BIOCHEMICAL VARIATIONS IN SOME *STRIGA* RESISTANT AND SUSCEPTIBLE MAIZE GERMPLASM.

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A thesis submitted in partial fulfillment of the requirement for the award of the degree of Master of Science in Biochemistry of Egerton University.

(EGERTON UNIVERSITY)

October, 2007

DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted in any other university

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DEDICATION

This work is dedicated to my husband Eddie, my son Louis Kim and my parents Mr. and Mrs. Abungu.

ACKNOWLEDGEMENT

The success of this work is due to the collaborative efforts of many individuals and institutions. A lot of gratitude to my supervisors Dr. Onek and Prof. Amole for introducing me to the field of plant biochemistry. Their prompt guidance and personal dedication from the beginning of this research work to its completion is highly acknowledged. I further thank Dr. Onek for his financial support, patience and encouragement during the experiments and production of this thesis.

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ABSTRACT

Rapid human population growth, increased demand for food supplies and intensified land use has led to reduced crop rotation and hence reduction of soil fertility.
The decline in soil fertility has in turn intensified the extent of *Striga* infestation, causing the parasite to become a threat to food production. The *Striga* species decimates tropical crops such as maize, sorghum, millet, rice and cowpeas. Maize is specifically attacked by *Striga hermonthica.* In this study, biochemical markers of *Striga* resistant maize such as protein profiles, amino acid profiles and total phenolic contents were examined in F4 generation.
Five lines of azide mutated maize, one line of pure inbred resistant maize, one line of pure inbred susceptible maize and the control H513 were grown in a *Striga*-infested area.
The protein analysis showed that the maize mutants had an additional low molecular weight protein. This was lacking in the control and in the susceptible cultivar that only had a high molecular weight protein present in all the cultivars. This protein band was very prominent and distinct in all the analysis carried out. The mutants and the resistant cultivar showed significantly higher concentrations ($P>0.500$) of phenolics in comparison to the control and the susceptible cultivar. This was shown by the high absorbance values obtained. The mutants, resistant and tolerant varieties cultivars had the amino acids Asparagine and Alanine during the early stages of growth. However the susceptible and control cultivars lacked Alanine during this stage. In the post pollination period the maize cultivars lacked Asparagine but their growth was normal. Attempts have been made to correlate variations in protein profiles, amino acids and phenolic concentrations with the resistance to *Striga hermonthica* or lack of *Strigal* attack.

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LIST OF ABBREVIATIONS

CYMMYT	International maize and wheat improvement center
EDTA	Ethylene diamine tetra acetic
HIF	Haustoria inducing factor
HPLC	High pressure liquid chromatography
NaN_3	Sodium azide
$Nha^{-1}RF$	Nitrogen per hectare
R_F	Retention factor
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T.E	Tris EDTA buffer
TEMED	N, N, N, N-tetramethyl diammine
TLC	Thin layer chromatography
Tris buffer	Hydroxymethyl amino methane buffer
Tris-HCl	Hydroxymethyl amino methane hydrochloric buffer
TvQR1	Transcript encoding the quinone reductase that catalyzes univalent reductions
TvQR2	Transcript encoding the quinone reductase that catalyzes two electron reductions
UV-VIS	Ultra –Violet Visible spectroscopy
V/V	Volume by volume
W/V	Weight by volume
W/W	Weight by weight

UNIT OF ABBREVIATIONS

μg	Microgram
μL	Microlitre
Kda	Kilo dalton
gm	Gram
M	Molar concentration
mg	Milligram
ml	Millilitre
mA	MilliAmpere
Mm	Millimolar
M_r	Relative molecular weight
Nm	Nanometer
pH	-Log of hydrogen ion concentration
r.p.m	Revolutions per minute

CHAPTER ONE

INTRODUCTION

Cereals are the major staple food crops in Kenya. Maize is one of the most important cereals in terms of tonnage, consumption and financial value (Kiruki, 2000). The parasitic weed *Striga hermonthica* (Del) Benth is however a major constraint to maize production, especially in the infertile semiarid areas and humid lowlands in Kenya. The grain –area in Africa that is infested by *Striga* is estimated at 21 million hectares and the overall loss in grain production amounts to 4.1 million tons (Graves *et al*, 1997). In Kenya, maize cultivation area is estimated at 1.6 million hectares. There is evidence that *Striga hermonthica* originated from the Nuba mountains of Sudan and in parts of Ethiopia (Haussmann et al, 2000) .The species is heavily dependent on the host for its survival. It derives mineral nutrients, water, carbohydrates and amino acids from the host plants through root connections. (Lagoke, 1998).

The structure involved in root connection is the haustorium, which attaches to the host plant roots, penetrates it and acquires the nutrients. Due to the highly specialized relationship with the host, *Striga* exhibits wide genetic variability and different physiological strains, subspecies, ecotypes and morphotypes exist (Lagoke, 1998). As a part of its adaptation to parasitism, *S. hermonthica* produces a large number of seeds with prolonged viability and special germination requirements (Ejeta and Butler, 1993). These requirements include an after ripening period, conditioning and exposure to exogenous germination stimulants (Shank, 2003).The stimulants are produced by host and non-host plant species. However, once all these requirements are met the seeds of *S. hermonthica*

germinate. Due to its devastating effects on grains, several control methods have been developed to eradicate the weed. For example, a judicious use of herbicides has been formulated as a control measure. Low dose herbicide (imazapyr) coated maize seeds have been developed. However, the imazapyr coated resistant maize seeds give complete control for only one season (Kanampiu *et al,* 1998). The small quantities of imazapyr delivered in this manner act at the time of *Striga* attachment to the maize root. Imazapyr prevents phytotoxic effect of *S. hermonthica* on the maize plant, which usually occurs even before emergence of the *Striga* plant from the soil. It further diffuses into the surrounding as it is not absorbed by the maize and kills the ungerminated *Striga* seeds.

Application of nitrogenous fertilizers increases the soil fertility and therefore reduces *Striga* infestation (Watson and Ciotola, 1999). Fertilizers are however expensive and uneconomical to resource- poor farmers. Mechanical weeding and hand pulling can control *Striga* to a certain extent, although it is tedious and may not increase the yield of already infected plants. Furthermore it does not reduce the seed bank of *S. hermonthica* in the soil. Catch and trap crops can also be used to control *S. hermonthica.* Such trap crops usually lure the *S. hermonthica* seeds to suicidal germination without becoming parasitized themselves. The common trap crops include soya bean, lucern and cotton (Terry, 1984). The catch crops on the other hand are parasitized as they lure the *S. hermonthica* seeds to suicidal germination, they include sorghum and Sudan grass (Terry, 1984). Intercropping maize with catch and trap crops thus increase the efficiency of land use through improved soil productivity and reduction of the witch weed (Kureh *et al*, 2000). Breeding for resistant host varieties combined with legume/cereal crop rotation is one of the most effective and economical control strategies (Shank, 2003). This is

because resistant cultivars reduce both the new seed production and the *Striga* seed bank in the soil. An ideal control method for the weed should therefore include identification and use of host varieties that are resistant to *S. hermonthica*. The resistant host crop genotype should be able produce normal yield in the presence of *Striga* attack while supporting significantly few plants of *S. hermonthica*. Resistance is ascribed to low stimulant production by some cultivars; production of chemical substances by the host plant roots which resist establishment of haustorium (Kiruki, 2000) and a hypersensitive response (Haussmann, 2000).

1.1 JUSTIFICATION

Striga hermonthica (Del) Benth causes yield reduction of very important cereals including maize in semiarid tropics and humid lowlands in Africa. In Kenya, most subsistence farmers in the western part of the country grow maize, whose major constraint is *S. hermonthica.* Several control methods are routinely used such as increasing soil fertility, use of trap crops, intercropping, crop rotation and weeding though none of these is completely effective (Shank, 2003). Some resistant varieties of maize have nevertheless been developed (Kanampiu *et al*, 2003) that show promising results. The azide mutation of maize has particularly been reported to be effective in enhancing resistance of maize to *S. hermonthica* (Hodgdon *et al*, 2000). Little is however known about the underlying biochemical and molecular mechanism responsible for the resistance, though it is known that mutations affect metabolism of various biomolecules.The mutations affect proteins and amino acid metabolism resulting to either overproduction or underproduction of the amino acids and proteins (Russel, 2002).

This study was therefore designed to determine variation in the leaf proteins, amino acids and phenolics of field- tested resistant maize cultivars and the susceptible maize cultivars as a first step in the elucidation of the resistance mechanism of the maize mutants.

1.2 OBJECTIVES OF THE STUDY

1.2.1 General Objective

The general aim of this study was to elucidate the mechanism underlying the maize host resistance to *Striga* by identifying biochemical and molecular markers responsible for this trait. This was to be used as a step towards the development of *Striga* resistant maize mutant varieties.

1.2.2 Specific Objectives

The specific objectives of this study were as follows:

1 To extract and screen leaf amino acids from *Striga* resistant maize using thin layer chromatography (TLC).

2 To extract and screen root phenolics and phenolic acids from *Striga* resistant maize using Ultra-violet-Visible (UV-VIS) spectroscopy.

3 To characterize leaf maize protein of resistant and susceptible plants using Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

1.3 EXPECTED OUTPUT

This study provided more information on existing biochemical variations between *Striga* resistant and susceptible maize varieties. This information will be vital for further

studies geared towards identification of genes conferring *Striga* resistance to maize and development of *Striga* resistant maize varieties.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biology of *Striga*

Striga is a parasitic angiosperm of the class *Scrophulariaceae* and genus *Striga.* This genus contains 41 species of which 11species are capable of attacking crops (Raynal-Roques, 1996). *Striga hermonthica* (Del) Benth and *Striga asiatica* (L). Kuntze are the most virulent species on cereals. *Striga* has been given the common name of "witchweed" because of the various and subterranean debilitating effects inflicted upon its host in addition to attaching to the roots and robbing the host of water and nutrients (Kuiper, 1997). It is a flowering obligate parasite requiring insect pollinators for fertilization and seed production (Musselman, 1987).

Striga seeds are contained in a structure known as the capsule. There are many seeds in the capsule, with the number varying from about 700 in *S. hermonthica* to 800 in *S. asiatica* per capsule (Joel *et al*, 1995). The numbers of capsules per plant also differ, but on average these are 60-70 in both *S. hermonthica* and *S. asiatica* (Musselman, 1987). Therefore the largest number of seeds to be produced being estimated up to 500, 000 seeds. These seeds are produced every season hence increasing the seed bank in the soil (Joel *et al*, 1995). Under specific soil conditions they may remain viable for as long as 20 years in soil (Kuiper, 1997).These factors make *Striga* a very noxious weed that is difficult to eradicate. Furthermore, *Striga* seeds are extremely small ranging from 0.10 to 0.40 mm in size and 3 - 15 µg in weight and can therefore be easily blown by the wind leading to their dispersal (Musselman, 1987).

The seeds also have a characteristic surface pattern of ridges, which plays a role in the uptake of germination stimulants (Kuiper, 1997). The highly efficient mechanism of seed production also contributes to the difficulty of its control (Musselman, 1987). The weed generally has highly specialized relationships with the hosts and exhibits wide genetic variability, resulting in different physiological strain, sub-species, ecotypes, and morphotypes (Kuiper, 1997). It also has a wide range of prolificity, longevity, and dormancy of seeds, and some species are able to undergo intra-specific and inter-specific hybridization (Musselman, 1987). This has resulted in a wide adaptation to environments and hosts, as well as different levels of virulence, thus making *Striga* a very serious but peculiar constraint in terms of difficulties encountered in its control (Kuiper, 1997).

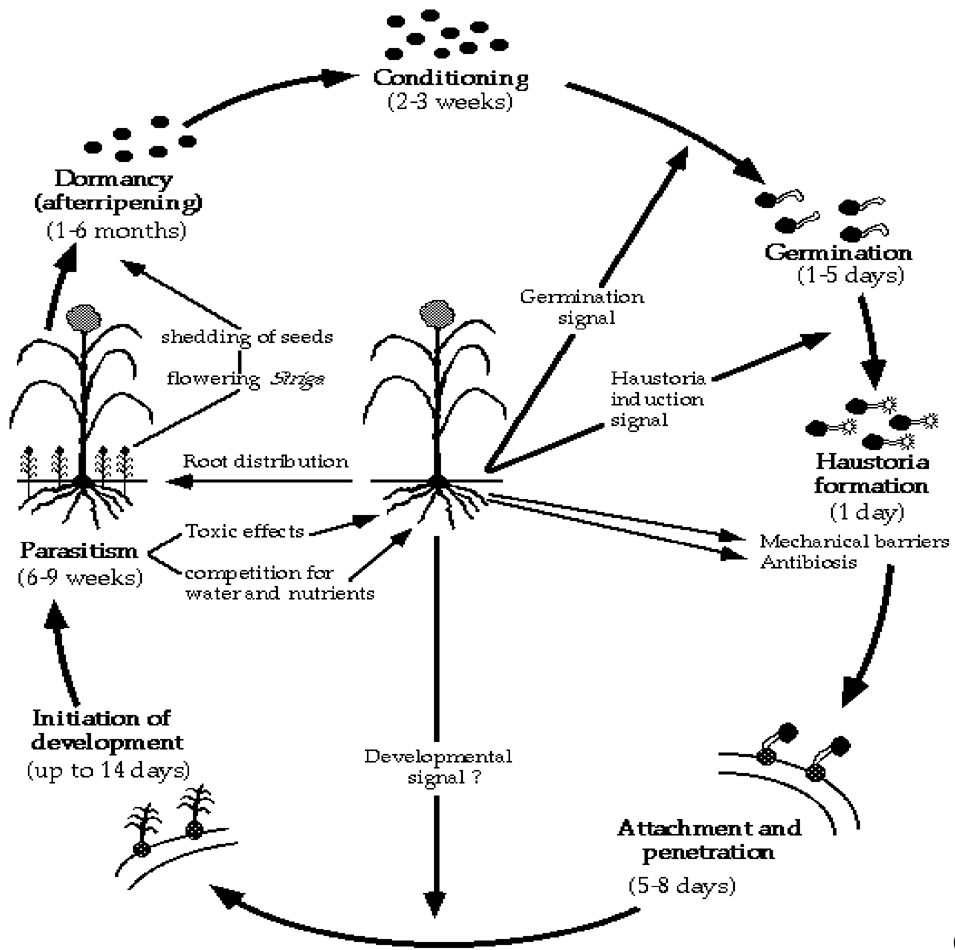
2.2 Distribution of *Striga hermonthica* in Africa

Striga hermonthica is distributed throughout northern tropical Africa, extending from semi arid areas of Ethiopia and Sudan, through to most savanna areas of West Africa and the lake basin in East Africa. In Kenya, *Striga* infestation is most severe in Nyanza and Western Provinces, where it occurs in about 180,000 acres and results in crop losses estimated between KSh 800 and KSh 2200 million per year (Woomer, 2004).

2.3 Life cycle of *Striga hermonthica*

The life cycle of *Striga* is composed of several stages which include; dormancy, conditioning, germination, haustorial initiation, penetration of host tissue, physiological compatibility and parasite growth and maturation.

Figure 1



(Kuiper, 1997)

In the complex life cycle of *S. hermonthica* which is completely adapted to that of its host, the seeds are the sole source of inoculum. They seeds are easily dispersed the wind and after dispersal they may remain dormant for several months, a condition referred to as primary dormancy. However if the seeds are conditioned then germination occurs.

2.3.1 Primary Dormancy

During the primary dormancy phase, the embryo lacks sufficient growth potential to push through the seed coat. However, this is also an evolutionary adaptation to prevent germination during the last rains of the season, when there are no hosts around. This period may last between two days to several years. For *Striga hermothica*, the minimal duration is six months (Joel *et al,* 1995). After this period the seeds will germinate but only if conditioned.

2.3.2 *Striga* Conditioning

This is the period when the seeds have to be imbibed for a period of 10 –21 days prior to a germination stimulant for germination to occur. During this period, major metabolic pathways are operating in the seed with a characteristic pattern of respiration and synthesis of DNA, proteins and hormones (Joel *et al*, 1995). This period necessitates the removal of phenolic compounds that act as germination inhibitors (Shank, 2003). The inhibitors are located on the seed coat and are leached out when the seeds are exposed to water and temperatures of 20-40°C, (optimum temperatures are 25-35°C) (Bupe *et al,* 1993).

However when conditioning exceeds the optimum period of two to three weeks, germination does not occur, hence secondary dormancy. Secondary dormancy could act as a safety mechanism, ensuring that in the absence of the germination stimulants the conditioned seed would not run out of respiratory substrates (Logan and Stewart, 1992).

2.3.3 *Striga* Germination

Due to their small size, of the *Striga* seeds lack sufficient reserves for sustained periods of growth before successful attachment to the host. Host attachment therefore

takes place within 3-4 nm of host root since *Striga* radicle can manage only 2-4nm of growth. This is also used as a survival strategy as those seeds that are too far from the host roots do not germinate. To compensate for this biological restriction *Striga* produces many seeds, that is up to 450,000 seeds per plant (Stump, 2000).

The major requirement after conditioning is a specific chemical germination stimulant from the host roots. Indeed *Striga* species only germinate in the presence of a stimulant exuded by the host (Parker and Riches, 1993). Several of these stimulant compounds have been found and identified in root exudates of host plants and non-host plants (Yongquing *et al*, 1998). These compounds very active at extremely low levels. They include;

Strigol: a highly unstable tetra cyclic sequesterpene (Figure 2a) isolated from cotton that has an activity in the soil at concentrations as low as 10-15 mol/m^3 (Vail *et al,* 1990)

Sorgoleone: an unstable dihydroquinone (Figure 2b) that is rapidly oxidized to a stable inactive quinone (Chang *et al,* 1986). It is hydrophobic with an activity of $10^{-7} mol/m^3$. The ephemeral nature and limited mobility of this stimulant is capitalized on by *Striga* to ensure germination in close proximity to the host plant (Stump, 2000).

Sorgolactone: was first isolated from water soluble sorghum root exudates. Previous studies have shown that it has a lot of similarity to strigol in terms of solubility, activity and even structure as depicted in Figure 2c (Hauck *et al*, 1992).

Alectrol: is a stimulant found in cowpeas *Vigna unguiculata* that is attacked by *Striga gesnerioides* (Figure 2d) (Lane *et al,* 1994).

In addition to natural host exudates, a number of chemicals such as ethylene, kinetins and zeatin also stimulate *Striga* growth (Worshum, 1987).

Figure 2(a)

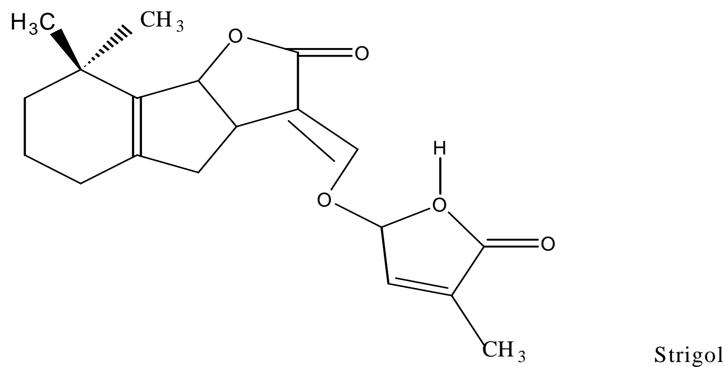


Figure 2(b)

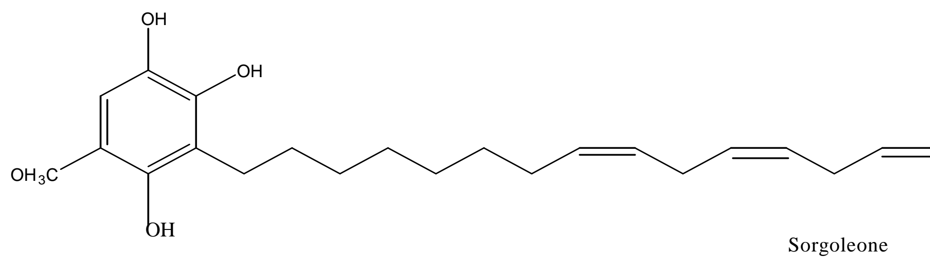


Figure 2(c)

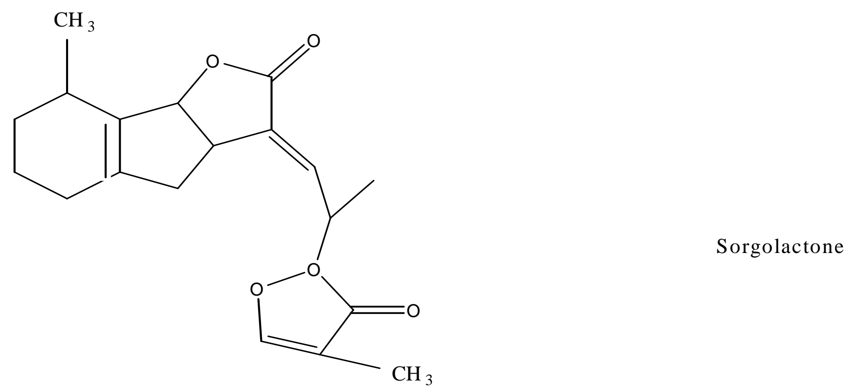
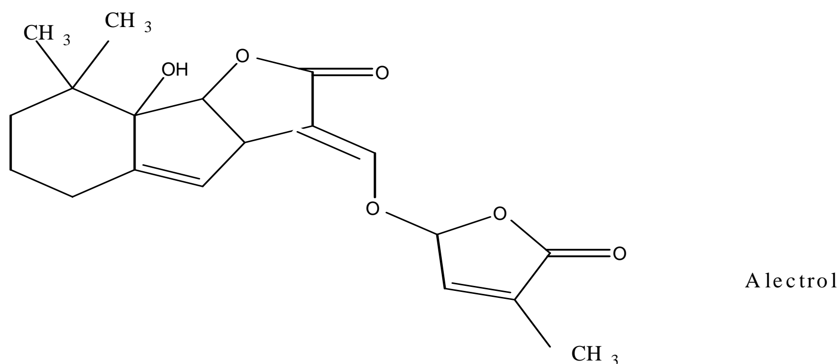


Figure 2(d)



2.3.4 *Striga* Attachment and Penetration

Once the *Striga* seeds have germinated, their radicle growth is directed towards the host root through chemotrophism. This means that chemical substances exuded by the host act as a direction seeking mechanism for the parasite's germ tube (Kuiper, 1997). When the germ tube gets into contact with the host roots, radicle elongation ceases and development of haustoria begins. The formation of the haustorium is also guided by host derived secondary metabolites (Mohammed *et al*, 2001). Finally, after haustorium formation, the host root xylem penetration is established with the involvement of hydrolytic enzymes produced by the penetrating parasite (Labrousee *et al*, 2001). Haustorium hairs aid the attachment and an open connection is formed between the parasite xylem and host xylem (Kuiper, 1997). The supply of resources from the host is mainly through secondary haustoria developed after attachment.

2.4 Resistance of Maize to *Striga*

There are several terminologies used to describe maize cultivars that grow under conditions of *Striga* infestation.

2.4.1 Types of Maize Cultivars

i) Resistant cultivar

This is a crop genotype which when grown under conditions of *Striga* infestation, supports significantly very few *Striga* plants and has a higher yield than a susceptible cultivar (Haussmann, 2000a).

ii) Susceptible cultivar

This is a crop genotype which when grown under conditions of *Striga* infestation succumbs to *Strigal* attack hence producing very low yield (Haussmann, 2000a).

iii) Tolerant cultivar

This is a crop genotype which gives normal yield in the presence of *Striga* attack.

2.4.2 *Striga* Resistance Mechanism

Due to the extraordinary plasticity of *Striga* plants, it is very difficult to study the mechanism of resistance. However Haussmann (2000b) proposed the mechanisms of *Striga* resistance consisting of:

- Low production of germination stimulant;
- Effect of mechanical barrier (e.g. lignification of cell walls);
- Inhibition of germ tube exoenzymes by host root exudates;
- Synthesis of low molecular weight compounds (phytoalexins) that protect the plant against attacking pathogenic organisms;

- Antibiosis i.e. reduced *Striga* development through unfavorable phytohormone supply by the host;
- Insensitivity to *Striga* toxin (e.g. maintenance of stomatal aperture and photosynthetic efficiency;
- Avoidance of root growth habit (fewer roots in the upper 15-20 cm);
- Post attachment hypersensitive reaction entailing the appearance of a necrotic region around the site of attempted infection followed closely by death of the affected host cells within hours of the attack (Ejeta and Butler, 1996). The necrosis of affected tissue is directly related to the accumulation, oxidation and polymerization of phenolic compounds (Ejeta and Butler, 1996).The hyper sensitive response at attachment sites is thought to discourage further penetration of the parasite into the host roots (Ejeta, 2002).

However the study of the resistance of azide mutated maize to *Striga* is however very interesting as it is centralized on the mutational changes caused by sodium azide. Such changes occur in the DNA, which is transcribed and translated into proteins. Generally the maize plant is a low protein plant with a total protein content of 10% (Loomis *et al,* 1992). The two main kinds of protein in maize are zein and glutein. These proteins are mainly found in the maize grain. Zein is found in the endosperm and makes up the greatest part (60%) (Loomis *et al,* 1992) but is deficient in the essential amino acids, tryptophan and lysine. Glutein occurs in lesser amounts in the endosperm and also in the germ and is a better source of these two amino acids.

2.5 Phenolics and Resistance to *Striga hermonthica*

Plants are usually in constant communication with a multitude of diverse organisms including other plants (Estabrook and Yoder, 1998). Such interactions are mediated by signal molecules that cue developmental and physiological events critical in the interaction (Baker *et al*, 1997). A more direct interaction is found between parasitic plants and their hosts (Press and Graves, 1995). The parasitic plants of the class *Scrophulariaceae* are able to use a variety of molecules as host recognition factors provided that they fulfill certain structural and electrochemical requirements (Yoder, 2001). These molecules include germination stimulants and phenolic compounds. The phenolics include xenognostic quinones, which are secondary metabolites produced by plants which do not play a role in the primary metabolic processes essential for the plant survival (Wu *et al*, 2002).

These metabolites however, make up a significant component of plant cell walls and are used for several functions including lignin biosynthesis and plant defense (Estabrook and Yoder, 1998). They are mainly prevalent in the roots and are therefore commonly found in root exudates (Siqueira *et al*, 1991). Studies of root exudates show that these molecules act as haustoria inducing factor (HIF). Estabrook and Yoder (1998) found out that in sorghum the phenolic HIFs are removed from the host cell walls of the roots and activated by parasite specific enzymes. Once removed the phenolic acids must be oxidized to the proper redox potential for haustoria induction. This suggests that HIFs initiate haustoria development through a redox mechanism, i.e. the transfer of electrons controlling the activity of proteins or other molecules (Smith *et al*, 1996). Recent studies by Yoder (2001) showed that the xenognostic quinones are oxidoreduced to

semiquinones. The semiquinones generated during xenognosin reduction are the likely candidates for the signaling charge (Yoder, 2001). However, the redox potentials of these biologically active quinones are within a range of about 300 mV, and molecules that fall outside of this window are largely inactive (Estabrook and Yoder, 1998). Host plants like maize, release xenognostic quinones as a component of root exudates. The quinones enter the *Striga* roots where they are oxidoreduced by two distinctive cytoplasmic quinone reductases, TvQR2 and TvQR1 (Yoder, 2001). Lynn and Chang (1986) also hypothesized that ligninolytic peroxidases, produced by the parasitic plant, extract phenolic molecules from the host cell walls and convert these to the appropriate quinone forms. It is interesting that some phenolics also inactivate the parasite's cell wall degrading enzymes (Patil and Dimond, 1967) hence preventing the attachment to the host.

Therefore paucity of the parasite specific enzymes due to resistance to *Striga* by the maize mutants may lead to lack of haustoria induction. Host resistance against plant pathogens is generally considered one of the best protection measures with regard to effectiveness, cost, implementation, and environmental soundness (Estabrook and Yoder, 1998). Although some resistances against parasitic weeds have been reported, the characterization, manipulation, and incorporation of these factors into crop plants have been difficult. Therefore, an elucidation of the mechanisms that limit self-parasitism might suggest novel strategies for engineering resistance against such devastating plants as *Striga.*

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Development and testing of the maize mutants

Hybrid maize (H513) seeds were mutated in 2000 by Kiruki. He observed that mutagenesis induced *Striga* resistance to maize (Kiruki, 2000). Birechi, (2001) further tested the azide treated maize in the field. It was observed that some of the mutants did not support *Striga* emergence while some supported very few (Birechi, 2001).

3.1.2 Chemicals

All the chemicals used during this work were of analytical grade obtained from Sigma Chemical Company, England.

3.1.3 Maize Samples

The maize samples used for the work included five different types (lines) of azide mutated maize (F_3) which were developed and tested by Kiruki, 2000 and further tested by Birechi (2001).One pure inbred line of tolerant maize and one pure in bred line of susceptible maize from International maize and wheat improvement center (CYMMYT) and a natural tolerant breed and the control line (H513) as shown on Table 3.0.

TABLE 3.0 List of Maize samples analyzed

NAME OF SAMPLE	CHARACTERISTICS
KBO100B14	*Striga* resistant pure line from CYMMYT
K9921-2	Mutant
K9920	Mutant
K9904	Mutant
K9920-1	Mutant
K9913	Mutant
UGANDA RED	Naturally *Striga* tolerant
KORANDO	*Striga* susceptible pure line from CYMMYT
H513	Control

The table above summarizes the maize samples used in this study, which comprised of *Striga* resistant and susceptible varieties.

3.2 Experimental Procedures

3.2.1 Growth of Plant Materials

The seeds of all the listed samples as in Table 3.0 were grown in a *Striga*-infested field. The planting holes were 7cm deep and spaced by 75cm x 30cm. Diamonium phosphate (DAP) was applied at a rate of 36kgNha^{-1}(i.e. 4.5g of the fertilizer per hole). Three weeks after seed germination, some of the leaves of the germinated seedlings were cut and stored in liquid nitrogen before protein analysis. Leaf collection was repeated routinely after every three weeks until the maize attained full maturity.

3.2.2 Protein Extraction and Analysis

Proteins were extracted from 5.0g liquid nitrogen frozen leaves by grinding using glass beads at 4^{0}C in 5 ml of T.E buffer (10Mm Tris-HCl, 1mM EDTA pH8.0), containing 1% Triton X-100. The resulting homogenate was then centrifuged in a micro centrifuge at 4^{0}C for 20 minutes at 14,000 revolution per minute and supernatant stored at –20^{0}C (Kiruki, 2000).

The proteins were then analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) as performed by Laemmli method (1970). A 12% resolving gel (acrylamide 30:0.8%) was used. The resolving gel was layered with isobutanol to remove air bubbles. After polymerization of the gel, the alcohol was poured out and a stacking gel of the concentration (3% acrylamide) cast on top of resolving gel fitted with combs. The supernatant from the protein extraction protocol was mixed with equal volumes of sample buffer (0.13M Tris-HCl, 20% glycerol, 0.002% bromophenol blue, 4% SDS, 1% β-mercaptoethanol pH 6.8). The samples were then boiled for five minutes in a hot water bath. The boiled samples were loaded in the wells of the gel. After

loading, electrophoresis was carried out at 30mA at room temperature until the tracking dye (bromophenol blue) was no longer on the gel matrix. After electrophoresis the stacking gel was cut off and the resolving gel stained for protein visualization with 0.6% Coomasie brilliant blue R-250 in acetic acid: methanol: distilled water in the ratios 9.2:50:40.8, respectively, overnight. The gel was then destained followed using a destaining solution made of acetic acid: methanol: distilled water in the afore-mentioned ratios at room temperature until destained. The destained gels were photographed immediately and stored in 7% acetic acid.

3.2.3 Amino acid Extraction and Analysis

Amino acids were extracted from1.0g of liquid nitrogen frozen leaves using the method of Dever *et al* (1995). The leaves were ground in 1ml of a mixture of Tris/HCl (10mM pH 8.4) buffer and absolute ethanol at the ratio $1{:}3^{v}/v$ using a mortar and pestle. The homogenates were then transferred into micro centrifuge tubes (Microcapped centrifuge tubes, Hughes and Hughes, Rumford, Essex, and U.K) and centrifuged at 10,000 *g* for 5 minutes. The supernatants were transferred into clean micro centrifuge tubes ready for spotting. This procedure was repeated after every three weeks in both the pre-pollination and post pollination periods.

A sample of 20µl of the supernatant of each sample was spotted on a TLC plate using a positive displacement pipette (PDM8, Boehringer Corporation (London) LTD U.K).

The chromatogram was then developed in a solvent system of n-butanol: acetone: diethylamine: water (70:70:14:35 v/v) until the solvent front reached the top of the plate.

The plate was removed from the tank and air dried for 30-45 minutes to remove traces of solvent. Volatile amines were removed by oven drying at 120^0C for one hour. The amino acids were visualized by spraying the plate in a fume cupboard with a 20% (w/v) ninhydrin /acetone solution. Using identified running standards, the positions of the individual amino acids were identified (Dever *et al*, 1995). The plate was then photographed. This procedure was repeated after every three weeks in both the pre-pollination and post pollination periods.

3.2.4 Extraction and Analysis of total phenolics

Phenolics were extracted from fresh roots of the maize samples using the method of Harbone (1998). Fresh roots were cut from the plants and 1.0g of roots from each sample weighed. The roots were plunged in boiling methanol for 5 minutes. The extracts were stored in sample bottles awaiting analysis.

The extracts were screened for total phenolics immediately after extraction using the Harborne (1989) method. A spectrophotometer (Varian DMS 80 UV Visible spectrophotometer) was set in the wavelength range of 263nm and used to obtain the absorbance values of the extracts. The readings were obtained in triplicates each time the analysis was done. According to Beer-Lamberts law absorbance is directly proportional to concentration. Therefore the absorbance values were correlated to concentration and significant differences analyzed using the statistical package SPSS 9.0

CHAPTER FOUR

RESULTS

4.1 Protein Analysis by SDS-PAGE

Proteins were extracted from leaf material frozen under liquid nitrogen and analyzed by SDS-PAGE as described in Chapter 3. The leaves were chosen for protein extraction as leaves are the center of all metabolic processes in the plant hence that assumption that they contained the highest protein amounts in the growing maize plants. A distinct variation in the resolution of the protein subunits in the resistant varieties (mutants) and the susceptible varieties was observed as depicted in Figure 3 on page 28.

The observed distinct variation in the resolution of protein subunits of resistant mutants and the susceptible varieties (control and CYMMYT line) was the presence of two protein subunits, a high molecular weight subunit and a lower molecular weight subunit in the putative mutants while only the high molecular weight subunit was present in the susceptible varieties.

4.2 Amino acid analysis by TLC

Amino acids were extracted from frozen leaf material and analyzed as described in Chapter 3. Variations in the resolution of several amino acids were observed among the maize varieties studied after every three weeks during the pre and post pollination period (Table 4.1, Figure 4 and Figure 5). The R_F values of the various amino acids detected are calculated and correlated with the experimental and literature values of amino acid standards as summarized in Tables 4.0 and 4.1. The choice of the standards used was based on previous studies.

Table 4.0 Retention factor values of amino acid standards analyzed by TLC

NAMES OF STANDARDS USED	R_F VALUES
Aspartate	0.46
Cysteine	0.75
Alanine	0.55
Tryptophan	0.69
Tyrosine	0.67
Glycine	0.44
Asparagine	0.65
Glutamate	0.48

Table 4.1 Retention factor values of samples analyzed by TLC before pollination of the maize.

NAMES OF SAMPLES ANALYSED BEFORE POLLINATION	R_F VALUES			
		Probable amino acid		Probable amino acid
CYMMYT Tolerant line	0.55	Alanine	0.65	Asparagine
Mutant 1-K9921-2	0.55	Alanine	0.65	Asparagine
Mutant 2 –K9920	0.55	Alanine	0.65	Asparagine
Mutant 3-K9904	0.55	Alanine	0.65	Asparagine
Mutant 4-K9920-1	0.55	Alanine	0.65	Asparagine
Mutant 5-K9913	0.55	Alanine	0.65	Asparagine
Uganda red	0.55	Alanine	0.65	Asparagine
Control-H513	n	_	0.65	Asparagine
CYMMYT susceptible line	n	_	0.65	Asparagine

The letter **n**, denoted not detectable.

The TLC analysis of the maize cultivars showed that the tolerant line from CYMMYT and the putative mutants had two bands at R_F values of 0.55 and 0.65.These bands correlated with the standards Alanine and Asparagine as shown in Table 4.0. However the control and the CYMMYT susceptible line both lacked the Alanine band. This was depicted by the letter **n** in Table 4.1. Figure 4 on page 29 further shows the bands as they appeared on the TLC plate.

The TLC plate (Figure 4) showed two bands in most of the lanes. Bands 1 and 2 on the plate corresponded to Alanine and Asparagine respectively. Lanes 1, 2, 3, 4, 6, and 11 were leaf extracts of putative maize mutants. They had two definite bands indicating that they contained Alanine and Asparagine. Lane 7 was the leaf extracts of Uganda red, a natural tolerant variety. It had two bands although the upper band was faint. Lanes 8 and 9 were the leaf extracts of CYMMYT tolerant variety. They showed two bands hence contained both Alanine and Asparagine. This was in contrast to lane 5, which was the leaf extracts of the control, which only had one band corresponding to Asparagine. Similar results were also seen with the CYMMYT susceptible variety of lane 10.

Post pollination analysis however showed loss of the upper band that corresponded to Asparagine in all the samples. The mutants, tolerant and resistant cultivars however retained the lower band (Alanine) at R_F 0.55 as shown in Figure 5 on page 30. There was variation between the putative mutants, the tolerant variety, susceptible variety and the control during the post pollination period. The only band observed that corresponded to Alanine was seen in lanes 2, 3, 4, 5, 6, 7 and 8 that were extracts of the putative mutants. Identical bands were also seen in lanes 9 and 10 that were leaf extracts of the natural tolerant variety, Uganda red and the CYMMYT tolerant

variety respectively. The extracts of the control however did not show any band as seen on lane 11. Similar results were seen with the leaf extracts of the CYMMYT susceptible variety on lane 1, with the exception of artifacts on these lanes.

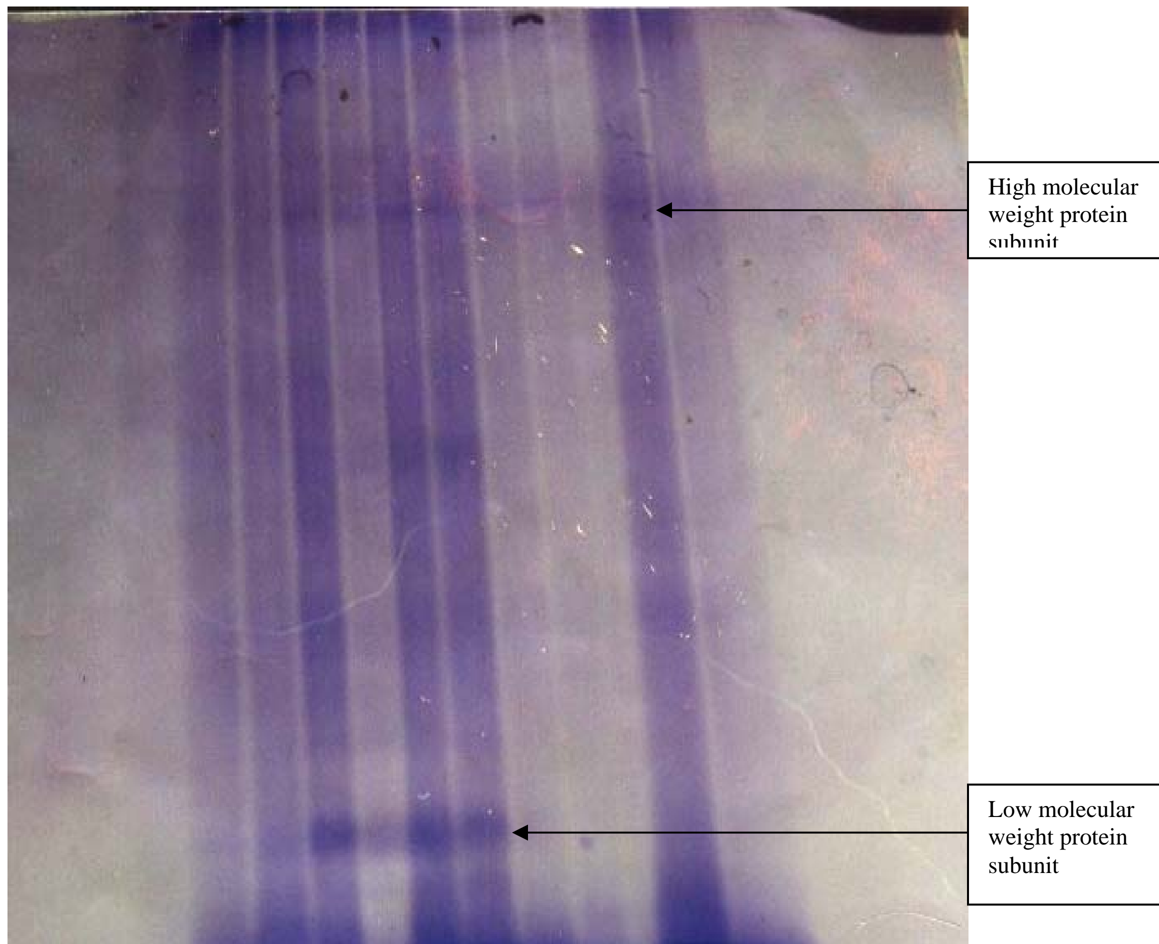
4.3 Total Phenolics Analysis of Root Extracts.

The root extracts of the putative mutants, tolerant varieties and the control were analyzed for their totals phenolics. This was done by determining the absorbancies of the root extracts. The absorbance values of the mutants were in the range of 0.511 to 0.786 as shown in Table 4.2 .The CYMMYT susceptible variety and the control had the values 0.325 and 0.337, respectively. There was a great variation in the absorbancies of the putative mutants, tolerant varieties and the control. This variation is shown by the graphical presentation on Figure 6. From the graph it is apparent that the putative mutants had higher absorbancies than the control and the susceptible variety. A test for statistical significance in the values showed that the absorbance values of the putative mutants and the tolerant varieties were insignificantly different as shown by the letter **a** as shown in Table 4.2. There was significant difference between the control and the putative mutants hence the letter **b** as shown in Table 4.2. The bars within the columns indicate the lower and upper limits of standard errors of the means.

Table 4.2 Absorbancies of total phenolics in root extracts

SAMPLES	ABBSORBANCE READINGS			
	Replicate 1	Replicate 2	Replicate 3	MEAN
Mutant1	0.510	0.512	0.511	0.511±0.144[a]
Mutant 2	0.715	0.713	0.717	0.715±0.286[a]
Mutant 3	0.785	0.787	0.788	0.786±0.144[a]
Mutant4	0.762	0.760	0.761	0.761±0.143[a]
Mutant5	0.722	0.721	0.723	0.722±0.144[a]
Uganda red	0.525	0.526	0.524	0.525±0.144[a]
Control	0.348	0.322	0.322	0.337±0.143[b]
Korando	0.326	0.324	0.325	0.325±0.143[b]
Kb0100b	0.716	0.714	0.718	0.716±0.287[a]

The significance level was P=0.05



1 2 3 4 5 6 7 8 9 10

Figure 3: Protein analysis by SDS-PAGE

Key: Lanes 1 and 10 were distilled water.

Lanes 2, 3, 4, 5 and 6 were mutants and the resistant line

Lanes 7, 8 and 9 were the control and susceptible line.

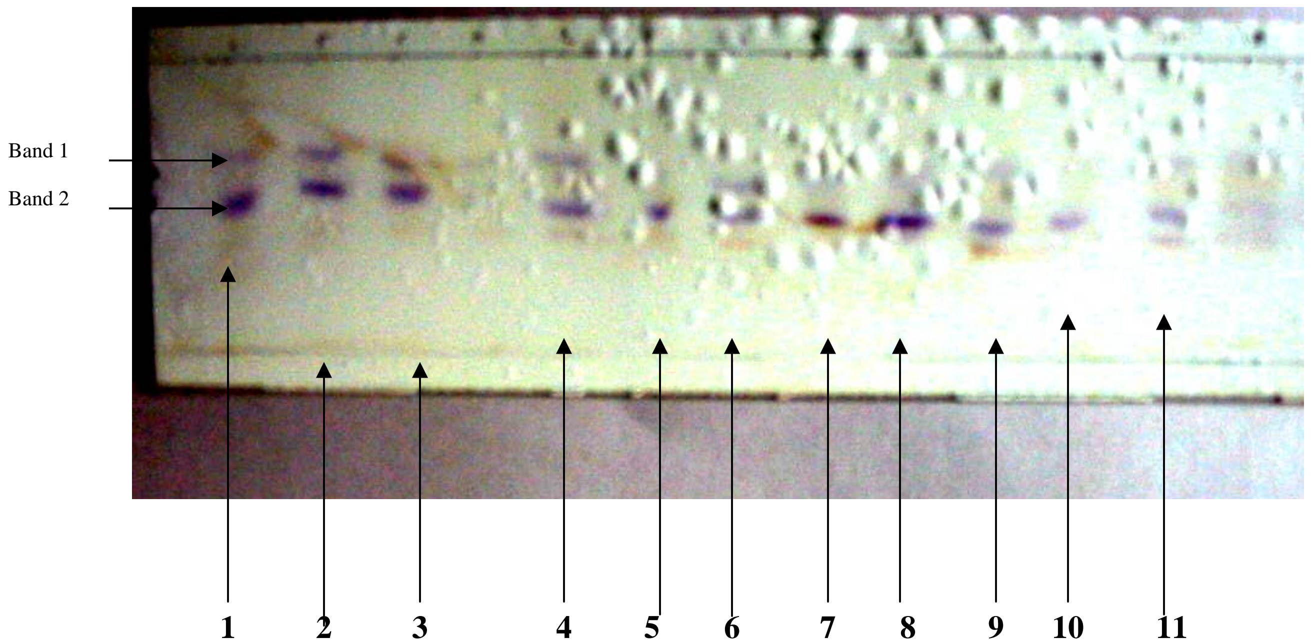


Figure 4: TLC plate of the maize cultivars analyzed before pollination.

Key: Band 1 and 2 corresponded to Alanine and Asparagine respectively.

Lanes 1, 2, 3, 4, 6, 11-Mutants

Lane 5-Control (H513)

Lanes 7- Uganda red

Lanes 8 and 9- CYMMYT tolerant variety

Lane 10- CYMMYT susceptible variety

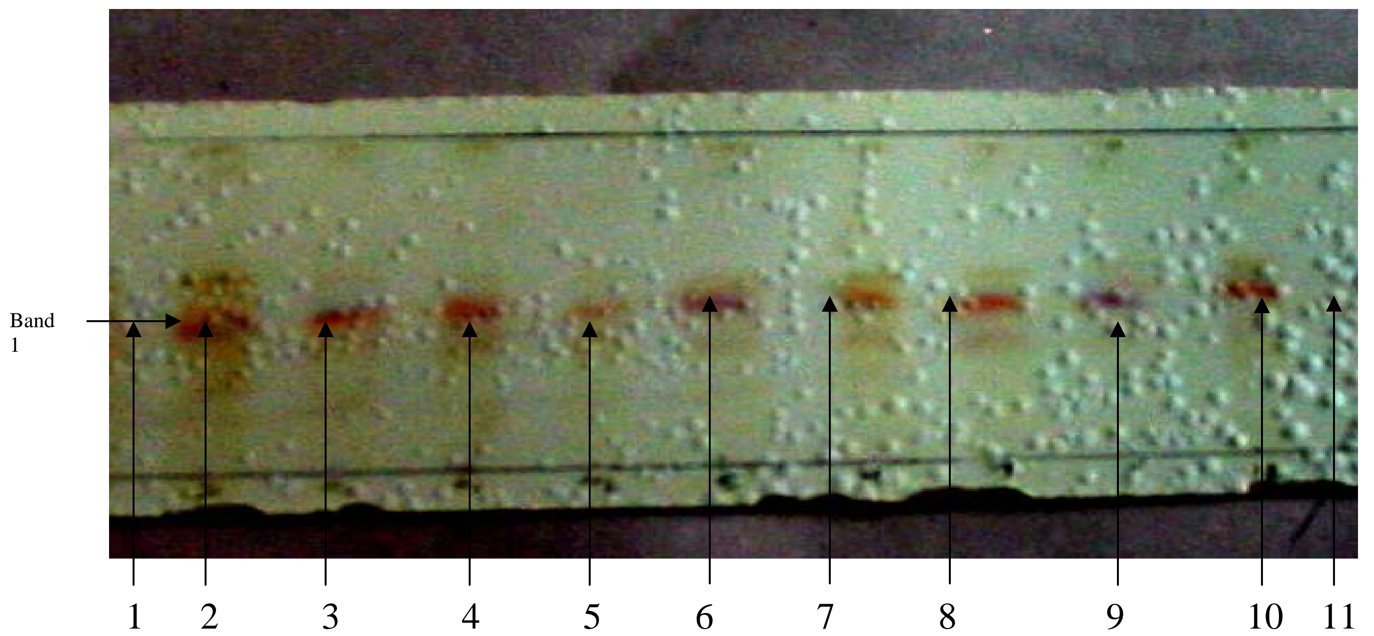


Figure 5: TLC plate of maize cultivars analyzed after pollination.

Key: Band 1on the plate corresponded to the amino acid Alanine.

Lanes 2, 3, 4, 5, 6, 7and 8-Mutants

Lane 1- CYMMYT susceptible variety

Lanes 9 - Uganda red

Lanes 10- CYMMYT tolerant variety

Lane 11- Control (H513)

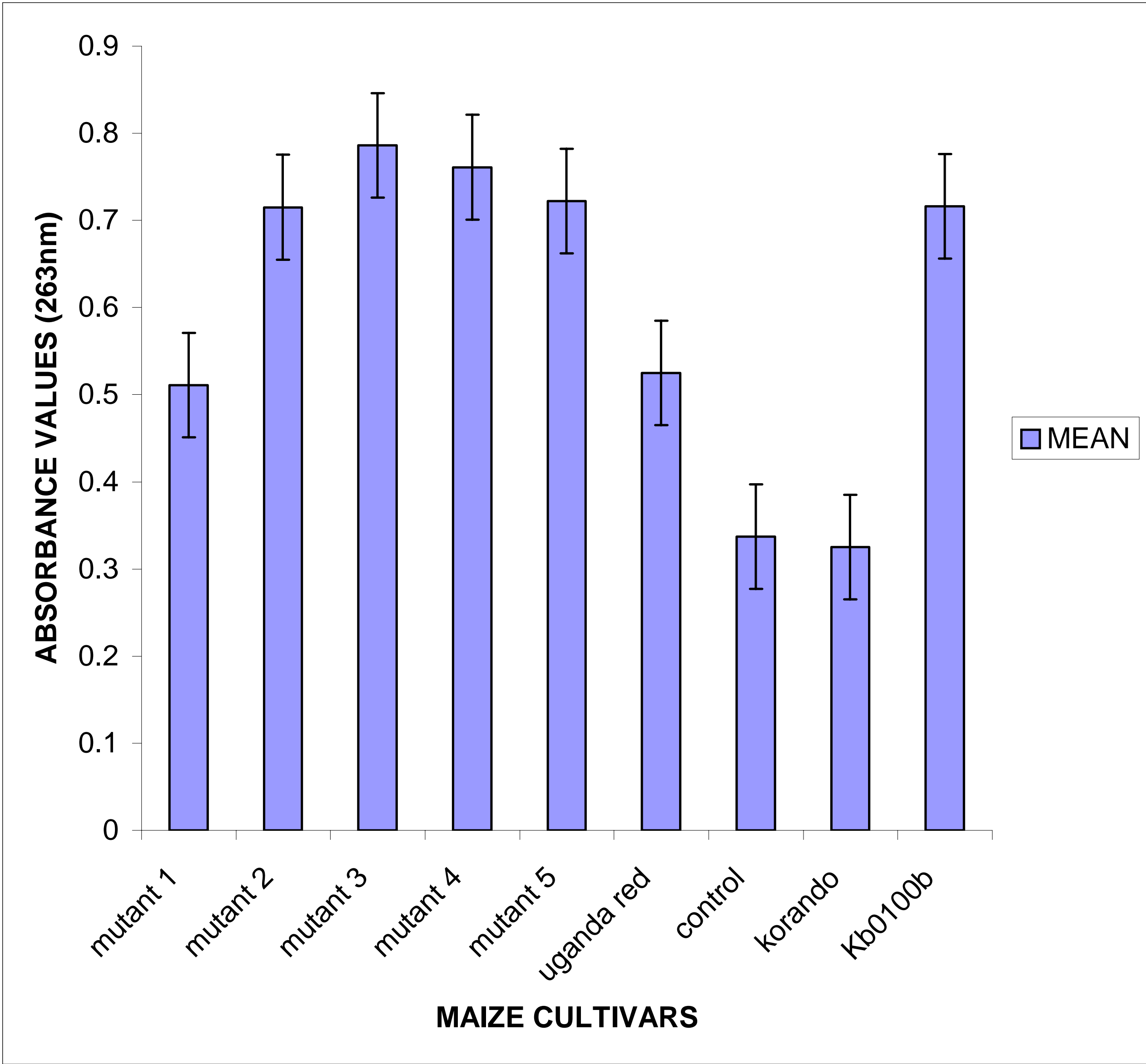


Figure 6: Comparison of root total phenolic absorbancies at 263nm

CHAPTER FIVE

DISCUSSION

5.1 Mutations

Mutation is any detectable and heritable change in the genetic material not caused by genetic recombination. During mutation, the sequence of base pairs in a DNA molecule is altered (Russell, 2002). This alteration may involve insertion, deletion or substitution of one or a few base pairs. This type of mutation that affects a single base pair of DNA is called a point mutation while mutation in the sequence of genes is called gene mutations (Modrich, 1987). Genes usually code for enzymes and their proteins, therefore certain sets of genes direct and control particular biochemical pathways (Beadle and Sturtevant, 1962). Gene mutations therefore can result in the loss of enzyme activity and lead to accumulation of precursors in the pathway (Beadle and Sturtevant, 1962). In the case of base pair deletion or addition, the reading frame of the mRNA can change downstream of the mutation. Such mutations result in nonfunctional proteins, which are either shortened or larger than normal. On the other hand, base pair substitutions may result in either an insignificant change or a noticeable change in the protein.

When the change is noticeable, the function of the protein may change (Russell, 2002). All these types of mutation may occur spontaneously or be induced. The induction may involve either physical or chemical agents. Induced mutation has played a significant role in the development of many crop varieties (Saddique *et al*, 1999). It has served as an important tool for creating usable genetic variability in crop improvement programmes. Bioassay guided biochemical assessment in combination with mutagenesis has been found to be the most promising method of controlling *Striga* (Bewabi, 1984).

The simplest way of mutating many seeds was found to be chemical mutagenesis. Common chemical mutagens used include N-methyl-N-nitrosourea, N-ethyl-N-nitrosourea, N-dimethyl-N-nitrosourea and sodium azide (Saddique *et al,* 1999). Sodium azide still remains the most potent mutagen in barley, maize, rice and beans (Hodgdon *et al*, 2000). Mutation breeding currently is proving to be a vital process for improving crops like rice, maize, soybean and tomatoes in Vietnam (Saddique *et al,* 1999). Studies have shown that sodium azide mutagenesis leads to point mutation in barley (Szarejko and Maluszynki, 1999). These mutations are exhibited phenotypically as dwarf characters, semi- dwarf characters, changes in root system development and structure.

5.2 Protein Analysis by SDS-PAGE

Polyacrylamide gel electrophoresis (Laemmli, 1970) is still the best method that gives high resolution and comparative protein subunit patterns. In this study, the technique was used to compare the protein profiles of *Striga* resistant maize mutants, tolerant natural variety and the control (H513). The electrophoretic pattern of the *Striga* resistant mutants revealed two interesting protein subunits. The same pattern was revealed by the natural tolerant variety Uganda red as seen in chapter 4. The subunits were distinct in that the resolution showed a high molecular weight and a low molecular weight protein subunit. Similar patterns were observed after several consecutive runs. Therefore, the results were reproducible and reasonably accurate. The lower molecular weight band was absent from the known susceptible line, namely Korando and the control H513.

Our results concur with Kiruki's findings (2000). He found that the F_1 generations of the mutants show a pattern of two protein subunits. A high molecular weight subunit

of 212.0 kDa and a low molecular weight subunit of 29.0 kDa. His studies linked the presence of this pattern to the ability of a line to support a few *Striga* plants. The presence of the additional protein subunit in the mutants shows probable accumulation of proteins in the plants. This finding gains support from the presence of resistance-conferring proteins that accumulate in disease and phytopathogen resistant plants that have been documented (Ja Choon *et al*, 2004). In this latter case, the accumulation is due to altered transport mechanisms (Mark Settles *et al,* 1998). A mutation in maize has been found to disrupt the localization of proteins transported through proton concentration difference (ΔpH) pathway in chloroplasts (Mark Settles *et al*, 1998). Consequently such mutations lead to accumulation of the ΔpH-transported proteins (Kumar *et al*, 2003).

On the other hand, tobacco mutants have been observed to over-express of some proteins which confer resistance to infection by tobamoviruses (Ja Choon *et al*, 2004). In similar studies by Kumar *et al* (2003), proteins that contain nucleotide binding site (NBS) and leucine rich repeats (LRR) have been shown to accumulate in tobacco plants and play a critical role in tobacco mosaic virus resistance (Kumar *et al,* 2003). Indeed, the involvement of genes containing these motifs in determining resistance responses to many groups of plant pathogens has been demonstrated (Kumar *et al,* 2003). Some of the processes associated with the resistance conferred by such genes include lignin deposition, hypersensitive response and localized cell death at the site of pathogen ingress (Kumar *et al*, 2003). Equally, Haussmann (2000b) also proposed these to be the mechanisms of *Striga* resistance in cereals.

It is therefore evident that proteins play an important role in resistance against pathogenic infections. This is further demonstrated by Hasegawa *et al,* (2002) in mutated

Arabidopsis thaliana. They found that mutation affects gene expression of certain proteins, in such a manner that signal pathways which control the expression of specific genes are turned on or off so that the plant can adapt to the pathogen and environmental stress (Hasegawa *et al,* 2002).

Based on these series of evidence, it can be concluded that accumulation of proteins in the mutants in the current study could be a result of an altered metabolic pathway. The presence of the lower molecular protein bands in the tolerant and mutant varieties support this conclusion. These findings would suggest that *Striga* resistance in the maize varieties studied is mediated through the alteration of the metabolic pathway of certain proteins.

5.3 Amino Acid Analysis

Analysis of amino acids of plants is a productive way of characterizing mutants in plants like barley and amaranthus (Hodgdon *et al*, 2000). In this study, the soluble leaf amino acids were analyzed by TLC as described in Chapter 3. The technique provided a qualitative screening method for the amino acid contents of the mutants and the other varieties. The variations showed a build up and absence of amino acids among some of the cultivars. The retention factors of the amino acids found in the mutants and tolerant variety were identical. They were 0.55 and 0.65, which correlated with the experimental and theoretical R_F values of Alanine and Asparagine. In contrast, the control lacked Alanine and only had Asparagine in its leaf extracts. This accumulation of the soluble leaf amino acids is due to altered allosteric regulation of amino acid metabolism (McCourt, 1997).

Studies of barley mutants that are resistant to *Hyp* -a toxic proline analog, showed an altered proline metabolism. The concentration of the soluble proline in the leaves was increased six fold (Bryan, 1990). Evidence of 44-fold increase in free tryptophan in the leaf extracts was also observed in *Datura innoxia* mutants (McCourt, 1997). These finding correlates well with McCourt's work (1997) in carrot mutants, which showed an accumulation of proline in the leaves due to an altered proline metabolism. Our results however show an accumulation of Alanine and Asparagine. The Asparagine was however lost in the post pollination period of the maize cultivars.

These two amino acids are very important in plant metabolism as they are amino donors in nitrogen metabolism in the leaf (Leegood *et al,* 2000). They can also be used, in addition to glutamate and serine, to provide nitrogen for the synthesis of glycine during

photorespiration (Ta and Joy, 1986). The glycine produced from photorespiration can then be used for glutathione synthesis. Glutathione provides protection in stressed plants, as it is an antioxidant that prevents photo -oxidation and photo inhibition. (Leegood *et al,* 2000). It has been proven that *Striga* predisposes maize to photo inhibition during periods of high irradiance (Leegood *et al,* 2000).

These findings are complex and difficult to interpret vis-à-vis the process of *Striga* resistance in maize. However it is clear that the mutation, which predisposes the plants to *Striga* resistance, do alter the profiles of the amino acids mentioned. More work is therefore needed to unequivocally establish a relation between the two effects.

5.4 Analysis of Root phenolics

The spectrophotometric analysis of the samples was based on the principle that optical density is directly proportional to concentration of phenolic substances. The root extracts analyzed for total phenolics displayed variation as seen in Table 4.2 The absorbance profiles showed characteristic differences between the mutant, tolerant, susceptible varieties and the control. The absorbance values of most mutants were very high ($\leq$0.786-$\geq$0.715). The resistant variety from CYMMYT also had a high optical density (0.716). The natural tolerant variety, Uganda Red and one mutant had moderately high optical density. In contrast, the control had a significantly low optical density value (0.337). In general, the total phenolic concentrations of the mutants and the tolerant varieties were comparatively higher than those of the susceptible variety and the control.

Our results showed that concentration differences between the *Striga*-resistant mutants were statistically insignificant. The tolerant variety also had insignificant concentration differences with the mutants. Cases of accumulation of phenolic

compounds in the roots of *Striga* host plants like maize have been reported (Patil *et al*, 1967; Yoder, 2001). Sorghum, under parasitic infection synthesizes stilbenes and anthrocyanidins that are essential in plants defense mechanism (Lo *et al*, 2005). These are found in high concentrations in root extracts (Yoder, 2001).

Although this study shows accumulation of phenolics in maize root extracts, it is not clear whether it can be linked to the observed *Striga* resistance characteristics of the mutants. Nevertheless the fact that sorghum accumulates phenolics in response to infection tends to point to a relationship. Could it be that the observed resistance in the mutants is related to the elevated concentration of phenolics? Only further work can provide a definite answer to this question.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

This work has provided tentative evidence that there are differences between the *Striga* resistant mutants and the *Striga* susceptible maize lines. These differences especially the protein profiles first appeared in the F_1 generation of the mutated maize seeds. This study has confirmed that they are still carried in the F_4 generation. The fact that such differences still exist in the F_4 generation attest to heritability and stability of the characteristics. The differences in the concentration of the total phenolics between the mutants and the control are a novel finding of this study. Furthermore, based on the difference on the protein profiles, it is reasonable to speculate that this will be an enduring and stable characteristic. This is because the experimental plants were inbred for four generations.

In this study, we attempted to relate the observed *Striga* – resistance of maize mutants to more than two factors as previously reported (Kiruki, 2000). It therefore appears that the phenomenon of *Striga*-resistance in maize is correlated to more than just one or two factors. Due to unavailability of equipment it was not possible to carry out a detailed analysis and identification of the accumulated phenolics. Further qualitative and quantitative analyses of these accumulated phenolics should be an incentive for the undertaking.

The identification of the proteins of interest and their sequences should provide the first step towards the isolation of the gene(s) that confer resistance to *Striga* in maize. When that point is reached, then the genes can be used to produce transgenic *Striga*

resistant maize (or other cereals). Such cereals can then be produced in bulk and distributed to farmers to combat the menace of this obnoxious weed.

This study has therefore opened the way for such optimism in that it has demonstrated a link between putative *Striga* resistant mutants and the differences in the profiles of proteins, amino acids and total phenolics in the plants studied.

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