333	2.08166600	12.0000000	1.00000000

Level of Family	Level of Inoc_Method	N	Dead_2 Mean	Dead_2 Std Dev	Dead_3 Mean	Dead_3 Std Dev
Family1	Control	3	4.6666667	2.51661148	4.6666667	2.51661148
Family1	Paste	3	15.3333333	8.14452782	15.3333333	8.14452782
Family1	Soaking	3	9.0000000	2.00000000	9.0000000	2.00000000
Family1	W-Paste	3	11.6666667	1.52752523	11.6666667	1.52752523
Family2	Control	3	0.3333333	0.57735027	0.3333333	0.57735027
Family2	Paste	3	14.6666667	1.15470054	14.6666667	1.15470054
Family2	Soaking	3	17.6666667	2.88675135	17.6666667	2.88675135
Family2	W-Paste	3	13.0000000	2.00000000	13.0000000	2.00000000

Level of Family	Level of Inoc_Method	N	Dead_4 Mean	Dead_4 Std Dev
Family1	Control	3	10.3333333	3.21455025
Family1	Paste	3	17.6666667	9.45163125
Family1	Soaking	3	14.3333333	7.57187779
Family1	W-Paste	3	17.6666667	8.14452782
Family2	Control	3	3.6666667	1.15470054
Family2	Paste	3	16.0000000	2.00000000
Family2	Soaking	3	18.0000000	3.46410162
Family2	W-Paste	3	15.3333333	0.57735027

Appendix 2: Lyophilization process

Sugarcane leaves were harvested from the green house. Young leaves without necrotic areas or lesions were used.

The thick tough mid were removed. The leaves were folded into 10-15 m sections and placed in a fiberglass screen mesh bag along with tag identifying the sample.

The bags were placed in ice chest or other container with ice to keep the sample cool. The samples were placed in a Styrofoam container or type of container able to hold liquid nitrogen. Liquid nitrogen was added to quick freeze the sample.

Frozen leaf samples were transferred to lyophilizer. The lyophilizer was set at a temperature (- 60 ^{o} c) puling a good vacuum (< 10 microns Hg) before loading samples. Samples were dried for 72 hours

Dried leaf samples were stored in sealed plastic bags at room temperatures for a few days

A harvesting record sheet was filled.

Lyophilized leaves were ground to fine powder with a mechanical mill and stored into appropriate plastic container that closed air tight

The ground samples tightly capped were stored at -20 ^{o} c.

Appendix 3: Genomic DNA isolation (Based on method of Saghai and Maroof et al., 1984).

35-40 mg of ground. lyophilized samples was weighed into a 15 ml polypropylene centrifuge tube. 1.0 ml of warm (65 ^{0} c) CTAB extraction buffer was added to the 35-40 g ground, lyophilized tissue and mixed several times by gentle inversion and incubated for 60-90 min, with continuous gentle rocking in 65 $^{\circ}$ c oven.

The tube was removed from the oven, left for 4-5 min for tubes to cool down and 4.5 ml chloroform/octanol added. The tube was rocked gently for 4-5 min and spinned in a table-top centrifuge for 10 min at 1200 x g ^{1} at RT.

The top aqueous layer was poured into a new 15 ml tubes. 4.5 ml chloroform/octanol was added and rocked gently for 5-10 min.

Table –top centrifuge was spinned for 10 min at 1200 x g ^{1} at RT.

Top aqueous layer was pipetted into a new 15 ml tubes containing 6 ul of 10mg/m Rnase and mixed by gentle inversion and incubate for 30 min at RT.

600 ul of isopropanol (2- propanol) was added and mixed by gentle inversion.

Precipitated DNA was removed with a hook and placed in a 2 ml plastic tubes containing 400 ul of wash 1 for 20 min. DNA was rinsed in wash 2 and transferred to 2ml microfuge tube containing 200 ul TE.DNA was stored at 4 $^{\circ}$ c.

Appendix 4: Master mix

Mixture	1 reaction	Final reaction
Template DNA (12.5 ng/ul)	2ul	~25ng
MgCl2 (25Mm)	2ul	2.5Mm
Dntp's(1Mm),1/10dCTP	4ul	200Um Datp, dttp, dgtp and 20uMDCTP
Primer F (6ul)	0.83ul	0.25Um
Primer R (6ul)	0.83ul	0.25
(&33 P) Dctp (200ulCi)	0.08	0.8uCi
10x PCR buffer	2ul	1x
Taq polymerase (10U/ul)	1.0 ul	0.5 U
water	7.26 ul	-
TOTAL	20ul	

Appendix 5: DNA Extraction Using the Sap Extractor (based on method of Clarke *et al.*, 19891)

1. Setting up and using the sap extractor. Make sure that the rollers are completely clean and that the flushing system for cleaning the rollers between samples is connected to a high pressure source of de-ionized water. If you can only use tap water to flush the rollers, make sure that you finally rinse them thoroughly with de-ionized or dH2O between samples. Always wipe the rollers dry using clean, soft tissue paper before initiating the following sample extraction. Position the buffer feeding tip over the upper half of the rollers to ensure that the buffer will mix effectively with the pressed tissue sample. Feed the tissue sample between the rotating rollers at a slight angle to ensure even pressure is applied to a single layer of the tissue (the tissue will wrap around one roller in a spiral).

2. Use 150-250 mg of freshly harvested leaf tissue kept in ice (within a tube) or frozen at -80°C (Within a tube). It is critical that as you feed the tissue into the extractor, between the rollers, the buffer should already be at that position in the rollers. So make sure that you synchronize this operation well with the pumping of the buffer; otherwise, the DNA will be degraded. Pump 1.0 ml of extraction buffer and collect the extract in 2 ml tubes at the tips of the rollers.

3. Incubate the extracts in a water bath or an oven at 65°C for 20-40 min; mix gently twice or continuously during this incubation. Remove the tubes from the heat and let cool for 5-10 min.
4. Extract the samples with 1 ml of octanol-chloroform (1:24). Mix by inversion for 5 min; then spin in a table-top centrifuge at 3200 rpm for 10 min.
5. Transfer the aqueous supernatant containing the DNA to 2.0 ml Eppendorf tubes. If the DNA has to be quantified precisely at the end of the extraction, add 10-20 µl of RNAse A + T1 (see other protocols) in the tube and incubate for 30 min at 37°C, or for one hour at RT.
6. Add 75 µl of 5M NaCl and precipitate DNA with 1 ml of cold absolute ethanol.
7. Spin DNA down, decant ethanol, and dry under a weak vacuum for 30 min.
8. Re-suspend overnight in the cold room in 200-500 µl TE, pH 8.0.
9. 9. Quantify using a gel method or a TKO fluorometer. With this method, a minimum of 15µg of DNA can be obtained.