Sudan University of Science and Technology

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Screening for Resistance to Smut Disease of Twenty Seven Sugarcane Varieties

مسح مقاومة مرض السويد في سبعة وعشرون صنف من قصب السكر

A thesis submitted in partial fulfillment of the requirements for the M.Sc. Degree in Plant Protection

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الآية

قال تعالي:

ِن السَّماءِ ماءً فَأَخْرَجْنا بِهِ نَباتَ كُلِّ شَيْءٍ فَأَخْرَجْنا
مِنْهُ حَبًّا مُتَراكِبًا وَمِنَ النَّخْلِ مِنْ طَلْعِها قِنْوانٌ دانِيَةٌ
وَالزَّيْتُونَ وَالرُّمَّانَ مُشْتَبِهًا وَغَيْرَ مُتَشابِهٍ انْظُرُوا إِلى
ثَمَرِهِ إِذا أَثْمَرَ وَيَنْعِهِ إِنَّ فِي ذلِكُمْ لَآياتٍ لِقَوْمٍ يُؤْمِنُونَ ((99))

صدق الله العظيم

سورة الأنعام((99))

Dedication

To my mother

To my father

To my sisters and brothers

To my family

To my teachers

To my colleagues and friends

With love and respect.

Mohammed

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Abstract

Sugarcane smut (*Sporisorium scitamineum*) is considered cosmopolitan in distribution, and has been important in nearly every sugarcane producing country of the world and in Sudan as well. The control of the disease depends mainly on resistance of cane variety to the pathogen. In the Sudan smut draws greatest attention as the disease is prevalent on all sugar estates and at same times it seriously threatened the industry resulting in that two of the highest yielding varieties for both cane tonnage and sugar (Nco 310 and Nco 376) have been phased out of commercial cultivation. Since then the search for an effective control measures was emphasised. pressent study was aimed of the carried out at the research field of the Guneid Research Centre in seasons 2014-2015 screen newly introduced sugarcane varieties for thier resistance to smut disease using two methods of inoculation (natural and dipping inoculation). The results revealed that the test varieties reacted differently to inoculation with smut disease and hence they gave significantly variable level of infection. The percentage of stool infection ranged from 0% in variety CP 99-1894 that rated as highly resistant (HR) and 76% in variety BSR97051 which was rated as highly susceptible (HS). Moreover, out of the 27 varieties tested, five ones were reacted as highly resistant (HR) in plant cane, namely, (CP 99-1894, FG 03204, B 89640, DB 66113, DP 71060) and one variety FG 04754 rated 2R compared to check variety Co 6806 which is known as highly resistant (HR). These five varieties maintained similar reactions to smut in first ratoon by the two methods. The remaining varieties which reacted and rated less than resistant (R), their reaction rated from moderately to highly susceptible. The smut infection in these varieties was higher in first ratoon than in plant cane. This study proved categorically, that there is positive correlation in the results of the two methods used which suggests that the dip method is suitable for future studies. The study also recommended studies in disease epidemiology.

ملخص البحث

يعتبر مرض التفحم في قصب السكر منتشر عالميا وذو أهميه في كل من الدول المنتجة لقصب السكر في العالم وفى السودان أيضا. تعتمد مكافحة المرض بصورة رئيسية على مقاومة القصب للكائن الممرض. في السودان حظي مرض السويد باهتمام كبير لان المرض متواجد في كل مشاريع السكر وفى وقت ما هدد الصناعة بشكل خطير مما نجم عنه خروج أعلى صنفين إنتاجية من حيث القصب والسكر و هما NCo 310 and NCo 376 من الإنتاج التجاري. ومنذ ذلك الحين تم التركيز على البحث عن طرق فعالة للمكافحة. هدفت الدراسة الحالية والتي نفذت بمزرعة بحوث مركز أبحاث الجنيدفى سنة 2014–2015 إلى فحص مقاومة أصناف من قصب السكر استجلبت حديثا باستعمال اثنين من طرق التلقيح (التلقيح الطبيعي والغمر). أظهرت النتائج أن رد فعل الأصناف المختبرة تجاه التلقيح بمرض السويد كان مختلفا وبالتالي أعطت مستويات إصابة مختلفة معنويا. تراوحت نسبة الإصابة ما بين 0% في الصنف CP 99–1894 والتي قدرت أنها ذات مقاومة عالية و 76%في الصنف BSR97051 والتي قدرت أنها ذات قابلية للإصابة عالية. أيضا من بين الـ 27 صنف التي تم إختبارها، خمسة أصناف كانت رد فعلها عالية المقاومة(HR) بالتحديد هم (CP 99–1894, FG 03204, B 89640, DB 66113, DP 71060) وصنف واحد FG 04754 تم تقديره 2R مقارنة بالصنف الشاهد 6806 والمعروف بمقاومة العالية (HR). هذه الأصناف الخمسة حافظت على رد فعل ضد السويد مشابه في الخلفة الأولى (R1) . بقية الأصناف كانت اقل مقاومة، اذ أن تفاعلهما قدر ما بين متوسط إلى شديد الإصابة. ألإصابة بالسويد في هذه الأصناف كانت أعلى في الخلفة الأولى من القصب الغرس. هذه الدراسة أثبتت بصورة مطلقة أن هنالك إرتباط موجب في نتائج الطريقتين اللذين استعملتا الأمر الذي يشير إلى ملائمة طريقة الغمر في الدراسات المستقبلية. الدراسة أيضا أوصت بدراسات في وبائية المرض.

CHAPTER ONE

INTRODUCTION

Sugarcane is one of the several species of tall perennial true grasses of the genus Saccharum, family Poaceae, native to the warm temperate to tropical regions of South Asia, and used for sugar production (Peter, 1998). Different species likely originated in different locations, with *Saccharum barberi* originating in India and *S. edule* and S. *officinarum* in New Guinea. Approximately 70% of the sugar produced globally comes from open pollinated and hybrids *S. officinarum* (Peter, 2000).

The crop is the world's largest crop produced. FAO (2015) estimated that sugarcane was cultivated on about 26.0 million hectares, in more than 90 countries, with a worldwide harvest of 1.83 billion tons. Brazil is the largest producer of sugar cane in the world. The next five major producers, in decreasing amounts of production, were India, China, Thailand, Pakistan and Mexico (Draycott, 2006).

In Sudan, sugarcane was first grown in about 29 hectares in Berber area. Trails to grow sugarcane were conducted in late 1940s in Zandi area, southern Sudan (Bacon, 1952). Commercial production of sugarcane as industrial cash crop started in 1962/63 with the establishment of Guneid sugar factory. The industry was then rapidly expanded to include New Halfa sugar factory 1964/65, West Sennar sugar factory 1976/77, Assalaya sugar factory1979/81, Kenana sugar company 1980/81 and white Nile sugar company 2011/2012 the total acreage under sugar production is 376.5 thousand acres. The earliest sugarcane cultivars used were NCo310, NCo376 and Co527 which were replaced later on by the present high yielding ones (Co 775, Co 997 and Co 6806) (Obeid, 2005).

Numerous pathogens infect sugarcane, such as sugarcane grassy shoot disease caused by Phytoplasma, sugarcane smut caused by *Sporisorium scitamineum*, pokkah boeng caused by *Fusarium moniliforme*, gumming disease *Xanthomonas axonopodis*, and red rot disease caused by *Colletotrichum falc*atum. Viral diseases affecting sugarcane include sugarcane mosaic virus, maize streak virus, and sugarcane yellow leaf virus. The major constraint facing the productivity of sugarcane crop worldwide is sugarcane smut. It is one of the most serious diseases of this crop. Affected cane is severely stunted and production losses of 30-100% are common in susceptible varieties (Ferreira and Comstock, 1989; Croft *et al* 2000 and Solomon *et al*., 2000).

In Sudan, smut disease was first reported in the Sudan at Guneid on variety NCo 310 (Abu Gideiri, 1965; Nasr and Ahmed, 1974). Currently, it occurs in all sugar estates and causes considerable losses in susceptible varieties. Nasr and Ahmed (1974) reported 100% incidence thus led to the withdrawal of several excellent varieties i.e. NCO 310, Co 527 etc. from production. The use of the resistant sugarcane varieties such as Co 997 and Co 6806 has maintained the disease under some good control. However; occasional infections by *S. scitamineum* especially on Co 6806 (the number one variety) has become common probably suggesting resistance deterioration or variation in the pathogen population Marchelo,*et al*(2008)Since planting of resistance varieties is of paramount importance for disease control the current study aimed for screening of sugarcane varieties for resistance to smut disease with the following objectives:-

- To evaluate the resistance of some newly introduced sugarcane genotypes to sugarcane smut disease caused by *Sporisorium scitamineum* under field condition.
- To compare different methods of screening of sugarcane genotypes for resistance to smut.

CHAPTER TWO

LITREATURE REVIEW

2.1. Sugarcane (*Saccharum* spp.)

Sugarcane is a robust, tall growing, perennial thick-stemmed and monocotyledonous grass crop cultivated in the tropical and subtropical regions of the world primarily for its ability to store high concentrations of sucrose or sugar in the stem (Rena, 1997). Sugarcane is a C4 plant having high efficiency in storing solar energy and most efficient converter of solar energy to sucrose. Sugarcane has essentially four growth phases: 1.Germination phase, 2. Tillering phase, 3. Grand growth phase, 4. Maturity and ripening phase.

The crop is a long duration clonally propagated plant. In the right climate, the cane will grow in 12-14 months (plant cane) and, when cut, will re-grow in another 12 months (ratoon crop). The typical sugar content of mature cane would be 10% by weight but, usually, this varies depending on variety, location and even the season (Peter, 2000). The genus Saccharum has five important species *viz*., *Saccharum officinarum, S. Sinense, barberi, S. robustum* and *S. spontaneum*. The first three species are the cultivated species and the last two are wild ones. *S. officinarum* species is widely cultivated worldwide because of high sucrose content.

2.1.1 Description of the crop

Sugarcane is a tropical, perennial grass that forms lateral shoots at the base to produce multiple stems, typically three to four meters high and about five cm in diameter. The stems grow into cane stalk, which when mature constitutes approximately 75% of the entire plant. A mature stalk is typically composed of 11–16% fiber, 12–16% soluble sugars, 2–3%

non-sugars, and 63–73% water. The crop is sensitive to the climate, soil type, irrigation, fertilizers, insects, diseases and the harvest period.

The plant is composed of four principal parts, root system, stalk, leaves and inflorescence. Sheath is green with red blotches; moderate to heavy bloom; scarious border prominent; sheath splitting occasional clasping; spines present on the middle of the sheath; deciduous. blade joint or transverse mark is purplish green; medium: fair bloom. Ligules': medium; crescent form; symmetrical; gradually tapering towards the edges. The growth pattern includes germination; tillering and the underground nodes of primary shoots give rise to secondary shoots which in turn give rise to tertiary ones. As internodes extension becomes marked, apical dominance exercised by the leading shoots discourages further tiller production (Schueneman, 2002). Initially, tillering is profuse but is normally followed by a wave of mortality as soon as the rows close in. Miller and Lentine., (2002) reported that tillering increases with increasing light intensity and duration and more than 50 % of the number of the initial stalks die, mainly, due to light competition. They pointed out that the most important external factors influencing tillering are light, temperature, nutrition, moisture and spacing. The grand growth phase is also known as the cane formation phase in which upper internodes are rapidly formed and the lower internodes extend length and diameter. This phase is accompanied by the onset of the maturity phase which starts in the lower internodes of the stalk and proceeds gradually towards the upper ones. The maturing /harvesting age of sugarcane varies in the world ranges between 10 to 24 month depending on climatic condition (Alam, and Khan, 2001).

Moreover, sugarcane is a perennial crop. Cane growth produced from the first year of cultivation is called plant cane whereas growth in subsequent year is called "ratoons" or "stubbles" The inflorescence of sugarcane

generally called 'arrow' which is an open panicle. It is long 30 centimeter or more and tapering. Cane is Medium-thick; slightly staggered; slightly oval in cross section, internal tissue yellow with purple tinge: rind hard; pith present as small cavity. Node and buds are slightly depressed; leaf scar slightly inclined. Buds are medium, plumpy, and ovate; occasionally hairs at the tip of the bud; inserted at leaf scar (Draycott, 2006).The sugarcane plant is a largely cross pollinated species with a low frequency of selfing and pollen is dispersed by wind and no insect vectors for sugarcane pollen are known (Mclntyre and Jackson, 2001).Pollen viability is a low under natural conditions and has a life of only 12 minutes and no viability after 35 minutes (More and Nuss, 1987). Modern sugarcane varieties that are commercially cultivated for sugar production are inter-specific hybrids between *Saccharum spontaneum* and *Saccharum officinarum*; and, are therefore generally referred to as Saccharum spp. (Anon, 1984; Cuadrado *et al*., 2004).

2.1.2 Sugarcane production worldwide

Three quarters of the world's sugar is made from sugar cane in tropical zones located in the southern hemisphere. Leading sugarcane producers are Brazil, India, China, Thailand, Pakistan, and Mexico (FAO, 2015).The remainder is processed from sugar beets grown in temperate zones of the northern hemisphere. France, Germany, U.S., Russia, Ukraine and Turkey produce it from sugar beets. Currently, 70% of the world's sugar is consumed in the country where are harvested; only 30% is traded outside country of origin. Global sugar consumption rises by about 2% per year, and has increased by 17%, from 128 million tons in the year 2000 to 150 million in 2006. The highest sugar consumption per capita is found in Brazil (59kg of sugar/annum), Mexico (53 kg sugar/annum) and Australia (50 kg of sugar /annum) (Workman, 2007).

2.1.3 Yield and quality

Yield and quality of sugarcane is a broad term making up the ultimate sugar yield per unit area. The cane yield is the outcome of the number of stalks per unit area together with the average stalk height and thickness. Mature cane, generally, consists of around 70% moisture and 30% dry matter. The dry matter consists of variable amounts of brix and fiber. The brix is the total soluble solids, which include mainly sugar (Pol) together with some salts and other organic non- sugar (Workman, 2007)

2.1.4 Nutritional value of Sugarcane:

The juice sugarcane per serving 28.35 grams contain energy-111.13 kJ (26.56 kcal), Carbohydrates-27.51 g, Protein-0.27 g, Calcium11.23 mg (1%), Iron 0.37 mg (3%), Potassium41.96 mg (1%), Sodium17.01 mg (1%) (Workman, 2007)

2.2 Sugar cane production in Sudan

In Sudan, sugarcane was first grown in about 29 hectares in Berber area. Trials to grow sugarcane were conducted in late 1940s in Zandi area, southern Sudan (Bacon, 1952). Commercial production of sugarcane as industrial cash crop started in 1962/63 with the establishment of Guneid sugar factory. The growing industry was then rapidly expanded to include New Halfa sugar factory 1964/65, West Sennar sugar factory 1976/77, Assalaya sugar factory 1979/81, Kenana sugar company 1980/81 and white Nile sugar company 2011/2012 the total acreage under sugar production 376.5 thousand acres. Farm were all irrigated and located in the central clay plain of Sudan. The projected annual output of these six schemes was 1195 thousand tons sugar (Obeid,. 2005). Sugarcane production in Sudan was supported by establishment of two research stations *viz*., Guneid sugarcane research station and Kenana sugar

company research stations. Sugarcane is grown under condition of high temperature, frequent irrigation, wide inter-row spacing (1.50-1.55m) and the crop receives substantial amount of fertilizers. The earliest sugarcane cultivars used were NCo310, NCo376 and Co527 which were replaced later on by the present high yielding main cultivars (Co775, Co797 and Co6806). Plant crop constituted generally 15% of the total cultivated area leaving the majority for ratoon crop (5-6 ratoons). The growth period of the crop is 14 month for plant cane and 11-12 month for rations. The crop is harvested using both manual and mechanical methods. Harvesting is normally done in winter months. Research finding coupled with management experience resulted in high improvement in cane varieties and cultural practices. These improvements, lately, boosted the average cane yield to reach 116 ton/ha in some sugarcane estates which is comparable to the international yield levels.

2.2.1 Constraints of sugar cane production

Sugar yield per unit area increases with optimum husbandry inputs, fertilizer, irrigation, weed control *etc*; However, it could adversely be affected by several other factors such as untimely planting, unsuitable harvest age and unfavorable climate. In practice, the planting period of sugar cane extends over many months with variable climatic conditions. Similarly, the harvest period extends over the variable weather conditions in the winter season. Lack of optimum planting times and harvest age in these periods constitutes major constraints for sugar cane production.

2.2 Sugarcane smut disease

Numerous pathogens infect sugarcane; bacterial, fungal, viral and nematodes diseases were reported worldwide. Among fungal diseases smut is the most devastating and threatening disease in all sugarcane growing areas World wide.

Sugarcane smut disease caused by the fungus *Sporisorium scitamineum* which was first reported from South Africa in 1877 on Chinese cane (*Saccharum sinense*); Thereafter, many observations were made in Africa, and Asia, in the following decades (Antoine, 1961; Presley, 1978). Smut remained confined to the Eastern hemisphere until 1940 when it was found in Argentina. It has since been recorded in most sugar cane producing countries of the world (Presley, 1978). Only, sugar industries of Eastern Australia, Fiji and Papua New Guinea are still free of the disease. Smut of sugarcane is reported to be a serious disease in most sugarcane producing countries and cane cause considerable yield losses and reduction in cane quality (Ferreira and Comstock, 1989). Croft *et al* (2000) reported yield losses between 20 – 100% depending on the cane variety. Some workers put losses at between 60 -70% (Fawcett, 1942; Raga *et al*., 1972; Alexander, 1995; Solomon *et al*., 2000).

Smut was first reported in the Sudan at Guneid (on variety NCo 310 in Wad-Surur minor) in 1964/65 Abu Gideiri, (1965); Nasr and Ahmed, (1974). Thereafter it was subsequently, confirmed on 35 of the 46 varieties in the variety collections unit at that time, with an incidence of 30-40%. Currently it occurs in all sugar estates and causes considerable losses in susceptible varieties. Nasr and Ahmed (1974) reported 100% incidence in Sudan. It has also led to the withdrawal of several excellent varieties i.e. NCO 310, Co 527 *etc*. from production. The most likely source of this initial infection was never determined, but could have come with imported sugarcane material or wind-blown spores from neighboring countries. The use of the resistant sugarcane varieties such as Co 997 and Co 6806 has maintained the disease under some good control. However, occasional infections by *S. scitamineum* especially on Co 6806 (the number one variety) has become common probably suggesting variety deterioration (resistance erosion) or variation in the pathogen population.

Since then, successful disease management strategies and control require an understanding of the level of diversity in the pathogen population. Information on the genetic variability of the smut pathogen present in Sudan is of paramount important as this will enable plant breeders and pathologists to adopt/select appropriate breeding and control strategies including germplasm selection for increased resistance both in the introduced and current sugarcane genotypes in the country. The disease is sometimes referred to as "culmicolous" smut of sugarcane because it affects the stalk of the cane. At one time or another, sugarcane smut has been important in nearly every sugarcane growing country in the world.

Sugarcane smut does not always pose a serious problem where it occurs. However, the disease may remain unnoticed for years, and then quickly devastate large areas of susceptible varieties. Hence, the disease has been called the "dread disease of sugarcane" by some and a "trivial disease with exaggerated yield losses" by others. Smut can cause significant tonnage losses as well as juice quality losses. Disease development is dependent on the environmental conditions and the resistance of the sugarcane cultivars (Royal Botanical Gardens, 2004).

2.3.1 Classification of sugarcane smut pathogen {*Sporisorium scitamineum* (Syd.) M. Piepenbr; Stoll, M. & Oberw, 2002}

Scientific classification	
Kingdom:	Fungi
Division:	Basidiomycota
Class:	Ustilaginomycetes
Order:	Ustilaginales
Genus:	*Sporisorium*
Species:	***S. scitamineum***
Binomial name	
Sporisorium scitamineum {(Syd.) M. Piepenbr., M.Stoll& Oberw. 2002}	
Synonyms	
Ustilago scitaminea	

2.3.2Symptoms

The most recognizable diagnostic feature of a smut infected plant is the emergence of a "smut whip" (Comstock, 2000). A "smut whip" is a curved, pencil-thick growth, gray to black in color that emerges from the top of the affected sugarcane plant (Figure, 1). These "whips" arise from the terminal bud or from lateral shoots on infected stalks. They can vary in length from a few inches to several feet long. The whip is composed partly of host plant tissue and partly of fungal tissue. Whips begin emerging from infected cane by 2–4 months of age with peak whip growth occurring at the sixth or seventh month.

Other smut symptoms may be evident before the characteristic whip is seen. Spindle leaves are erect before the whip emerges. Affected sugarcane plants may tiller profusely with the shoots being more spindly and erect with small narrow leaves (i.e., the cane appears "grass-like"). Less common symptoms are bud proliferation and leaf and stem galls. Recent modern techniques, molecular and serological based were used to diagnose the disease in early states. Technological advances in PCR-based methods, such as real-time PCR, allow fast, accurate detection and quantification of plant pathogens and are now being applied to practical problems. Singh *et al*., (2004) demonstrated that PCR assay was extremely sensitive in detecting the presence of the pathogen and yielded a positive response in plantlets inoculated with sporidia and observed that PCR assay was significantly better for smut detection than microscopy (Comstock, 2000).

Dry and hot spring weather favors the disease. Plants grown under stress conditions are more prone to develop smut. In 1997, smut was observed on a resistant cultivar CP70-1133 which was grown on sand land under stressed conditions. Low incidences (less than 5%) of smut have been

observed in a few sand land fields of cultivars CP78-1628 and CP73-1547 under stressed conditions. In 2014, several smut affected plants of cultivar CP88-1762 grown on muck soil were seen in an experimental field at the UF/IFAS Everglades Research and Education Center in Belle Glade, Florida (Figure 1).

Plate 1: Sugarcane smut whips on sugarcane cultivar CP88-1762
Credits: Philippe Rott, UF/IFAS

2.3.3 The pathogen:

Although occurrence of several races of *S. scitamineum,* the sugarcane smut fungus, has been reported, the race picture is poorly defined at this time. Part of the problem is due to sugarcane variety-environment interactions causing test-to-test variability regarding pathogenicity. Breakdown of resistance to smut due to appearance of new rust races has been confirmed in locations such as Hawaii and Taiwan, and worldwide genetic variation possibly linked to various pathotypes of *S. scitamineum* has also been reported (Sundar *et al.*, 2012).

2.3.4 Variability

Information on the prevalence and distribution pattern of races/pathotype in S. *scitamineum* in an area is required for effective deployment of host resistance. Schenck (2003) recorded incidence of smut in one variety (H78-7750), considered to be completely resistant in several seed fields on Maui, indicating the possible emergence of a new race of the smut fungus in Hawaii. The new smut race was included in breeding program susceptibility screening, keeping in mind, that smut resistant varieties should also be treated and monitored even though the appearance of new smut races was presumed to be quite rare. The use of differential hosts is a viable option for the evaluation of pathogenic variability. However, not much of information on the use of differential hosts is available in sugarcane against the smut pathogen. Gillaspie *et al*., (1983) used seven sugarcane clones (*Saccharum* interspecific hybrids) for inoculation with *S. scitamineum* isolates collected from Argentina, Florida, Hawaii, Taiwan, and Zimbabwe. Six different isolates (races) could be differentiated on five of the clones under greenhouse conditions and it was concluded that this method is a valid, rapid method for isolate separation when the correct differential clones are used. It was also observed that the environment effects on the teliospores might be confounded with genetic differences amongst the test isolates which might probably complicate breeding for smut resistance. Smut pathogen being biotrophic, the inoculuma henceforth was to be maintained in the standing cane as teliospores. Slow growing fluffy white mycelia was observed from actively growing meristem tips cultured under aseptic conditions, which was further used for molecular characterization of pathogen variability. Smut isolate collection is made from different representative sugarcane growing areas in India and the pathogen

variability is being investigated using differential hosts and molecular markers *viz*. RAPD, SSR *etc* (Ramesh Sundar *et al*., 2002 - personal communication).

The 20th century saw the steady spread of sugarcane smut to almost all sugar industries of the world reviewed by Presley, (1978). A widely adapted, stable smut pathotype may have been involved in this spread, explaining the lack of genetic variation in isolates collected from countries outside of Asia. Pathogenic races of sugarcane smut have been observed in several countries including two races (A and B) from Hawaii (Comstock & Heinz, 1977) and three races (1, 2, 3) reported in Taiwan (Leu *et al*, 1976). However, Ferreira and Comstock (1989) considered the true prevalence of races to be controversial. Many claims were based on the reaction of the same cultivar in different countries, but the interpretation of these claims was confused by test-to-test variation and the use of different inoculation methods in different countries.

Xu *et al*., (2004) studied the genetic diversity of sugarcane smut fungus representing different provinces in Mainland China applying RAPD. Dendrogram of UPGMA cluster analysis revealed that 18 isolates of the fungus were clustered into six groups according to the dissimilarity coefficient of 0.70. The results of cluster analysis suggested that the molecular variation and differentiation could be associated with geographical origin to some extent, but not applicable to all isolates. It might be due to the frequent exchange of sugarcane varieties and clones in the recent years. Molecular diversity analysis observed no relationship between pathogen variability and host origin. Singh *et al.*, (2005) estimated interspecies diversity within *Ustilago scitaminea* isolates from South Africa (SA), Reunion Island, Hawaii and Guadeloupe using RAPDs, *b*E mating-type gene detection, DNA sequence analysis, and spore morphological studies. Mycelial DNA of the South African isolate

shared 100% sequence identity with that of mycelial DNA cultured from *in vitro* produced teliospores of the parent cultivar. Overall, the ITS1 and ITS2 regions were found to have 96.1% and 96.9% sequence identity with a total of 17 and 21 base changes, respectively, amongst the isolates. The Reunion Island isolate was shown to be most distantly related by 3.6% to the other isolates, indicating a single clonal lineage. The lack of germination in teliospores from Guadeloupe might be attributed to changes in temperature and humidity during transportation.

Raboin *et al*., (2007) investigated the genetic diversity and structure of different populations of the smut fungus worldwide using microsatellites by subjecting 77 distinct whips (sori) collected in 15 countries worldwide. Results indicated that the genetic diversity of either American or African *S. scitamineum* populations was found to be extremely low and all strains belonged to a single lineage. This lineage was also found in some populations of Asia, where most *scitamineum genetic diversity S.* was detected, suggesting that this fungal species originated from this region. The results obtained in this study thus suggested that the use of resistant cultivars to *S. scitamineum* might be an efficient and durable strategy to control sugarcane smut outside Asia. Comstock et al., (2007) comprehensively reviewed the status of genetic diversity in *S. scitamineum* and summarized in line with the results presented during the International Sugarcane Technologists workshop 2006. It was concluded, that the fungus originated in Asia and was disseminated to other continents on rare occasions. It was also indicated that, the resistance reaction of sugarcane clones tested in various countries was strongly influenced by the environment. The possibility of using Near Infra Red spectroscopy (NIR) in prediction of disease resistance rating for smut disease was investigated. The results were promising and the model provided acceptable predicted ratings for all the clones. Munkacsi *et al*.,

(2007) suggested that domestication and cultivation of crop plants did not drive divergence and speciation of smut species on maize, sorghum, and sugarcane. The results obtained greatly weakened a hypothesis, that the speciation of crop pathogens is the necessary result of agricultural practices, and further, showed that these fungi diverged in natural populations of the fungus and host. Most importantly, the findings demonstrated that the domestication process very likely retained symbioses between the crops and scores of microbes, which had co-evolved in ancestral, natural populations. Fattah *et al*., (2009) attempted genotyping of the races of *Ustilago* species in Egypt using the chitinase gene primers. The study concluded that chitinase genes are the most suitable for genotyping study between sugarcane smut fungal isolates. The results obtained by differential display techniques showed that there were at least 10 different races from the *Ustilago sp.* In Egyptian field. Nzioki *et al*., (2010) attempted to identify presence of physiological races of sugarcane smut and the results suggested possible existence of smut races in Kenya.

2.3.5 Disease cycle

Sugarcane smut is disseminated via teliospores that are produced in the smut whip. These teliospores located either in the soil or on the plant, germinate in the presence of water (Waller, 1969). After germination they produce promycelium and undergo meiosis to create four haploid sporida. Sugarcane smut is bipolar and therefore produces two different mating types of sporida. For infection to occur, two sporida from different mating types must come together and form a dikaryon. This dikaryon then produces hyphae that penetrate the bud scales of the sugarcane plant and infect the meristematic tissue. The fungus grows within the meristematic tissue and induces formation of flowering structures which

it colonises to produce its Teliospores (Croft and Braithwaite, 2006). The flowering structures, usually typical grass arrows, are transformed into a whip like sorus that grows out between the leaf sheaths. At first it is covered by a thin silvery presidium (this is the host tissue) which easily peels back when desiccated to expose the sooty black-brown teliospores. These teliospores are then dispersed via wind and the cycle continues. The spores are reddish brown, round and subovoid and may be smooth to moderately echinulate. The size varies from 6.5 to 8 um. Sugarcane cultivars intended for distribution to other geographical areas should be tested for susceptibility to *S. scitamineum* populations in each area (Que *et al., 2012).*

2.3.6 Dissemination of spores

Sugarcane smut is spread by microscopic spores. The spores are particularly adapted to aerial dispersal and can be spread over great distances by wind currents (Ferreira and Comstock 1989). The whip serves as a source of spores. It has been shown that approximately one billion spores per whip per day can be released into the air. Standing sugarcane plants become infected in the buds. Since many infected buds remain dormant until sugarcane stalks are cut for seed (stalks cuttings) and planted, the use of infected seed cane is another important way that the disease is spread. Strict quarantine measures are necessary in affected areas.

Windborne spores may settle on the soil of cropped or newly prepared fields. Disease-free seed pieces may become infected if planted in soil containing viable spores. The spores, however, only survive for a short time in the soil under normal soil moisture regimes. Several species of insects have been consistently associated with smut whips and spores have been found on their bodies. These observations suggest insects

could play a role in spore dissemination. Although sugarcane smut has been reported on a few other members of the grass family, there are probably no important naturally occurring alternative hosts outside the *Saccharum* species.

2.3.7 Environment

Sugarcane smut is a very widespread disease and is prevalent in Central and South America, Africa, and South-Western Asia. Sugarcane smut has been reported in all countries that lie between 20 degrees north and south of the equator (Marin *et al*., 1961). The pathogen does well in hot dry weather (Riley and Jubb et al., 1999). for most of the disease cycle but requires wet conditions for teliospores to germinate.

2.3.8 Economic Impact

It is difficult to make precise assessment of the economic importance of *S .scitamineum* since most estimates of yield losses are based on observation and experience rather than rigorous experimentation. It is certain, however, that losses may be quite sever in susceptible varieties under conditions suitable for disease development. There are reports of yield losses of 50-73%in addition to cane yield losses. *S. scitaminea* also appears to reduce cane quality. Decrease in both sugar extractability and recovery, as estimated by reductions in juice purity, have been reported. *S. scitaminea* is also known to cause decrease in the number of millable stalks as well as in stalk diameter. In Hawaii, highly susceptible varieties showed cane yield losses of 10-15% in severely infected commercial ratoon field, while losses in sugar processing were an additional 5-7 %.(Ferreira and Comstock,.1989)

Descriptions of *S. scitaminea* epidemics in various countries suggest that severe disease losses are associated with hot dry climates where crops

may experience water stress. Additionally, crop age and growth stage at the time of infection becomes more severe at the number of ratoons increases. *S. scitaminea* does not always pose a serious problem where it occurs. One unexplained aspect of the disease is that both incidence and severity appear to be cyclical. Severe epidemics are often followed by periods when smut can be difficult to find. To date there has been no reasonable explanation of this behavior.

2.3.9 Prevention and Control

Growing of resistant sugarcane cultivars is the best approach to smut control and has been used successfully in all sugarcane growing areas worldwide. There is a strong genetic basis for resistance. Resistant varieties have been readily available and used to control outbreaks of smut in several countries.

Rouging diseased stools has been successful in some instances usually in foreign countries where the lower wages allow repeated rouging. However, it is not practical for severe outbreaks involving commercial acreage. Rouging may be effective in seed nurseries where smut incidence is generally low.

Using disease-free seed cane is also very important for disease control. Care should be taken because this disease can be latent and show up only after planting. Disease-free planting material can usually be obtained by subjecting seed to a hot water treatment.

Various hot water treatments have been reported to be effective in controlling the smut pathogen residing in the planting setts, but it may not be practical on large scale cultivation and its effectiveness may be subject to varietal differences. The loss of bud germination due to in appropriate temperature settings needs to be handled properly (Srinivasan & Rao,

1968). Hardening of setts prior to hot water treatment was observed to considerably improve upon the germination of the buds. The efficacy of moist hot air treatments have been reported by Misra *et al*., (1978). Gupta *et al*., (1978) reported production of thicker and heavier canes with an increased number of millable canes due to hot water treatment.

Joyce *et al*., (2008) attempted to utilize smut resistant varieties in genetic modification research programs leading to commercial GM crop development in Australia. Protocol optimization was done for selecting an efficient tissue culture medium to produce embryogenic celli with high transformation efficiency.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Location of study:

This study was conducted in the Sugarcane Research Center, Guneid; located approximately at latitude 15°N, longitude 33°E in season 2014/2015. The soils in the experimental site are of the typical heavy clay vertisols with about 64% clay, 0.09%N, 2-8ppm available P and alkaline in reaction with pH of 8.2. Mean annual rainfall is about 112 mm falling mainly in July and August.

3.2. Test varieties

The experiments conducted included 27 newly introduced sugarcane varieties obtained from bulking plots of varieties at Gunied Sugarcane Research Station that passed quarantine periods in addition to variety Co 6806 which used as highly resistant check.

3.3. Collection and maintenance of *S. scitamineum* inoculum

Typical sugarcane smut whips or sori were collected from Sennar, Assalaya, Newhalfa, Gunied commercial sugarcane fields. Whips were then shade dried for 72 hrs. Thereafter, smut teliospores were extracted using a 200 mm diameter (500, 250 and a final 106) micro aperture or mesh) laboratory sieves mounted on an Endecott's sieve shaker model EFL 2000. the teliospores were then maintained in sealed polythene bags in the laboratory prior to use for artificial inoculation trials.

3.4. Seedbed preparation and planting materials

For all field experiments , the land was prepared according to the standard practice; by a disc plough then harrowed , leveled and ridged . The spacing between ridges was 1.5 m. plot size was I or 2 rows of 5 or 10 meter length .

3.5. Preparation of sugarcane differentials:

Three-node cuttings were prepared from each of the twenty-seven introduced varieties and commercial cultivar Co 6806. All varieties were 8-10 month old. The tested varieties were: TCP 93-4241, FG 066700, CP 99-1894, FG 03425, DP 71060, FG 03318, DB 66113, FG 03487, FG 03418, FG 03520, FG 04463, FG03372, VMC 95173, DB 70047, FG 03204, FG 06729, FG 04754, PSR 97092, BSR 97051, BJ85-34, BBZ 951034, B 89640, BJ 82118, B 93775, B 93712, B 04996, B 041291. The secured cuttings from each variety were given a long hot water treatment (HWT) at 50^0C for 2 hr before being artificially inoculated by two methods; namely, (a) the dip methods (DM) and (b) natural spreader row method (NSRM) as described by Marchelo *et al*., (2008).

3.6. Inoculation protocol/ methods and field design:

All test materials were inoculated by each of two methods

3.6.1. Dip methods (DM):

The seed setts inoculated by dipping or immersion into a smut spore suspension at a concentration of 1 g smut teliospores/L of water for 15-20 minutes. The inoculated seed setts were then kept under humid condition in polythene bags for 24 hr prior to planting in the field. The plot size was 1 furrow of 10 m length and furrows spaced 1.5 m apart. A 20 cane setts were planted per plot and the plots were arranged in a randomized

complete block design with three replications. Sixty buds planted in each plot.

3.6.2 Natural spreading method (NSM):

In this protocol, healthy setts of the tested materials were inter-planted with susceptible cultivar NCO 376 as inoculums spreader. Spreader row material was prepared by dipping method as mentioned in 3.6.1., and planted in one speeder row (furrow) between two tested genotypes. The plot size was 1 furrow of 5 m length and furrows spaced 1.5 m apart. A 20 cane setts were planted per plot (Sixty buds in each plot) and the plots were arranged in a randomized complete block design with three replications.

3.7. Disease incidence and assessment of resistance

Disease incidence was determined from the proportion of diseased stools expressed as percentage of the total number of the stools in each plot. Resistance as expressed by reaction types was evaluated with a numerical rating scale of 1-9 where, 1=highly resistant and 9=highly susceptible as described by Satya Vir Beniwal (1978) table(1). The cumulative number of whips as an infection index was also recorded. The final genotypic reaction types in all trails were determined at the age of 16 months for plant cane (PC) and ratoons cane (RC).

3.8. Data collection

Data on (i) infection index such as (a) smut incidence on stools basis (SI%) and (b)cumulative number of smut whips (CNSW) during the growing season was determined; and (ii) epidemiological parameters namely,(a)the latent infection period (= time period from inoculation to disease symptom expression) in days (LIP/D); (b)sustained disease

duration (=time from inoculation to disease symptom expression)or LIP/D to harvest in days (SDD/D).

3.9. Statistical analysis

The collected data in both methods were subjected to an analysis of variance by either the statistical software MSTAT-C or SAS and Duncan's Multiple Ranges Test was used to locate differences between the treatment means.

3.10. Evaluation of sugarcane germplasm against smut:

Table 1: Numerical rating system for sugarcane smut according to (Satya Vir Beniwal, 1978)*

Percentage infection	Rating	Definition /Reaction type
0 to3%	1	Highly Resistant (HR)
4 to6%	2	Resistant (R)
7 to 9%	3	Resistant (R)
10 to12%	4	Resistant (R)
13 to25 %	5	Moderately susceptible (MS)
26 to 35%	6	Susceptible (S)
36 to 50%	7	Highly susceptible (HS)
51 to 65%	8	Highly susceptible (HS)
66 to 100%	9	Highly susceptible (HS)

CHAPTER FOUR

RESULTS

4.1 Reaction of varieties inoculated with natural infection method

Table, 2, figure, 1 and figure 2, showed that the reaction of test varieties to smut disease of sugarcane inoculated with natural infection method. The results obtained showed that the percentage of stool infection ranged from 0% in variety CP 99-1894 that rated as highly resistant (HR) and 76% in variety BSR 97051 which rated as highly susceptible (HS). Out of 27 varieties tested, five varieties reacted as highly resistant in plant cane, namely, (CP 99-1894, FG 03204, B 89640, DB 66113, DP 71060 and one variety FG 04754 rated 2R compared to check resistant cultivar Co 6806 which was rated HR. five varieties maintained similar reactions to smut in first ratoon. The remaining varieties which reacted and rated less than resistant (R), their reaction ranged from moderately to highly susceptible according to Satya Vir (1978) scale of resistance. The smut infection in these varieties was higher in first ratoon than in plant cane. However, other than the varieties which rated as HR or R, the percentages of stools infection was observed to be higher in first ratoon (R1) than plant cane.

Table 2: Rating varieties of smut disease reactions to smut inoculated with natural infection method in plant cane (PC) and in first ratoon (R1).

Natural infection methods		
	Natural infection methods (P.C)	Ratoon, 1 cane(p.c)
Cultivars	Rating	Rating
CP 99-1894	1HR	1HR
FG 03204	1HR	1HR
B 89640	1HR	1HR
DB 66113	1HR	1HR
DP 71060	1HR	1HR
CO 6806	1HR	1HR
FG 04754	2R	2R
BJ 82118	2R	6S
FG 03418	4R	7HS
FG 03425	4R	7HS
BBZ 951034	4R	6S
VMC 95173	5MS	9HS
FG 03372	5MS	8HS
DB 70047	5MS	8HS
B 93712	5MS	5MS
FG 04463	5MS	6S
B 93775	5MS	5MS
FG 066700	6S	7HS
FG 03318	6S	7HS
FG 03520	6S	8HS
TCP 93-4214	6S	7HS
FG 03487	6S	9HS
FG 06729	6S	9HS
PSR97092	7HS	9HS
BJ 85-34	7HS	9HS
B 041291	8HS	9HS
B 04996	9HS	9HS
BSR 97051	9HS	9HS
SE	0.90	0.91
CV%	34.81%	26.83%
LSD	2.57	2.58

Figure 1: Percentage of smut stool infection in plant cane (PC) of sugarcane varieties inoculated with natural infection method

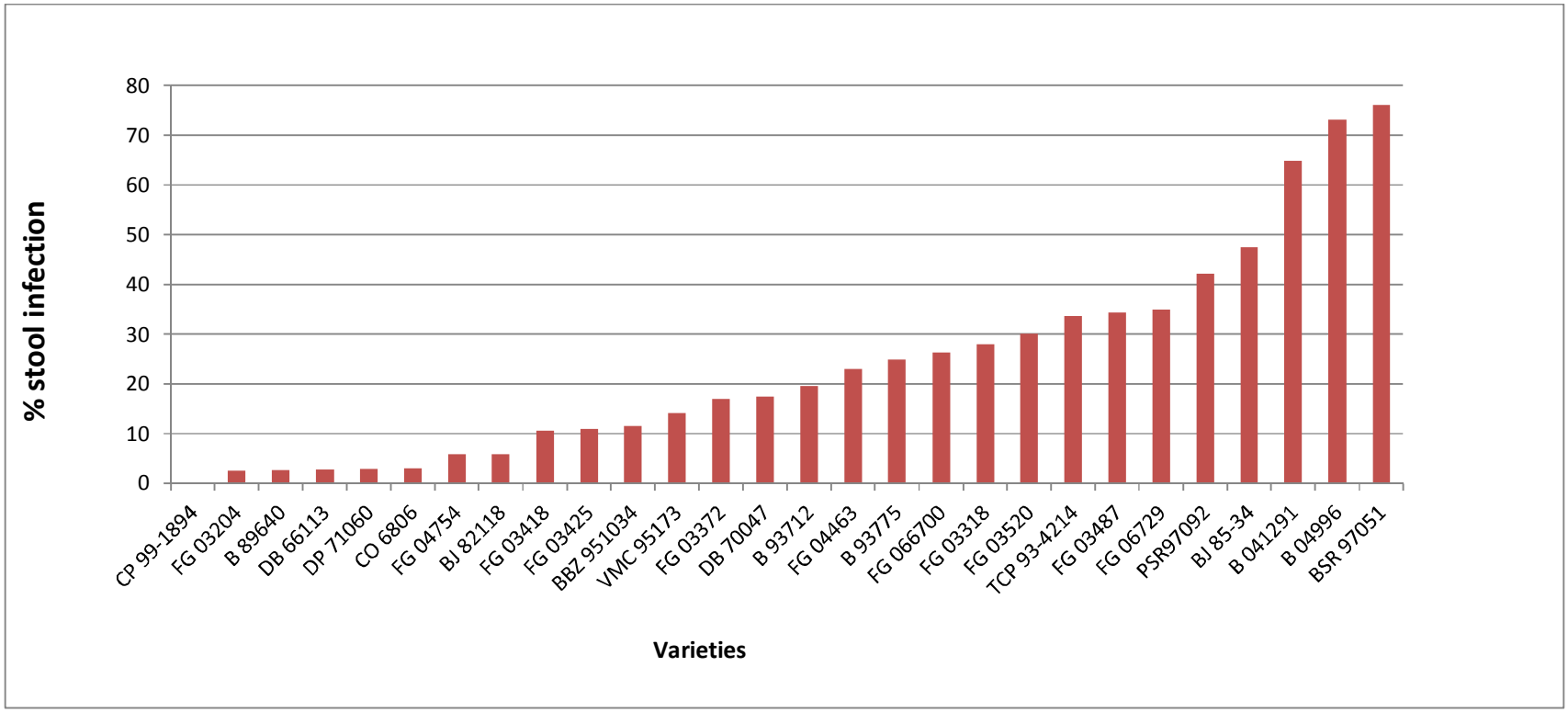
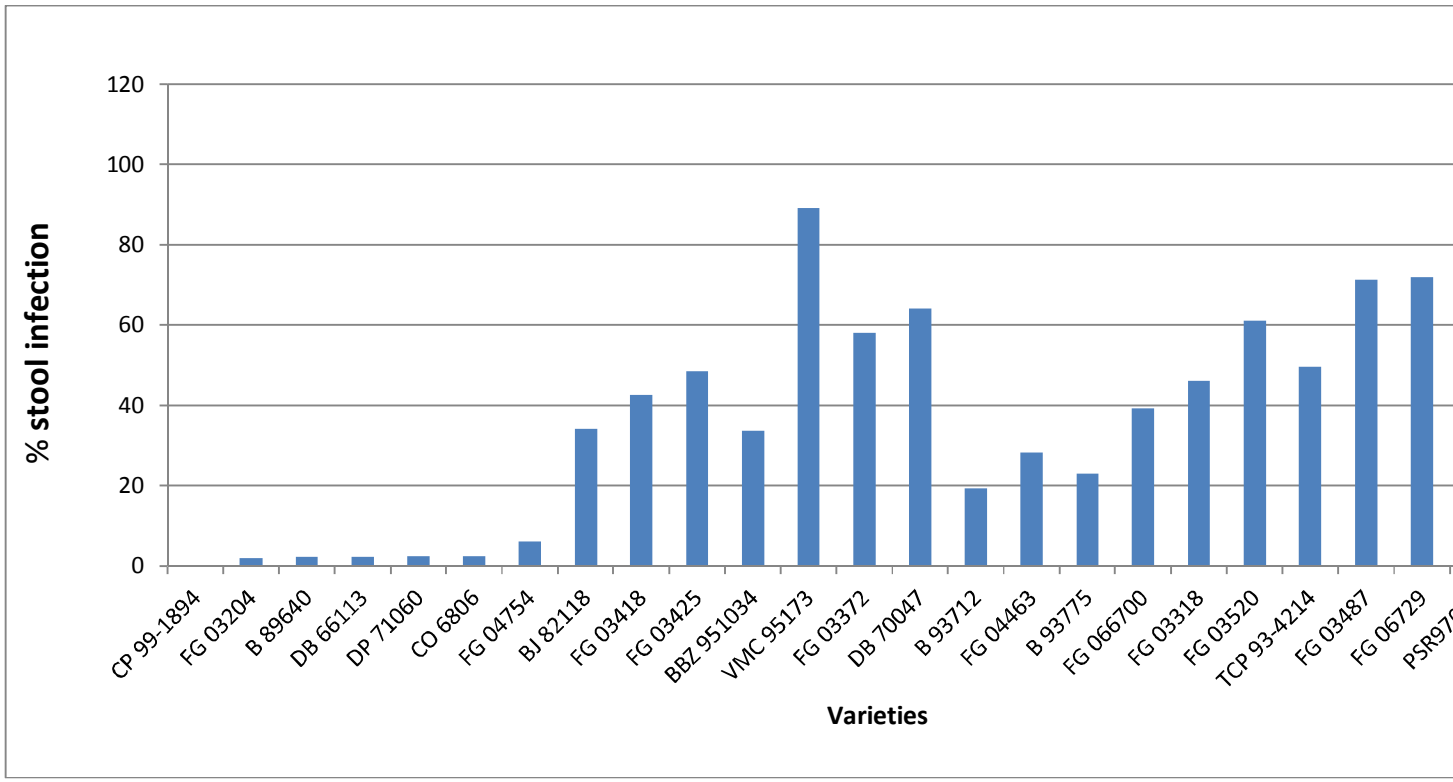


Figure 2: Percentage of smut stool infection in first ratoon (R1) of sugarcane varieties inoculated infection methods



4.2 Reaction of varieties to smut inoculated with dip inoculation method

The reaction of test varieties to smut disease of sugarcane inoculated with dip inoculation method was presented in Table, 3, figure 3 and 4. The data revealed that the percentage of stool infection ranged from 1% in variety CP 99-1894 that rated as highly resistant (HR) and 87% in variety VMC 95173which are rated as highly susceptible (HS). The reactions of the varieties inoculated with dip method are more or less similar to that of natural method in plant cane (Table, 4 and fig. 5). Moreover, there is a positive correlation (0.56) in the results of the two methods. The remaining varieties which reacted and rated less than resistant (R), their reaction ranged from moderately to highly susceptible according to Satya Vir (1978) scale of resistance. The smut infection in these varieties was higher in first ratoon than in plant cane. However, other than the varieties which rated as HR or R, the percentages of stools infection was observed to be higher in first ratoon (R1) than plant cane.

Table 3: Rating varieties of smut disease reactions to smut inoculated with dipping infection method in plant cane PC and in first ratoon R1

Dipping infection methods		
plant cane(P.C)		Ratoon cane (R1)
Varieties	Rating	rating
CP 99-1894	1HR	1HR
CO 8606	2R	2R
B 93775	4R	6S
FG 04754	5R	5R
DP 71060	5MS	7HS
FG 066700	5MS	8HS
BBZ 951034	5MS	7HS
BJ 82118	5MS	5MS
B 89640	6S	7HS
FG 03425	6S	7HS
DB 66113	6S	6S
B 93712	6S	7HS
B 04996	6S	8HS
BJ 85-34	6S	9HS
FG 03204	6S	6S
TCP 93-4214	7HS	8HS
FG 04463	7HS	8HS
FG 03418	7HS	7HS
FG 06729	8HS	9HS
DB 70047	8HS	8HS
FG 03372	8HS	8HS
B 041291	8HS	9HS
FG 03318	8HS	8HS
FG 03520	8HS	8HS
PSR97092	8HS	9HS
BSR 97051	8HS	9HS
FG 03487	9HS	9HS
VMC 95173	9HS	9HS
SE	0.74	0.72
CV%	21.69%	19.40%
LSD(0.05)	2.09	2.03

Figure 3: Percentage of smut stool infection in plant cane (PC) of sugarcane varieties inoculated with dip infection methods.

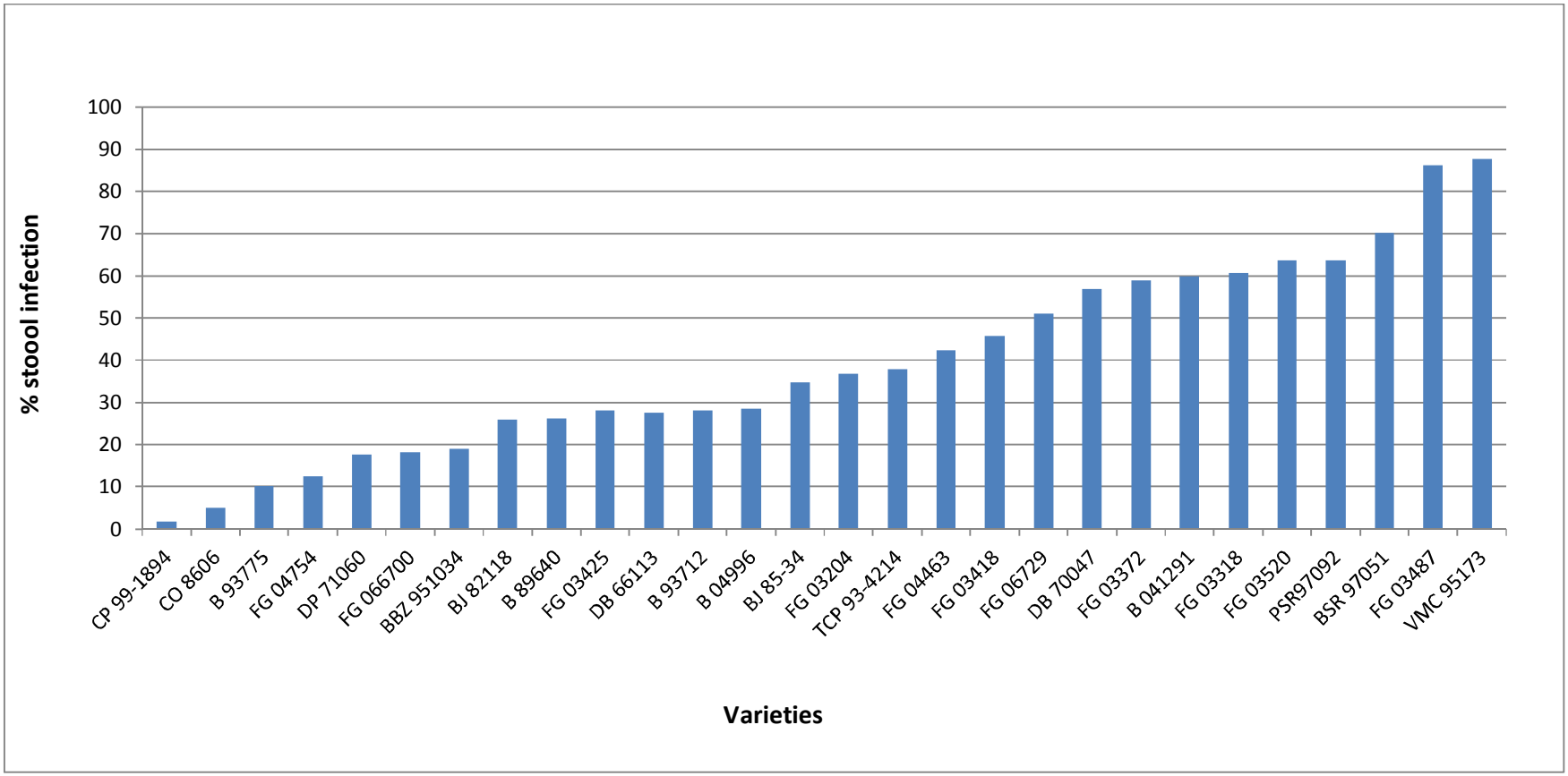


Figure 4: Percentage of smut stool infection in fist ratoon (R1) of sugarcane varieties inoculate methods

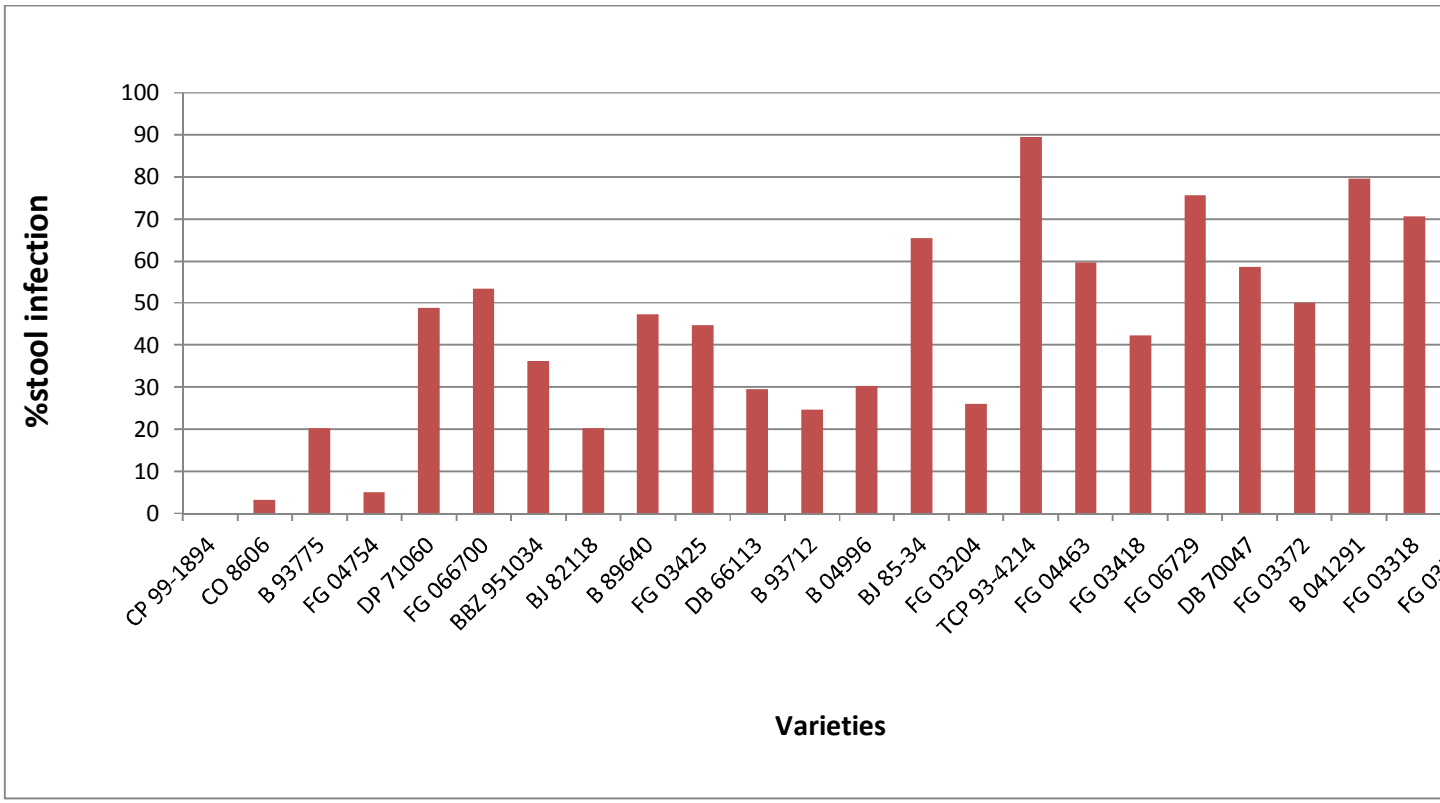


Table 4: Percentage of stool infection showed the correlation between the natural infection methods and dipping infection methods in plant cane.

Cultivars	%stool infection in natural (P.C)	% stool infection in dipping (P.C)
CP 99-1894	0.71	0.71
FG 03204	1.55	5.69
B 89640	2.39	5.18
DB 66113	2.82	5.26
DP 71060	2.9	4.2
CO 6806	2.9	2.1
FG 04754	3.21	3.36
BJ 82118	3.21	4.94
FG 03418	3.24	6.67
FG 03425	3.36	5.2
BBZ 951034	3.67	4.38
VMC 95173	4	9.38
FG 03372	4.26	7.56
DB 70047	4.35	7.5
B 93712	4.48	5.29
FG 04463	4.53	6.49
B 93775	4.54	3.2
FG 066700	5.14	4.27
FG 03318	5.15	7.75
FG 03520	5.22	7.96
TCP 93-4214	5.29	6.15
FG 03487	5.75	9.27
FG 06729	5.8	7.09
PSR97092	6.37	8.04
BJ 85-34	6.76	5.68
B 041291	7.36	7.73
B 04996	7.8	5.34
BSR 97051	7.82	8.45
Correlation	0.56	

Figure 5: Percentage of smut stool infection in plant cane (PC) of sugarcane varieties inoculated with natural infection methods

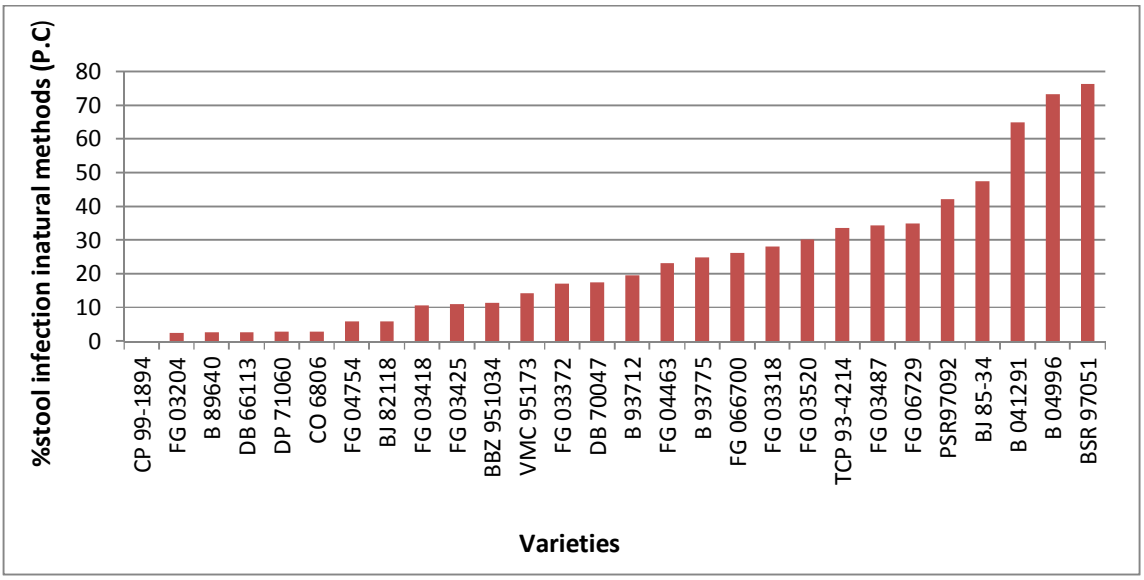
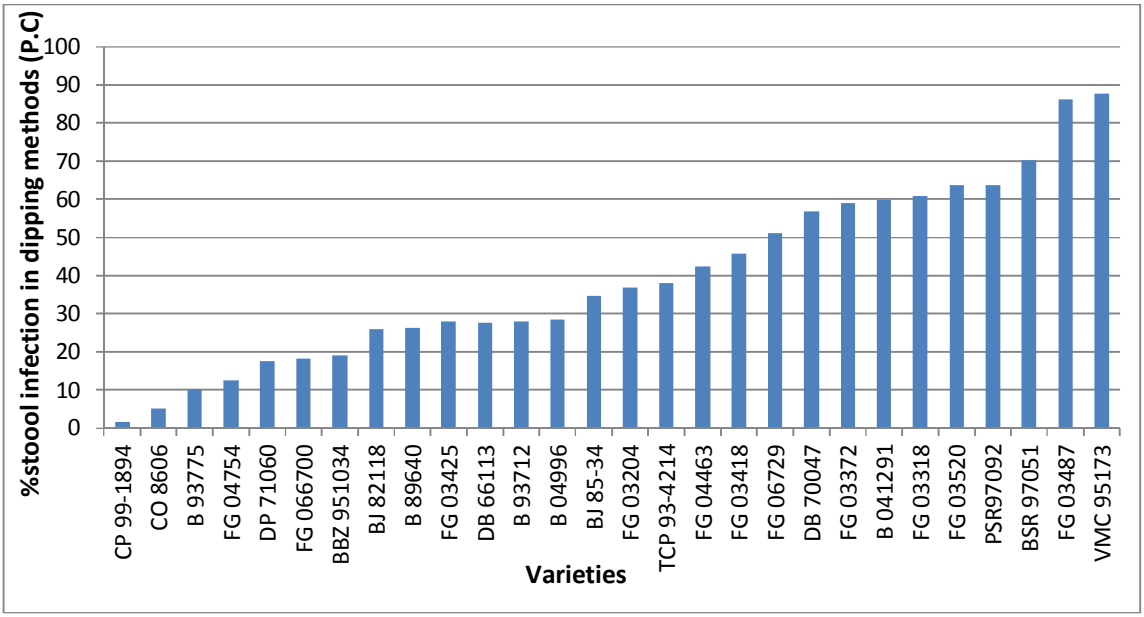


Figure 6: Percentage of smut stool infection in plant cane (PC) of sugarcane varieties inoculated with dip infection methods



CHAPTER FIVE

DISCUSSION

The stem or culmicolous smut (*Sporisorium scitamineum*) of sugarcane is cosmopolitan in distribution, and at one time or another has been important in nearly every sugarcane producing country of the world (Ferreira *et al*., 1980 and Peter, 1998). Management of the disease depends mainly on resistance of cane variety to the pathogen where in absence of resistant varieties losses up to 100% is expected (Croft *et al.,* 2000). In the Sudan smut disease draws greatest attention because the disease is prevalent on all sugar estates and at times it seriously threatened the industry resulting in that two of the highest yielding varieties for both cane tonnage and sugar (Nco 310 and Nco 376) have been phased out of commercial cultivation (Nasr and Ahmed, 1974). Since then the search for an effective control measures was emphasised.

Obviousyl, the use of resistant varieties is one of the best approach to control smut disease. Infact, the aspect of evaluation of varietal disease reaction has been adopted a number of recently introduced varieteis to the Sudan and locally bred ones (Marchelo *et al*., 2008). In fact, reflection of the negative relation between the distribution pattern of the fungus and resistance to the disease is an important aspect in the host-pathogen interaction as it suggests the existance of a form of resistance that was different and independant of factors governing bud infection. The observation could also account for variation in incubation period for whip development.

Accordingly, this study was under taken to screen 27 newly introduced varieties of sugarcane for resistance to smut disease using two methods of

inoculation, dip infection and natural infection. The results revealed that out the 27 varieties tested, five ones reacted as highly resistant, |Namely, (CP 99-1894, FG 03204, B 89640, DB 66113, DP 71060 and one variety FG 04754 rated 2R compared to check resistant cultivar Co 6806 which was rated HR according to the scale Satya (1978). The categorization of sugar cane based on their reactions to inoculation with smut disease using dip and natural infection methods was also used by Nasr and Ahmed (1974); Croft *et al*., (2000) and Marchelo *et al*., (2008). The remaining varieties which reacted and were rated less than (R) are not suitable for commercial cultivation.

The results revealed that the test varieties expressed slightly higher percentage of stool infection in first ratoon than plant cane in the two methods of inoculation. These results were in line with Nasr and Ahmed (1974) and Marchelo *et al*., (2008) who reported the buildup of smut in successive ratoons of cane especially in susceptible varieties.

Obviously, the dip inoculation is the most widely used method for screening varieties in sugarcane breeding programs (Lee-Lovick, 1978; Ferreira *et al*., 1980). In this study, the results obtained from natural infection trials indicated that the dip inoculation method showed a good correlation with natural infection method. This result is in line with Ferreira *et a*l. (1980) who reported a good correlation between the two methods in Hawaii. They reported two main constraints for the natural infection trial, compared with dip inoculation. This is because natural infection requires (i) at least two ratoon cycles to get a reliable result (Ferreira *et al*., 1980) and (ii) more than double the area for trial establishment an observation that encountered in this study.

Conclusions and Recommendation

Conclusions

Based on the results of this study it can be concluded that:-

- The reflection of the negative relation between the distribution pattern of the fungus and resistance to the disease is an important aspect in the host-pathogen interaction as it suggest the existance of a form of resistance that was different and independant of factors governing bud infection.
- Out of 27 varieties tested using dip and natural inoculation methods, five ones were proven to be as highly resistant to smut disease, namely, (CP 99-1894, FG 03204, B 89640, DB 66113, DP 71060 and one variety FG 04754 rated 2R compared to check resistant cultivar Co 6806 which was rated HR
- Variety CP 99-1894 demonstrated a very high degree of resistant to smut disease in plant cane and first ratoon in the two methods of inoculation.
- The experiment obsreved that the level of smut infection in susceptible varieties was observed to be higher in first ratoon than plant cane and hence should be excluded them from commercial planting
- The study revealed that the dip inoculation method is suitable for screening sugarcane varieties for resistance to smut disease as it was positively correlated with natural infection method which requires more time and space.

Recommendation

- To continue research pertaining to disease expression and rates of disease increase in ratoon crops.
- More research was needed to choose whether to use percentage of stool infection or whip percentage as parameter for evaluating the level of resistance in sugarcane.
- To make use of the variety CP99-1894 this expressed high level of resistance in commercial and breeding programmes.

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Appendices

Appendices (1): infection (natural p.c)

Grand Mean = 4.514 Grand Sum = 379.200 Total Count = 84

T A B L E O F M E A N S

1	2	3	Total
1	*	4.529	126.800
2	*	4.860	136.080
3	*	4.154	116.320
*	1	5.290	15.870
*	2	5.140	15.420
*	3	0.710	2.130
*	4	3.363	10.090
*	5	2.907	8.720
*	6	5.153	15.460
*	7	2.827	8.480
*	8	5.750	17.250
*	9	3.240	9.720
*	10	5.220	15.660
*	11	4.530	13.590
*	12	4.260	12.780
*	13	4.000	12.000
*	14	4.353	13.060
*	15	1.553	4.660
*	16	5.800	17.400
*	17	4.000	12.000
*	18	6.377	19.130
*	19	7.820	23.460
*	20	6.760	20.280
*	21	3.670	11.010
*	22	2.397	7.190
*	23	3.213	9.640
*	24	4.547	13.640
*	25	4.487	13.460
*	26	7.800	23.400
*	27	7.367	22.100
*	28	3.867	11.600

Appendices (2):

A N A L Y S I S O F V A R I A N C E T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	6.981	3.491	1.4138	0.2521
2	Factor A	27	249.008	9.223	3.7356	0.0000
-3	Error	54	133.316	2.469		
	Total	83	389.305			

Appendices (3):

Infection (dipping p.c)

Grand Mean = 5.911 Grand Sum = 496.550 Total Count = 84

T A B L E O F M E A N S

1	2	5	Total
1	*	6.726	188.320
2	*	5.668	158.710
3	*	5.340	149.520
*	1	6.150	18.450
*	2	4.270	12.810
*	3	1.273	3.820
*	4	5.207	15.620
*	5	4.200	12.600
*	6	7.753	23.260
*	7	5.267	15.800
*	8	9.277	27.830
*	9	6.673	20.020
*	10	7.967	23.900
*	11	6.493	19.480
*	12	7.560	22.680
*	13	9.383	28.150
*	14	7.507	22.520
*	15	5.697	17.090
*	16	7.097	21.290
*	17	3.367	10.100
*	18	8.043	24.130
*	19	8.457	25.370
*	20	5.680	17.040
*	21	4.387	13.160
*	22	5.183	15.550
*	23	4.947	14.840
*	24	3.203	9.610
*	25	5.297	15.890
*	26	5.347	16.040
*	27	7.733	23.200
*	28	2.100	6.300

Appendices (4):

A N A L Y S I S O F V A R I A N C E T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	29.365	14.682	8.9320	0.0004
2	Factor A	27	341.844	12.661	7.7022	0.0000
-3	Error	54	88.765	1.644		
	Total	83	459.974			

Appendices (5):

Natural infection methods (R1)

Grand Mean = 6.341 Grand Sum = 532.672 Total Count = 84

T A B L E O F M E A N S

1	2	3	Total
1	*	6.815	190.810
2	*	6.332	177.302
3	*	5.877	164.560
*	1	7.623	22.870
*	2	6.517	19.550
*	3	0.710	2.130
*	4	6.727	20.180
*	5	6.627	19.880
*	6	7.327	21.982
*	7	5.423	16.270
*	8	7.963	23.890
*	9	6.673	20.020
*	10	7.787	23.360
*	11	7.773	23.320
*	12	7.133	21.400
*	13	9.097	27.290
*	14	6.940	20.820
*	15	5.547	16.640
*	16	7.860	23.580
*	17	2.270	6.810
*	18	7.753	23.260
*	19	8.130	24.390
*	20	8.167	24.500
*	21	5.963	17.890
*	22	6.757	20.270
*	23	4.780	14.340
*	24	4.467	13.400
*	25	4.830	14.490
*	26	6.160	18.480
*	27	9.000	27.000
*	28	1.553	4.660

Appendices (6):

A N A L Y S I S O F V A R I A N C E T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	12.308	6.154	4.0681	0.0226
2	Factor A	27	352.517	13.056	8.6307	0.0000
-3	Error	54	81.689	1.513		
	Total	83	446.514			

Appendices (7):

Infection dipping methods (R1)

Grand Mean = 5.891 Grand Sum = 494.820 Total Count = 84

T A B L E O F M E A N S

1	2	4	Total
1	*	6.197	173.510
2	*	5.492	153.790
3	*	5.983	167.520
*	1	7.223	21.670
*	2	7.040	21.120
*	3	0.710	2.130
*	4	6.597	19.790
*	5	1.610	4.830
*	6	6.653	19.960
*	7	2.453	7.360
*	8	7.763	23.290
*	9	6.330	18.990
*	10	7.403	22.210
*	11	4.960	14.880
*	12	6.490	19.470
*	13	8.940	26.820
*	14	8.223	24.670
*	15	1.553	4.660
*	16	7.970	23.910
*	17	2.063	6.190
*	18	9.007	27.020
*	19	8.880	26.640
*	20	9.267	27.800
*	21	6.000	18.000
*	22	2.827	8.480
*	23	5.347	16.040
*	24	3.257	9.770
*	25	4.487	13.460
*	26	7.307	21.920
*	27	10.033	30.100
*	28	4.547	13.640

Appendices (8):

A N A L Y S I S O F V A R I A N C E T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	7.301	3.650	1.4612	0.2410
2	Factor A	27	571.544	21.168	8.4735	0.0000
-3	Error	54	134.901	2.498		
	Total	83	713.746			