DEVELOPING *STRIGA* RESISTANCE IN MAIZE THROUGH MAIZE-SORGHUM HYBRIDIZATION

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JULY 2018
DECLARATION

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

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To Assumpta and Stephen
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# ACRONYMS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AATF</td>
<td>African Agricultural Technology Foundation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CIMMYT</td>
<td>International Maize and Wheat Improvement Center</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>DAP</td>
<td>Day after pollination</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>F₁</td>
<td>First filial generation</td>
</tr>
<tr>
<td>F₂</td>
<td>Second filial generation</td>
</tr>
<tr>
<td>F₃</td>
<td>Third filial generation</td>
</tr>
<tr>
<td>g/L</td>
<td>Gram per liter</td>
</tr>
<tr>
<td>ICRISAT</td>
<td>International Crops Research Institute for the Semi-Arid Tropics</td>
</tr>
<tr>
<td>IITA</td>
<td>International Institute of Tropical Agriculture</td>
</tr>
<tr>
<td>KALRO</td>
<td>Kenya Agriculture &amp; Livestock Research Organization</td>
</tr>
<tr>
<td>LB</td>
<td>Luria and Bertani</td>
</tr>
<tr>
<td>Mg/L</td>
<td>Milligram per liter</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>Mya</td>
<td>Million years ago</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SAR</td>
<td>Systemic acquired resistance</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical analysis software</td>
</tr>
<tr>
<td>Spp</td>
<td>Species</td>
</tr>
<tr>
<td>SSA</td>
<td>Sub-Saharan Africa</td>
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**ABSTRACT**

*Striga hermonthica* is an obligate hemi-parasite that belongs to the family Orobanchaceae. Crop infestations by this parasitic weed pose a great threat to agriculture in Sub-Saharan Africa. The most commonly affected crops include cereals such as sorghum, maize, and rice, which are important to subsistent livelihoods in the region through food production. Some of the control measures currently used include weeding and herbicide seed dressing using Imazapyr (StrigAway®) maize and crop rotation with non-host plants. For effective control, these strategies will need to be combined with other promising integrated *Striga* management approaches. This study focused on a *Striga* resistant sorghum cultivar N-13 whose mechanism of resistance is cell wall fortification by lignin synthesis. In the first approach, cloning of a key lignin biosynthesis gene, *Hydroxycinnamoyl transferase* (*HCT*) was carried out. *HCT* gene has a nucleotide sequence of 1489 bps and codes for an enzyme made of 496 amino acid sequence. The HCT protein consists of two domains, and the active substrate-binding site is located between these two. The probability of successfully hybridizing maize and sorghum was evaluated by reconstructing phylogenetic relationship of *Sorghum bicolor* HCT protein and its orthologs in the grass family. The *Sorghum bicolor* HCT protein was aligned with 7 other orthologs and revealed a 92% identity to *Zea mays* HCT protein. The second approach involved crossing maize inbred line E04 with a *Striga*-resistant sorghum cultivar N-13. A total of 9 out of 540 maize cobs formed embryos representing a 1.67% success rate. The embryos were rescued and cultured *in vitro* and after that regenerated. Molecular characterization of hybrids was done using the Polymerase Chain Reaction (PCR). The screening strategy involved amplification of the CEN38, a repetitive marker unique only to sorghum. Hybrid plants expressing CEN38 marker were screened for resistance to *S. hermonthica* using a soil free laboratory assay. The F₁ hybrids had a 74.19% survival rate and a death rate of 25.81%. Sorghum cultivar N-13 served as a positive control for post-*Striga* germination resistance. The mean count, length and biomass of *S. hermonthica* plants growing on F₁ and F₂ hybrids roots were significantly lower compared to the sorghum cultivar N-13 and the susceptible maize inbred line E04 according to Tukey’s HSD test (*p*<0.05). The hybrids exhibited mechanical resistance against *S. hermonthica* similar to the sorghum cultivar N-13. This research shows that (i) lignin biosynthesis gene, *HCT* (1489 bps) from sorghum cultivar N-13 can be isolated and cloned into a vector. Therefore, this gene can be transformed into susceptible host plants to confer resistance to *Striga* spp. (ii) Maize-sorghum hybrid can be used as a strategy to alleviate yield loss associated with *Striga* infestation.
CHAPTER ONE

INTRODUCTION

1.1 Background information

Maize is a major staple food for more than 300 million people in Sub-Saharan Africa. It was introduced to Africa in 1500s, and since then, it has become one of the most important cereal (Gurney et al., 2003). Fifty percent of the Sub-Saharan (SSA) population relies on maize for 20% of their caloric intake (Gianessi, 2014). About 28 million hectares of maize in Africa are cultivated yearly (IITA, 2009). Its production in developing countries is about 100 million hectares (Suleiman & Rosentrater, 2015).

Maize production faces a broad range of challenges in Sub-Saharan Africa which includes poor soil fertility and drought (Reynolds et al., 2015). One of the major maize production constraints has been infestation by parasitic weeds such as *Striga hermonthica* (Rich & Ejeta, 2008; Teka, 2014). Twenty to eighty percent maize yield loss is associated with *Striga hermonthica* damage (Teka, 2014). In western Kenya, moderate and severe *Striga* infestation results in maize crop yield loss of 50% and 80% respectively. *Striga hermonthica* causes economic losses exceeding US $1 billion annually (Parker, 2012).

To date, there is no known complete *Striga* resistance found in maize. Maize did not co-evolve with *Striga* due to its exotic origin. Therefore, it lacks the desired systemic acquired resistance (Amusan et al., 2008). *Striga hermonthica* is difficult to control because its life cycle is greatly synchronized to its host (Yoder & Scholes, 2010). Host and non-host plants
produce chemical signals called strigolactones that induce germination of *Striga* seeds followed by haustoria formation. The *Striga* radicle makes contact with the host roots, penetrates the root cell and differentiates into vessels that act as a bridge to the xylem for the supply of water and nutrients, resulting in the host plant exhibiting severe drought symptoms (Rich & Ejeta, 2008). *Striga* weed manifestation appears to be aggravated by drought, poor soil fertility and degradation of soil minerals, a problem predominant in SSA making the atrocious weed flourish in Africa (Teka, 2014).

Different strategies have been used to curb the *Striga* menace such as hand weeding, sanitation, crop rotation, use of trap crops, catch crops, intercropping, use of herbivorous insects and Imazapyr-resistant maize technology without substantive effects on its control (Teka, 2014). Development of host plant resistance remains the most feasible technique to overcome this *Striga spp* menace (Gurney *et al.*, 2003). Although there is no complete genetic host resistance reported in maize, some wild relatives of sorghum varieties have shown *Striga* resistance (Gurney *et al.*, 2002). These wild relatives of sorghum varieties are believed to have co-evolved with *S. hermonthica* in Sudano-Ethiopian region in Africa, hence, the tolerance acquired towards *S. hermonthica* (Gurney *et al.*, 2006; Mbuvi *et al.*, 2017).

The main goal of this study was to develop a *Striga* resistant maize inbred line by crossing with the *Striga* resistant sorghum cultivar N-13. Secondly, this study was designed to characterise and clone one of the systemic acquired
resistance gene, *Hydroxycinammoyl Transferase (HCT)* that codes for a critical enzyme that enhances lignin deposition on sorghum cultivar N-13.

### 1.2 Problem statement

Maize is a major food crop globally ranked third after wheat and rice (Olaniyan, 2015). It is an important staple food for more than 1.2 billion people in SSA and Latin America. In the SSA, maize cultivation is severely limited by drought and poor soil fertility, which are the main abiotic constraints (Reynolds et al., 2015). One of the major biotic constraints is infestation by *S. hermonthica*, which accounts for an annual crop loss of US $53 million in Africa (Atera et al., 2013). In Kenya, *Striga* infestation is particularly severe in the western region where about 180,000 acres of arable land are affected (Woomer, 2004). Effective control of *Striga* weed is difficult because by the time it emerges above the ground, it has already detrimentally affected the growth and development of the plant (Teka, 2014). Additionally, the *Striga* seeds remain viable in the soil for more than ten years, thereby building a reservoir for subsequent cropping (Emechebe et al., 2004).

Conventional strategies such as hand weeding, animal ploughing and burning of fields towards curbing its spread and control have not been effective as they are not sustainable. The sowing of herbicide-treated seed utilizing Imazapyr® has proven effective but only in cases where mono-cropping is practised (Ransom et al., 2012). Imazapyr® intolerance is exhibited in other crops like common beans, hence the likely effective strategy would be the development of host resistant inbred maize lines (Teka, 2014).
1.3 Justification

Due to the ineffectiveness of the existing strategies for control of *Striga* in SSA and the absence of *Striga* resistance in maize, it is almost certain that wide hybridization between a resistant sorghum cultivar and a susceptible maize inbred line with the help of embryo rescue technique, among other biotechnological improvement techniques, is the most promising resolution. Developing *Striga*-resistant maize germplasm is imperative since it will contribute to reducing losses due to *Striga* infestation incurred by maize farmers in SSA thus increasing yields. The use of *Striga*-resistant sorghum germplasm as a source of systemic acquired resistance (SAR) and its subsequent transfer into maize inbred line may be the ideal approach for finally controlling the witch weed. This will aid in alleviating the maize yield and economic losses due to *Striga* infestation and henceforth improve food security in Sub-Saharan Africa. Secondly, characterization and cloning a key systemic resistance gene, *HCT*, which is involved in lignin biosynthesis, will depict the phylogenetic relationship of the gene to the other members of the grass family.

1.4 Research Questions

i. Do orthologs of *HCT* gene in the grass family have a close phylogenetic relationship?

ii. Does intergeneric hybridization between maize and sorghum produce a viable hybrid?

iii. Does intergeneric hybridization between *Striga* susceptible maize and *Striga* resistant sorghum produce a *Striga* resistant hybrid?
1.5 Objectives

1.5.1 General objective

To develop a Striga resistant maize-sorghum hybrid based on high lignification of sorghum cultivar N-13’s systemic acquired resistance gene (*Hydroxycinnamoyl transferase*).

1.5.2 Specific objectives

i. To characterize and clone sorghum systemic acquired resistance gene, *Hydroxycinnamoyl transferase*.

ii. To develop an intergeneric hybrid between maize and sorghum.

iii. To screen for Striga resistance in maize-sorghum hybrids.
CHAPTER TWO
LITERATURE REVIEW

2.1 Striga Biology

The Striga genus comprises of 30 species of obligate root hemi-parasites that infests cereal and legume crops in Sub-Saharan Africa. It belongs to the family Orobancheaceae and originated from Sudano-Ethiopian region of Africa (Gurney et al., 2003). There are three economically important Striga species which include; hermonthica, asiatica and gesneroides. Most Striga species infests maize, sorghum, sugar cane, rice and millet but gesneroides has evolved and parasitizes dicots like cowpea, tobacco and sweet potato (Spallek et al., 2013).

Striga has developed ways of successful reproduction by producing over 50,000 seeds per plant that are very minute dust-like seeds making their dispersion easy by wind, water and animals. Moreover, the seeds can stay viable for more than ten years in the soil (Spallek et al., 2013).

Striga has evolved and devised three key mechanisms for its survival. First, the presence of a host and production of the necessary Striga germination stimulants (strigolactones). Second, the development of the Striga haustorium required for the establishment of the xylem connection with the host xylem. Lastly, the maintenance of the parasitism with the host plant by establishing a xylem to xylem connection with the host enabling siphoning of water and nutrients for the survival of Striga till seed set (Spallek et al., 2013). Factors
such as nitrogen deficiency, water stress and well-drained soils which are predominant in Sub-Saharan Africa have aggravated the weed by making a friendly environment for the thriving of the weed (Haussmann et al., 2004). This makes control strategies ineffective in promising a lasting solution aggravated by adverse climatic and edaphic factors (Teka, 2014).

2.2 Striga life cycle

Striga seeds can survive for more than ten years, but once the seed germinates, the Striga endosperm can support its life for only 3 to 7 days. For that reason, the geminated Striga seed must establish a parasitic relationship with a host failure to which it dies (Spallek et al., 2013). Host plants produce strigolactones for regulation of shoot and root branching and symbiotic relations with arbuscular mycorrhizal fungi. However, these strigolactones act as Striga germination stimulants too (Runo et al., 2012). Once the Striga seeds are in the proximity of the host plants roots, they encounter these stimulants resulting to their germination. Their root tips start growing chemotropically towards the host roots for attachment (Spallek et al., 2013; Ichihashi et al., 2015).

The minute root tip establishes contact with the host root, the host produces haustorium inducing factors which include various quinones, flavonoids and phenolic acids supporting the formation of the Striga haustorium (Westwood et al., 2010). The root tip develops swelling and forms haustorial hairs that act as attachment peg to the host root (Yoshida & Shirasu, 2009).
Once the haustorium penetrates the host cell, the host and parasite are connected via xylem-bridge (Ichihashi et al., 2015).

Penetration is usually completed within 48 to 72 hours once contact has been made. *Striga* starts growing upwards, and the developed adventitious roots may form a secondary attachment with other host roots (Ichihashi et al., 2015). In 4 to 8 weeks, *Striga* emerges above the soil and starts photosynthesis. Figure 2.1 shows the life cycle of *Striga* before and after emergence. Although *Striga* carries out photosynthesis, the carbon fixation occurs at insufficient levels, so it continues depending on the host plant for sufficient carbon supply (Teka, 2014). *Striga* flowering occurs 4 weeks post-emergence, and pollination takes place, and the seeds mature in seed pods for 4 weeks. The seedpods crack and further deposit the seeds back to the soil (Spallek et al., 2013). The host plant is significantly affected by *Striga* parasitism, and exhibits stunted growth, drought stress characteristics, wilting and low yield (Rich et al., 2004).
2.3 Economic importance of *Striga* in Africa and other parts of the world

*Striga* ranks top among the biotic constraints to cereal production with an infestation of up to two-thirds of the arable land in Africa, an economic loss estimated up to US $7 billion yearly and the most severely affected are the small-scale farmers in SSA (Teka, 2014). *Striga* infests about 50 million hectares of arable land cultivated with legumes and maize in Sub-Saharan Africa, and to date, this has resulted in annual yield loss exceeding US $10 billion dollars (Zhen *et al.*, 2014). *Striga* infestation on cultivated land results in 20% to 80% of crop yield loss depending on the severity of the infestation (Atera *et al.*, 2012).

**Figure 2.1**: The lifecycle of *Striga*. Signalling and penetration are the *Striga* pre-emergence stages while flowering and seed rain are the post emergence stages of the lifecycle of *Striga*. Source: (https://ars.els-cdn.com/content/image/1-s2.0-S000398611000322X-gr1.jpg).
Striga hermonthica is the most common and pernicious parasite predominant in the high maize production western regions in Kenya. Striga is estimated to be about 180,000 acres in western Kenya with crop loss estimated at Ksh800 to Ksh2000 million per year (Woomer, 2004). This has led to 12.3% loss of the 2.4 million metric tons of maize production annually in Kenya (Mohamed et al., 2014).

Striga asiatica was accidentally introduced to Eastern USA in the 1950s and infested about 200,000 hectares of arable land (Mohamed et al., 2008; Parker, 2009). However, the US government funded a US $ 250 million project that successfully eradicated the weed. The project involved the use of high doses of 2, 4 Dichlorophenoxyacetic acid (2, 4-D) herbicide and the application of ethylene gas to induce suicidal germination in Striga infested farms. This project successfully reduced Striga infestation to 2800 hectares (Eplee, 1992).

Striga asiatica widely occurs in India but does not cause acute problems as its African relative (Parker, 2009). In the 1970s, Striga caused sorghum yield losses ranging from 25% to 100% depending on the severity of infestation. However, the application of high dosages of 2, 4-D herbicide proved to be an effective Striga control and therefore, to date, Striga does not threaten food security in India (Korwar & Friesen, 1984).

2.4 Striga control strategies and their limitations

Striga control has posed a great challenge due to its ability to produce thousands of seeds that remain in the soil dormant and viable for more than ten
years creating a reservoir for next cropping. *Striga* infestation can be reduced through improved soil fertility. Other techniques involve the use of herbicides, nitrogenous fertilizers, irrigation and intercropping with allelopathic legumes (Runo & Kuria, 2018).

Hand weeding is a common technique to alleviate seed set in the soil and dispersion. However, it is labor intensive and ineffective as the weed emerges above the ground long after weeding has ceased. Also, it does not improve the yield of the already infected crop (Teka, 2014). In Kenya, farmers try to control the weed by hand weeding and burning fields, but these still do not destroy the weed since a single *Striga* plant produces over 50,000 seeds that remain viable and dormant in the soil for more than ten years (Woomer, 2004; Teka, 2014).

Another commonly used technique is sanitation. It involves noting *Striga* infested farms and isolating them preventing further cultivation (Teka, 2014). Even though this method prevents further deposition of the *Striga* seeds into the soil, it does not tame the *Striga* problem because *Striga* seeds already deposited on the ground will remain in the soil viable for over ten years and it will be a waste of land since it is set aside. Crop rotation with non-host plants like wheat and barley results in a decrease of further *Striga* seeds deposition into the soil (Parker, 2009). However, this requires a three consecutive year’s exercise to be able to reduce *Striga* infestation by 50% as it has been shown in Ethiopia limiting the small-holder farmers that maximise their production (Teka, 2014).
Intercropping with trap crops like groundnut and soybeans has been practiced where they induce suicidal germination of the Striga seeds (De Groote et al., 2003). The witchweed seedlings are then ploughed off early before they deposit their seeds into the soil (Kroschel & Elzein, 2006). However, farmers do not practise this method despite its ability to slow Striga virulence in low-density infestation (Randrianjafizanaka et al., 2018). The use of Imazapyr herbicide coated on maize seed diffuses into the soil and kills germinated Striga seed thereby preventing the attachment of Striga on the host plant (Thomson et al., 2010). Despite that, this technology doesn’t discriminate and destroys any other crop in the field (Teka, 2004).

The push-pull strategy has been applied to maize farming and proven effective in reducing the Striga infestation. It involves intercropping the host plant with a repellent crop that disguises the presence of the host plant and a border crop around the intercrop (Atera et al., 2012). This strategy protects the host plant as the parasite is repelled away and attracted to the trap crop. Desmodium uncinatum is the commonly used repellent crop as it produces root exudates rich with Striga germination stimulants and post-germination inhibitors that destroy the Striga germinated plants (Teka, 2014). Another commonly used trap crop is the nappier grass. However, this strategy needs to be integrated with other Striga management techniques for effective control (Khan et al., 2010).

Edaphic factors are a major concern in Striga nourishment majorly due to its resilience in poor soil fertility, a factor contributing to the wide-spread and
flourishment in the Sub-Saharan Africa (Rich et al., 2004). It has been shown that nitrogen and phosphorous deficiency accentuates *Striga* damage on host plants (Teka, 2014). Therefore, the application of nitrogen and phosphorous manure has proved to improve the performance of the host plants at the same time alleviating the effect of *Striga* infestation. However, resource-poor farmers carry out monoculture farming with low input of manure and fertilizer promoting *Striga* infestation (Gurney et al., 2002; Teka, 2014).

### 2.5 Natural Sources of *Striga* resistance

*Striga* exists in savannah lands and their distribution is sparse, and there might be the existence of resistance exhibited by grasses growing in Savannah lands. In agricultural systems, *Striga* is dense due to monoculture which elevates *Striga* infestation (Spallek et al., 2013).

Identification of source germplasm will aid in developing resistant crop varieties. Breeding for post-attachment resistance seems to be a promising long-term control for *Striga* spp. The major *Striga* resistance among the Gramineae family lies in the *Sorghum* genus (Mbuvi et al., 2017). Host plants develop resistance against *Striga* after a long time exposure to the parasite, a process known as systemic acquired resistance (Gurney et al., 2003).

Sorghum co-evolved with *Striga* and underwent selection for *Striga* resistance genes and eventually developed resistance to parasitism resulting to some wild sorghum varieties such as *Sorghum arundinaceum* exhibiting resistance to *S. hermonthica* by having low levels of Strigolactones (Gurney et al., 2003).
Sorghum cultivar N-13 is a good source for *Striga* resistance by inhibiting penetration of *Striga spp* into the endodermis and to date, it is used as a donor of SAR (Ngugi *et al.*, 2015).

There exists a paucity of known *Striga* resistance in *Zea mays*, and it is speculated that it might be due to the absence of the interaction between *Striga* and maize to develop systemic acquired resistance since maize has an exotic origin. However, the wild relatives like *Zea diploperennis* and *Tripsacum dactyloides* have been reported to impair *Striga* intrusion (Timko *et al.*, 2012). To date, there is no known post-attachment resistance existing in maize cultivars (Amusan *et al.*, 2008).

There has been some level of resistance seen in rice cultivars. For example, New Rice for Africa cultivars have a broad spectrum resistance towards *Striga* species (Cissoko *et al.*, 2011). Besides, rice cultivar nipponbare harbours resistance to *Striga hermonthica* inhibiting vascular connection (Gurney *et al.*, 2006). Moreover, resistance has also been observed in dicots, for examples, cowpeas varieties 58-57 and B301 that exhibit resistance to *Striga gesnoroides* (Westwood *et al.*, 2010).

### 2.6 Mechanisms of natural resistance against *Striga*

To date, there are five documented resistance mechanisms that tolerant host cultivars exhibit against *Striga*. These are: low strigolactone production, *Striga* germination inhibitors, low *Striga* haustorium initiator signal, hypersensitivity reaction and mechanical resistance (Mohamed *et al.*, 2010). These
mechanisms have been classified into two categories: Pre-attachment resistance that occurs before *Striga* makes contact with host roots and post-attachment resistance that occurs after *Striga* makes contact with the host roots (Rodenburg & Bastiaans, 2011).

Production of lower levels of strigolactones by the host plants results to reduced *Striga* germination, and hence this acts as a natural resistance mechanism against *Striga* (Ezeaku & Gupta, 2004). This form of resistance has been characterized in some rice and sorghum cultivars (Cissoko *et al.*, 2011). The resistant rice cultivars; Super Basmati, TN 1, Anakila and Agee, produce low strigolactones levels resulting to low *Striga* germination, attachment and dry biomass. These cultivars have shown to perform well in yield production, and the few attached *Striga* do not affect the performance of the cultivars (Jamil *et al.*, 2012). The sorghum cultivars harbouring this mechanism are SRN 555 and SAR lines IS 15401 and IS 9830. These cultivars have good yields even in the presence of *Striga* infestation (Haussmann & Mauck, 2008).

Hypersensitivity reaction is the second mechanism of *Striga* resistance whereby the attached root cell undergoes necrosis and dies resulting in the death of the attached *Striga*. This mechanism was observed on wild sorghum accession (P47121) and two sorghum cultivars (Framida and Dobbs) in which the *Striga asiatica* parasite died after necrosis occurred at the site of attachment (Mohamed *et al.*, 2003). Hypersensitivity reaction was also observed on wild sorghum accession WSE-1 whereby deposition of secondary
metabolites occurred at the point of attachment of *Striga* at the roots (Mbuvi *et al*., 2017).

Some host plants produce sufficient levels of strigolactones for *Striga* germination but low levels of haustorial initiator factor (HIF). The germinated *Striga* seed is impaired and cannot form haustorium (Mohamed *et al*., 2010). Therefore the parasite fails to establish parasitism with the host and dies off, once it has exhausted the food stored on its endosperm. The signals for haustorium formation have not yet been identified, but some phenolic compounds have been shown to induce haustorial formation. For example, a quinone like 2, 6 dimethoxy1, 4-benzo quinine (DMBQ) induces haustorial formation, but it has not been detected in host plants (Runo & Kuria, 2018).

*Striga* tolerant host plants produce haustorium initiator factor inhibitor that impairs the development of the *Striga* haustorium (Gurney *et al*., 2003). The production of haustorium initiator factor inhibitor has been observed in some wild sorghum line PQ434 but none has been detected on cultivated sorghum (Rich & Ejeta 2008). Furthermore, *Tripsacum dactyloides*, a wild relative of maize, has been shown to produce an inhibitor of haustorial development when it was exposed to germinating *Striga hermonthica* seed (Gurney *et al*., 2003). Additionally, similar findings were reported in maize inbred lines derived from a backcross with *Zea diploperennis* (Amusan *et al*., 2008).

Mechanical resistance to *Striga* has been attributed to the strengthened secondary cell wall of plant roots, which acts as a mechanical barrier to
haustorial establishment (Mohamed et al., 2010). It involves increased deposition of lignin on the secondary walls of plant cells resulting in impairment of *Striga* haustorium penetration to the root cells (Maiti et al., 1984). This form of mechanism has been studied on Sorghum cultivar N-13 in which the *Striga* haustorium could not penetrate the root cell endodermis due to high deposition of lignin and silica on the endodermal and pericycle cells by the host (Maiti et al., 1984).

Other *Striga* hosts exhibit resistance, but the mechanism is yet to be characterised. For example, rice cultivars Nipponbare and NERICA are substantially resistant, but their mechanism of resistance is not yet known (Cissoko et al., 2011).

### 2.7 Genetic analysis of resistance to *Striga*

Information on the genetics of *Striga* resistance has been limited due to the paucity of *Striga* resistance germplasm. Genetic analysis of resistance has been extensively researched on wild relatives of sorghum, maize and rice and their progenies especially of F₁ and F₂ generation (Haussmann et al., 2004). Resistance to *Striga* is usually species-specific and site-specific (Rodenburg et al., 2017). By way of illustration, resistance to *Striga hermonthica* does not necessarily confer resistance to *Striga asiatica*. Moreover, it has been shown in sorghum cultivars exhibiting resistance in a certain location but susceptibility in different locations. This has been due to site-specific *Striga* races (Haussmann et al., 2000).
In the determination of the genetic basis of low production of strigolactones, a study was done on resistant sorghum cultivar SRN-39 and susceptible cultivars Shanqui red, P954063 and IS4225 (Reddy et al., 2006). It was deduced that the low germination stimulant lgs, a single recessive gene located at the nuclear region, controlled the low production of Striga germination stimulant according to segregation ratios (Timko et al., 2012).

However, agar gel assays of recombinant inbred populations between sorghum cultivar IS 9830 and E 36-1 revealed that production of strigolactones is controlled by one major gene and several other minor genes (Haussmann et al., 2000).

Haustorial initiator signal gene was investigated by crossing Striga resistant wild sorghum accession P78 and susceptible line. Their progenies were analysed, and the trait was discovered to be controlled by a dominant allele of a single gene (Mohamed et al., 2010).

Hypersensitivity mechanism of Striga resistance trait was determined by crossing Sorghum arundinaceum that exhibits hypersensitivity reaction to Striga with susceptible varieties. It was elucidated that the hypersensitivity resistance mechanism is controlled by the presence of two nuclear genes with dominant action, HR1 and HR2 gene (Hess & Gupta, 2004).
2.8 Interspecific crosses as *Striga* control mechanism

2.8.1 Wide hybridization to confer desired traits in plants

Wide hybridization is the crossing between two genetically distant plants (Smith & Deng, 2015). The major types of hybridization involve interspecific and intergeneric crosses. This technique is done largely to confer the desired traits into the hybrids to overcome abiotic and biotic stress factors, and in such a case it is called introgressive hybridization. It improves crops by transferring specific preferred traits such as pest and stress resistance, to crops from their wild relatives (JinFeng & Adelberg, 2000).

Wide hybridization occurs naturally or induction by crossing distant taxa resulting in the generation of new species (Orians, 2000). This technique started as early as 1800’s when Rimpu produced the first inter-generic hybrid from a cross between rye and wheat cross that exhibited drought and disease resistance (Chaudhary *et al.*, 2015). To date, so many wide crosses have been achieved and their challenges overcome (Soltis & Soltis, 2009).

2.8.2 Sorghum cultivars as a source of systemic acquired resistance (SAR).

Wide crosses have been made to confer resistance in susceptible host plants (JinFeng & Adelberg, 2000). Breeding for *Striga* resistance in sorghum has been extensively exploited (Mbuvi *et al.*, 2017). Sorghum cultivar N-13 widely known for its mechanical resistance against *Striga* has been used as a source of systemic acquired resistance (SAR) donor in many wide crosses. For example, Rwandan farmer preferred variety IS8193 was crossed with sorghum
cultivar N-13 (Niyibigira et al., 2013). The F$_1$ and BC$_1$F$_1$ progenies were screened for markers associated with *Striga* resistance linkage groups. A similar study was done successfully to transfer the *Striga* resistance from sorghum cultivar N-13 to Kenyan farmer preferred sorghum variety, Ochuti (Ngugi et al., 2015).

The crossing of sorghum N-13 with susceptible host plants has proved to transfer the systemic acquired resistance to the susceptible host. This has been seen in a backcross between sorghum cultivar N-13 with three farmer preferred sorghum cultivars Tabat, Wad-Ahmed and AG-8. The progenies were confirmed to be resistant, once *Striga* field resistance screening was done (Gamar & Mohamed, 2013).

Sorghum cultivar SRN 39, the second most used SAR donor harbours *Striga* resistance by low production of strigolactone (Haussmann et al., 2000). It has been used in crosses with high yielding sorghum varieties such as CS 95, Gaya Early, KSV 4, ICSV 400, CS 54, and ICSV 111. The crosses were resistant to *Striga* and had a high yield (Ngugi, 2014). Sorghum cultivar SRN 39 has also been crossed with a susceptible cultivar P954063 and the resulting backcross generation was resistant, and it was elucidated that the resistance was heritable (Hess & Ejeta, 1992).

### 2.9 CEN38 as an evolutionary marker

CEN38 marker is a repetitive sequence located at the centromere region of chromosomes of sorghum and sugar cane but absent in maize (Wang &
Bennetzen, 2012). It is present in the genomes of *Sorghum laxiflorum*, and *Sorghum bicolor* but it occurs in low copy numbers in sugarcane (Zwick *et al.*, 2016). It is rich in A-T nitrogenous base, and its protein is made of a dimer of 280 bps with two 140 bps monomers (Anderson, 2005). CEN38 marker occurs in different sizes ranging between 75 bps to 2000 bps. It has been previously used to determine the evolutionary relationships among different sorghum species (Anderson, 2005; Price *et al.*, 2015).

Furthermore, in studies where *Sorghum bicolor* has been hybridized with another species, the CEN38 marker has been used to verify the transfer of the *Sorghum bicolor* genome into the other species. For example, the marker was used to authenticate the hybrid resulting from hybridization between *Sorghum bicolor* and *Sorghum macrospermum* (Miller *et al.*, 1998).

### 2.10 Lignin and the genetic control of its synthesis

Lignin is an abundant polymer and comprises 30% in vascular tissues. It is comprised of three monolignols alcohols namely; p-hydroxyphenyl (H), guacyl (G) and syringyl (S) alcohols which constitute the three types of lignin. Hydroxyphenyl (H-type lignin) is the least lignin type in angiosperms comprising of < 2% of the lignin. Therefore lignin is highly composed of guacyl and syringyl lignin (Zhao *et al.*, 2013). It is deposited on the secondary walls of specialized cells where it contributes to plant growth and development. Lignin is found in xylem fibers and tracheids of xylem tissue, sclerenchyma, periderm and the phloem.
Lignin deposition occurs at the cell wall once the growth of the cell is completed. It strengthens the cell walls, conducts water and acts as a defense structure against pathogens (Harakava & Harakava, 2005). Mostly, lignin is synthesized due to wounding and presence of pathogens (Hawkins et al., 1997). It waterproofs plant cells from dehydration, and its indigestibility hinders herbivores. It is believed that lignification contributed to the colonization of land plants (Rogers & Campbell, 2004).

The synthesis of lignin spatial and temporal control is regulated according to the plant development (Wagner et al., 2007). The presence and activities of lignin enzymes, general phenylpropanoid pathway, and monolignol synthesis pathway determine the availability of phenylalanine, hydroxycinnamoyl-CoA thioesters, and monolignol precursors. The availability of lignin synthesis genes is controlled at the level of transcription (Lina et al., 2010). Also, the timing and localization of the transcription regulation are consistent with when and where lignification occurs. The regulation of lignin synthesis must conform to the plant status (Rogers & Campbell, 2004).

Some of the essential genes of lignin synthesis include *PAL, 4 CL, CAD, HCT, CCR* and *C4H*. They function under the control of a circadian clock (Harmer, 2000). A study in *Arabidopsis thaliana* showed that during the night; cell elongation stopped while cell lignification was initiated and it continued until dawn (Rogers & Campbell, 2004). It was proposed that the circadian regulation of the lignin biosynthesis genes was equivalent to the elongation of hypocotyl relative to cell wall biogenesis (Harmer, 2000).
Most of the promoters controlling the expression of the lignin biosynthesis genes have AC elements commonly known as H-box that function as regulatory motifs within the promoters for direct gene expression related to lignification. The AC elements are recognized by the N-terminal DNA binding domain of plant R2R3-MYB transcription factors resulting in the regulation of the activity of the lignin synthesis genes (Rogers & Campbell, 2004).

2.11 HCT family of genes

Hydroxycinnamoyl-CoA: shikimate/ quinate hydroxycinnamoyltransferase (HCT) gene is one of the key systemic acquired resistance genes of Striga resistant sorghum cultivar N-13. HCT gene codes for an acyltransferase enzyme involved in lignin biosynthetic pathway HCT (Weng & Chapple, 2010). The enzyme is located on the outer and inner cells of phloem and xylem tissues of vascular plants (Hoffmann et al., 2004; Walker et al., 2013). HCT enzyme consists of two domains as shown in Figure 2.2. Domain 1 consists of N-terminus and C-terminus residues while Domain 2 consists of C-terminus residues only. The active substrate-binding site is located between the two domains of the enzyme (Eudes et al., 2016).
Figure 2.2: Molecular structure of HCT protein. Derived from Berkley Lab’s Advanced Light Source. Key: p-coumaryl-shikimate and HS-CoA at the binding site (multicolored). The purple and green colored areas are the two domains of HCT protein.

The enzyme converts p-coumaroyl-CoA and caffeoyl-CoA to their corresponding shikimate or quinate esters and catalyses the reverse reaction as seen in Figure 2.3 below. HCT enzyme has been highlighted in red colour. The shikimate and quinate esters act as substrates for p-coumarate 3-hydroxylase (C3H) during the phenylpropanoid pathway for lignin synthesis (Harakava & Harakava, 2005). HCT is a member of the BAHD acyltransferase superfamily. The superfamily has extensively diversified within the plant kingdom, and it is involved in the biosynthesis of different secondary metabolites (D’Auria, 2006).
Figure 2.3: Monolignol biosynthetic pathway (Whetten & Sederoff, 1995). *HCT* gene is the metabolic entry point for G and C lignin units.

The BAHD acyltransferase superfamily constitutes groups of enzymes which include acetyltransferases and benzoyl/hydroxycinnamoyl-CoA acyltransferases (Lina *et al.*, 2010). The acetyltransferases include benzyl alcohol O-acetyltransferase (BEAT) which forms benzyl acetate, a floral volatile and deacetylvindoline 4-O-acetyltransferase (DAT) involved in the synthesis of alkaloid vindoline. The benzoyl/hydroxycinnamoyl-CoA acyltransferases include anthranilate N-hydroxycinnamoyl/benzoyltransferase (HCBT) which synthesize a class of phytoalexins known as anthramides and anthocyanin O-hydroxycinnamoyltransferase (AHCT) which is involved in the synthesis of acylated anthocyanins (D’Auria, 2006).
The BAHD family members are monomeric enzymes with a molecular mass ranging from 48 to 55 kD with an average of 445 amino acids (Walker et al., 2013). They occur within the cytosol of the cells (D'Auria, 2006). Furthermore, they share two conserved domains which include HXXXDG situated at the center of each enzyme and a DFGWG motif located at the C terminus of the enzymes (Walker et al., 2013). The BAHD family has only five clades, and HCT gene belongs to a subgroup within clade V that use hydroxycinnamoyl/benzoyl CoA to donate acyl groups (Harakava & Harakava, 2005).

Down-regulation of some major enzymes in the phenylpropanoid synthesis pathway results to lower levels of lignin. A study showed that Shikimate-hydroxycinnamoyl transferase (HCT) is a major enzyme that acts as the metabolic entry point for the synthesis for G-type and S-type lignin (Eudes et al., 2016). RNAi silencing of HCT resulted in a decrease of G-lignin and up to 42% of lignin content in tracheary elements of the xylem in Pinus radiata, and it compensated the decrease by increasing the H-lignin (Wagner et al., 2007).

It has been reported that silencing of this gene in Zea mays resulted in the reduction of coumaric acid-syringyl alcohol (S-lignin) conjugate but the plant had a mechanism for compensating the decrease to maintain a certain lignin threshold by increasing the G-lignin (Marita et al., 2014). In another study, in alfalfa plant, HCT gene was silenced through RNAi, and it resulted in a decrease in both S and G lignin, but the H-lignin increased to compensate for the reduction (Gallego-Giraldo et al., 2011).
2.12 Prospect of using biotechnology in controlling *Striga*

According to Smale & Groote (2003), promising biotechnology innovations in Sub-Saharan Africa include those tackling crop constraints that are not easily controlled by conventional techniques, they do not threaten international trade through exports and lastly make a positive impact economically for the welfare of small-holder farmers. Over the years some cultivars of sorghum, cowpeas and rice have exhibited resistance to *Striga* with low yield loss, and this has led to the idea that plant-wide hybridization using such *Striga* resistant germplasm has the potential for overcoming the witchweed (Teka, 2014).

To date, none of the discussed management techniques can completely eradicate this pernicious weed. Developing improved crop varieties resistant to *Striga* seems to be the most feasible technology to overcome this atrocious weed (Rich *et al.*, 2004).
CHAPTER THREE
MATERIALS AND METHODS

3.1 Plant materials
Maize inbred line E04 seeds and sorghum cultivar N-13 seeds were obtained from Kenya Agriculture and Livestock Research Organization (KALRO, Nairobi) and International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, Nairobi). The Maize inbred line E04 and sorghum cultivar N-13 were used as the parental taxa for intergeneric hybridization. *Striga hermonthica* seeds were collected from *Striga* infested fields at Kibos (KALRO centre) in western Kenya. The *Striga* seeds were sieved to remove the chaff and packaged for *Striga* resistance screening assays.

3.2 Phylogenetic characterization of HCT protein
The target amino acid sequence for *Sorghum bicolor* HCT protein was obtained from GenBank repository, accession number XM_002454651.2 (Altschul et al., 1997). Additionally, seven other HCT gene orthologs involved in lignin synthesis were accessed for alignment with the target amino acid sequence from *Sorghum bicolor* (Table 3.1). A multiple sequence alignment of HCT orthologs amino acids sequences was performed using EMBL EBI’s Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustal/0) and DNAMAN (Lyonnon Biosoft LLC, USA). The generated identity matrix (Appendix 2) was used to generate a neighbour joining phylogenetic tree using MEGA version 6 (Tamura et al., 2013).
Table 3.1: HCT protein ortholog sequences used for multiple sequence alignment and phylogenetic analysis.

<table>
<thead>
<tr>
<th>Source organism</th>
<th>Gene bank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorghum bicolor</td>
<td>XM_002454651.2</td>
</tr>
<tr>
<td>Zea mays</td>
<td>NM_001175656.1</td>
</tr>
<tr>
<td>Hordeum vulgare</td>
<td>AK360537.1</td>
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<tr>
<td>Aegilops tauschii</td>
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<tr>
<td>Oryza sativa (Japonica)</td>
<td>KX430016.1</td>
</tr>
<tr>
<td>Brachypodium distachyon</td>
<td>XM_003560941.3</td>
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<tr>
<td>Setaria italic</td>
<td>XM_004964598.3</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>NM_124270.4</td>
</tr>
</tbody>
</table>

3.3 RNA extraction

Sorghum cultivar N-13 RNA was extracted according to Honaas & Kahn (2017). Mortar and pestle for grinding plant material were sterilized by wiping with a cotton wool soaked in 3.85% m/v sodium hypochlorite and rinsed with sterile water. They were then wrapped with aluminum foil and autoclaved for 15 minutes at 121°C. A total of 100 to 200mg of roots of sorghum cultivar N-13 at flowering stage, growing in potted soil at the research field of Plant transformation laboratory, Kenyatta university were collected, frozen in liquid nitrogen at -196°C and thereafter crushed using mortar and pestle. The finely crushed root powder was placed into 2ml Eppendorf tubes for RNA extraction using the ZR Plant Miniprep™ Kit (Zymo Research, USA). The presence of RNA was confirmed by performing agarose gel electrophoresis that consisted of 2µl of RNA mixed with 1µl sybr green and 1µl loading dye loaded on the wells of 1% agarose gel that ran for 45 minutes at 80V. The agarose gel was
visualized under UV transilluminator box and recorded using a Nikon digital camera.

3.4 Source of sequence and primer design

Sorghum HCT gene sequence (XM_002454651.2) was obtained from NCBI database (appendix 1) and used to design a sets of gene-specific primers using NCBI’s Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The two primers are; forward 5’ATG GCG GTG GAG ATC 3’ and reverse 5’TCA GTT GGC CAT GCA 3’ primers. The primer pair was used for amplification of the HCT gene from cDNA synthesized from sorghum cultivar N-13.

3.5 cDNA synthesis and HCT gene amplification

Total RNA was used as the template for synthesis of cDNA according to Silva et al. (2016) and subsequent amplification of the HCT gene according to the manufacturer’s protocol for the One Step RT-PCR kit (Thermo Scientific, USA). Briefly 1µl of RNA was used in a reaction mix that contained: 25µl One Taq One-Step Reaction Mix (2X), 2µl One Taq One-Step Enzyme Mix (25X), 2µl HCT Forward primer (10µM), 2µl HCT Reverse Primer (10µM) and 17µl Nuclease-free H2O to a total reaction volume of 50µl.

The reaction mixture was subjected to reverse transcription followed by HCT gene amplification in a thermocycler (Eppendorf AG, Hamburg). The thermocycler was programmed to 7 steps including; reverse transcription at 48°C for 30 minutes, initial denaturation at 94°C for 60 seconds, denaturation
at 94°C for 15 seconds, annealing at 62.86°C for 30 seconds, extension at 68°C for 60 seconds, final extension for 68°C for 60 seconds and hold at 4°C for ∞. Presence of the \textit{HCT} gene was confirmed by visualization of the 1% gel in a UV transilluminator box and the image documented using a Nikon digital camera.

3.6 Cloning of \textit{HCT} gene in \textit{pJET} 1.2 vector.

The \textit{HCT} gene was ligated to a \textit{pJET} 1.2 vector using ClonJET PCR cloning kit (Thermo Scientific, USA) according to Choi & Meyerson (2014).

3.6.1 Ligation of \textit{HCT} gene to \textit{pJET} 1.2 cloning vector

The ligation reaction comprised the following reagents: 10µl reaction buffer, 1µl \textit{HCT} gene PCR product, 1µl \textit{pJET} 1.2 cloning vector, 7µl nuclease free water and 1µl T4 DNA ligase. The ligation mixture was mixed by vortexing briefly followed by centrifuging for 5 seconds and stored at 22°C for 5 minutes in an incubator.

3.6.2 Transformation of the \textit{Escherichia coli} cells

Two microliters (2µl) of this ligation mixture was transformed into chemically competent \textit{Escherichia coli} cells using a protocol as described by Tu \textit{et al.} (2008). This involved mixing 2µl of the ligation mixture with 250µl of chemically competent \textit{Escherichia coli} DHa-5 cells in a 2ml Eppendorf tube and freezing on ice for one hour. The cells were then heat-shocked at 42°C for 60 seconds followed by culturing in LB broth for 1 hour at 37°C. A volume of 100µl of the transformed \textit{E.coli} cells were streaked on LB agar supplemented
with 100mg/l of ampicillin antibiotic and incubated for 12 hours at 37°C. Different growing colonies were selected and different loopful of bacteria inoculated in LB broth supplemented with 100mg/l of ampicillin, incubated at 37°C with vigorous shaking overnight.

3.6.3 Plasmid DNA extraction

Plasmid DNA was extracted from the growing colonies of the transformed *E.coli* cells using the Alkaline denaturation method (Sambrook *et al.*, 1989). One milliliter (1ml) of the culture was transferred into 2ml Eppendorf tubes and the bacterial cells centrifuged at 6000 rpm for 15 minutes. The supernatant was discarded and the bacterial pellet re-suspended in 500µl of re-suspension buffer (Appendix 6) and kept at room temperature for 5 minutes. The lysis buffer (500µl) (Appendix 6) was added, mixed and kept at room temperature for 5 minutes. Neutralization buffer (700µl) was added, kept for 5 minutes at room temperature and centrifuged at 10000 rpm for 10 minutes. The supernatant was transferred to a 1.5 ml sterile Eppendorf tube, mixed with 70µl of cold isopropanol and incubated at –20°C for 30 minutes.

The samples were centrifuged at 10000 rpm for 30 minutes and the supernatant discarded. The DNA pellet was finally washed by adding 1ml of 70% (v/v) analytical grade ethanol followed by centrifuging for 5 minutes at 10000 rpm. This step was repeated twice. The supernatant was discarded carefully and the DNA pellet air dried for 2 hours. The DNA pellet was then dissolved in 30µl of sterile distilled water and stored at -20°C.
3. 6.4 Confirmation of extracted plasmid DNA and cloned gene

Presence of plasmid DNA was confirmed by mixing 2µl of the extracted DNA with 1µl of loading dye (New England Biolabs, USA) and 1µl of Sybr green (Invitrogen, USA). A 1% agarose gel electrophoresis was run for 45 minutes at 80 V and visualized on a UV Transilluminator (Bio-Rad, USA). The extracted plasmid DNA was subjected to PCR to confirm presence of *HCT* gene in pJET 1.2 vector using *HCT* specific primers (Table 3.2) and *Taq* DNA polymerase kit (New England Biolabs, USA).

3.7 Intergeneric Hybridization between Sorghum and Maize

Ten maize inbred line (EO4) and ten sorghum cultivar (N-13) seeds were planted in potted soil at the research field of plant transformation laboratory at Kenyatta University bi-weekly for the establishment of high population and at different intervals to synchronize flowering. In this study, EO4 was selected because it is susceptible to *Striga* infestation and N-13 is a *Striga*-resistant sorghum cultivar. At the silking stage, pollen from sorghum was collected and sprinkled on the maize silk for pollination during morning hours between 9 am to 11 am.

A negative control was included in the experimental setup by self-pollinating fifteen (15) maize inbred line EO4. Precaution was taken to avoid cross-pollination with other plants on the farm by covering maize ears with khaki bags before the emergence of silk and once pollination was done. The maize tassel was cut off to avoid self-pollination.
Different concentrations of 2, 4-Dichlorophenoxyacetic acid; 10mg/l, 20mg/l, 30mg/l, 40mg/l, 50mg/l, 60mg/l, 70mg/l and 80mg/l were sprayed on maize silk pollinated with sorghum pollen after 24 hours to promote pollen tube formation and embryo development (Wędzony & Lammeren, 1996). Sterile distilled water was sprayed on 30 sorghum pollinated maize silks as a negative control to determine the significance of 2, 4-D hormone on intergeneric hybridization. A sample size of 30 flowering maize plants was set for each 2, 4-D concentration. This was replicated three times to ensure reproducibility of results.

3.8 In vitro maize germination and regeneration

At 32 days post-pollination, the sorghum-pollinated maize having kernels on their cobs and self-pollinated maize were selected, and their ears cut off, de-husked and surface sterilized in 70% ethanol for 3 minutes followed by dipping in 3.85% m/v sodium hypochlorite for 20 minutes. This was followed by rinsing in sterile distilled water under a laminar flow hood. The embryos were rescued by excision and placed on Murashige and Skoog (MS) culture solid medium (pH 5.8) containing 8% phyto agar (Murashige & Skoog, 1962; Ombori et al., 2008). The cultured embryos were incubated in a growth room at 28ºC under 16h/8h day/night photoperiod. After one week, the germinated embryos were transferred in a bigger culture bottle with fresh media and incubated in the growth room at 28ºC for 7 days. On the 14th day post-germination, the hybrid plantlet had formed a well-developed root system.
3.9 Hardening and acclimatization

Fourteen-day old putative hybrids and maize plantlets with well-developed roots were transferred to a sterilized mixture of vermiculite and peat moss in a ratio of 1:1 in pots. These plantlets were placed in the glasshouse for 7 days and then transferred to potted soil in the glasshouse and allowed to grow to maturity and watered daily.

3.10 Analysis of the maize-sorghum hybrids

Validation of the obtained maize-sorghum hybrids was done by targeting a sorghum specific marker, CEN38 in a PCR reaction using CEN38 primers. Quantification and visualization were performed on a 1% agarose gel electrophoresis at 80V for 45 minutes.

3.10.1 DNA extraction for authentication of hybrids

Plant genomic DNA was extracted according to Zidani et al. (2005). Fresh young leaf tissues (100mg to 200mg) from 46 out of 62 putative hybrid regenerants that survived, including 1 sorghum wild-type and 1 maize wild-type were placed in 2ml Eppendorf tubes with two metal beads, frozen in liquid nitrogen and broken into a fine powder by vortexing. Thereafter, 3% of CTAB extraction buffer containing 0.2% mercapto-ethanol and 1% PVP was added just before use and incubated at 65°C. Five hundred microliters (500µl) of CTAB was added to the 2ml Eppendorf tubes containing the freshly broken leaf tissue powder and incubated at 65°C for 30 minutes with mixing by inversion after every 10 minutes. Then 500µl of Chloroform: Isoamyl alcohol
(24:1) was then added to the Eppendorf tubes and centrifuged at 13000 rpm for 10 minutes. The top aqueous layer (350µl) was pipetted into clean 2ml tubes, and the organic layer discarded.

DNA was precipitated by incubation in 700µl of isopropanol at -20°C for 1 hour followed by centrifugation at 13000 rpm for 10 minutes and the supernatant discarded. The DNA pellet was washed with 500µl of 70% ethanol and centrifuged for 10 minutes at 13000 rpm. This step was repeated and the ethanol discarded. The DNA pellet was air-dried, then dissolved in 50µl of sterile water and stored at -20°C.

3.10.2 Quantification of DNA by gel electrophoresis

Five micro litres of putative hybrid, sorghum and maize DNA samples were separately mixed with 1µl of loading dye (New England Biolabs, USA) and 1µl of Sybr green (Invitrogen, USA). The DNA was quantified on a 1% (w/v) agarose gel against GeneRuler 1kb plus DNA Ladder (Thermo Scientific, USA) at 80V for 30 minutes.

3.10.3. Screening for CEN38 marker gene by PCR analysis.

DNA isolated from the putative hybrid, sorghum and maize plantlets were subjected to endpoint PCR analysis for the CEN38 marker using Taq DNA Polymerase via CEN38 marker specific Primers. The primers for CEN38 marker used were (Forward Primer 5’ TCACATGGAATCTTGCTTCG3’) and reverse primer 5’GCCTGCAAATTGTGCAACTA 3’ obtained from Anderson (2005).
*Taq* DNA polymerase kit (New England Biolabs, USA) comprising of 25µl reaction mixture consisting of 2.5µl 10X standard Taq Reaction Buffer, 0.5µl 10µM Forward primer, 0.5µl 10 uM Reverse primer, 0.5µl 10 mM dNTPs, 2µl of DNA sample, 18.875µl of nuclease-free water and 0.125µl Taq DNA polymerase was made for each reaction set.

The putative hybrid, maize and sorghum DNA samples were then subjected to amplification using an Eppendorf Mastercycler Pro (Eppendorf AG, Hamburg). The amplification reaction involved the following steps: initial denaturation at 95°C for 30 seconds, denaturation at 95°C for 30 seconds, annealing at 53.35°C for 60 seconds, extension at 68°C for 60 seconds, final extension at 68°C for 5 minutes and hold at 4°C. After the amplification, the PCR products were subjected to gel electrophoresis using 1% agarose. The PCR products were stained with Sybr green and visualized on a UV Transilluminator box and documented using a digital camera.

### 3.11 Determination of the hybrid fertility and viability

The hybrid fertility was determined by comparison of all the F₁, F₂ and F₃ hybrid cobs with the wildtype maize in terms of the number of the kernels formed in each cob. The viability test was determined by comparing the survival rate of the hybrids from the germination stage to maturation stage.

The F₁ hybrids were grown to maturity in potted soil at the research field of Plant Transformation Laboratory, Kenyatta University. At flowering stage, a sample size of 3 F₁ hybrids were self -pollinated to generate the F₂ hybrids.
The formed F$_2$ seeds were grown to maturity in potted soil and a sample size of 3 F$_2$ hybrids were self-pollinated to generate F$_3$ hybrids. These experiments were replicated three times.

3.12 Screening for *Striga* resistance in soil free assays

3.12.1 Preconditioning of *Striga* seeds

Twenty five milligrams of *Striga* seeds were weighed and surface sterilized using 10% (v/v) commercial bleach for 10 minutes according to Gurney *et al.* (2003). The seeds were then rinsed in 500 ml of sterile distilled water, placed on filter paper in a petri dish and soaked with 5 ml of sterile distilled water. The Petri dishes were sealed with parafilm, wrapped with aluminum foil and incubated at 30°C for 14 days.

The preconditioned *Striga* seeds were prepared for host infection by treating with 5ml of 0.1 ppm GR24 (synthetic strigolactone) per petri dish containing *Striga* seeds, to induce *Striga* germination and incubated at 30°C for 18 hours. The germination frequency (GF) was calculated as follows;

\[
GF = \left( \frac{x}{y} \right) \times 100 + \left( \frac{x}{y} \right) \times 100 + \left( \frac{x}{y} \right) \times 100 / 3
\]

Where, x value is the number of germinated *Striga* seeds and y value is the total number of *Striga* seeds exposed to germination stimulant. Only the *Striga* seeds having a germination percentage of over 70% were used for host infection.
3.12.2 Screening for *Striga* resistance in hybrid plantlets

F₁ hybrid lines, F₂ hybrid lines, wild-type maize and wild-type sorghum plantlets were transferred to rhizotrons (root observation chambers) filled with vermiculite and watered with 100 ml of 40% (v/v) Long strength Ashton’s solution containing 2 mM Ammonium Nitrate daily. The rhizotrons were covered with aluminum foil to provide a dark environment for the roots (Gurney et al., 2006). Eighty milligrams of preconditioned *Striga* seeds were aligned onto the secondary roots using a paintbrush. The rhizotrons were placed in a glasshouse with controlled conditions of 10h/14h day/night at 28/25°C at 70% relative humidity.

3.12.3 Screening of post-germination *Striga* resistance

In the determination of successful *Striga* attachment to host roots, the infected plantlets were examined on days 3 and 9 post-infection according to Gurney et al. (2003) and images of the attached *Striga* radicle taken using a Leica MZFL stereomicroscope. Successful infection of the hosts’ roots systems at 21st day, were documented using a Canon EOS 300D digital camera.

At 21 days post-infection by *Striga* on hybrids, wild-type maize and wild-type sorghum roots, all the attached *Striga* were detached from the roots of hybrid, wild-type maize and wild-type sorghum per rhizotron and placed on fresh Petri dish layered with filter paper. Images of the harvested *Striga* per rhizotron were taken using Canon EOS 300D digital camera and the Petri dishes placed in laboratory incubator for drying for 3 days at 30°C. The dry biomass of *Striga* for each hybrid plant was weighed using analytical
weighing balance. The *Striga* length and number were then determined by analysis of the harvested *Striga* images using Image analysis software (Image J, Media Cybernetics).

### 3.12.4 Histological screening of *Striga* resistance

Root tissues of *Striga* infected hybrids, maize and sorghum plantlets were collected on days 3 and 9 post-infection according to Mbuvi *et al.* (2017). The tissues were then placed in Carnoy’s fluid which consisted of 100% ethanol: acetic acid (4:1) for a day in 2 ml Eppendorf tubes at room temperature. Infiltration was then done by replacing the Carnoy’s fluid with 100% Technovit® 1 for 15 minutes at room temperature as stated by the manufacturer’s instructions (Heraeus Kulzer GmbH, Germany). This was subsequently replaced with fresh 100% Technovit® 1 and kept for a day at room temperature.

A pinch of neutral red was added to the tissues and left for 5 minutes and then discarded. Hardener 2 and Technovit® 1 were then mixed in a ratio of 1:15 ratio in 1.5 ml Eppendorf lids according to manufactures instructions (Heraeus Kulzer GmbH, Germany). The stained tissue samples were then placed in the Eppendorf lids in a vertical position at the center of the lids, one tissue per lid. The lids were then kept at room temperature overnight. After 24 hours, they were covered in aluminum foil and placed at 37°C in an incubator for two hours for hardening.
The fixed tissues were then mounted onto wooden histoblock mounting blocks using Technovit 3040 kit according to manufacturer’s instructions (Heraeus Kulzer GmbH, Germany). The mounted hybrid, maize and sorghum tissues on histoblocks were cut using Leica RM 2145 microtome into small 5µm sections. The sections were then placed on microscopic slides, dyed with 0.1% toluidine blue O (BHD Poole, UK) in 100 mM phosphate buffer (PH 7) for 2 minutes at 65°C. The dye was washed off using distilled water and dried at 65°C for 30 minutes. It was then mounted with De-Pex (BDH, Poole, UK) directly on the sections and covered with a coverslip, observed and photographed using the Olympus BX51 microscope.

3.13 Determination of lignin content in maize, sorghum and hybrids

Lignin content was evaluated by staining with 1% phloroglucinol. A sample size of 3 maize plantlets, 3 sorghum plantlets, and 3 hybrid lines root tissues were collected and sectioned into 1 mm cross-sections. This was replicated three times for reproducibility of results. They were then placed on microscopic slides and a drop of 1% phloroglucinol was used for staining the tissues for 3 minutes (Preisner et al., 2014). The slides were then observed under a microscope to determine structure of the root cell and the staining of the lignin of the hybrid, maize and sorghum plants and pictures taken (Alakonya et al., 2012).
3.14 Statistical data analysis

Statistical Analysis Software (SAS version 9.1.3) was used for analysis of *Striga* resistance data from soil free assays. Analysis of variance (ANOVA) was used to compare the means of dry biomass, length, and count of infecting *Striga* to determine the resistance levels of hybrids vis-à-vis the parental lines (Appendix 5). The means were separated by Tukey’s HSD test at 95% confidence interval. All values $p \leq 0.05$ were considered statistically significant. This data is presented as means ± SEM and presented in the form of graphs using graph pad prism version 6 (http://www.graphpad.com).
CHAPTER FOUR

RESULTS

4.1 Target gene analysis

The *Sorghum bicolor* HCT gene has a nucleotide sequence of 1489 bps that codes for a 496 amino acid sequence. Searches with BLASTP and multiple sequence alignment (MSA) using sequences from the 7 grass family members revealed close similarity of (91.6%) between *S. bicolor* and *Zea mays* HCT proteins. There was a close similarity between *S. bicolor* HCT protein and HCT proteins from *Hordeum vulgare, Aegilops tauschii, Oryza sativa* (Japonica), *Brachypodium distachyon* and *Setaria italic* (Table 4.1).

To avoid bias in family-specific conserved protein regions, *S. bicolor* HCT protein was compared with *Arabidopsis thaliana* HCT protein as an outgroup (Figure 4.2). The MSA identified conserved regions between compared sequences (indicated in black colour) when the grass family members were aligned with *Arabidopsis thaliana*. Out of the 496 amino acid sequence, there was a consensus at 110 amino acid positions with the HCT protein of *Arabidopsis thaliana* (Figure 4.1).
Table 4.1: Percentage identity of *Sorghum bicolor* HCT protein with 7 other orthologs from different species.

<table>
<thead>
<tr>
<th>Source organism</th>
<th>Accession number</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Zea mays</em></td>
<td>NM_001175656.1</td>
<td>91.6 %</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>AK360537.1</td>
<td>76.1 %</td>
</tr>
<tr>
<td><em>Aegilops tauschii</em></td>
<td>XM_020317496.1</td>
<td>75.2 %</td>
</tr>
<tr>
<td><em>Oryza sativa (Japonica)</em></td>
<td>KX430016.1</td>
<td>76.1 %</td>
</tr>
<tr>
<td><em>Brachypodium distachyon</em></td>
<td>XM_003560941.3</td>
<td>74.9 %</td>
</tr>
<tr>
<td><em>Setaria italic</em></td>
<td>XM_004964598.3</td>
<td>61 %</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>NM_124270.4</td>
<td>37.6 %</td>
</tr>
</tbody>
</table>
Figure 4.1: Multiple sequence alignment of the Sorghum bicolor HCT protein with 6 other grass family HCT proteins. GenBank accession numbers: *S. bicolor* (XM_002454651.1), *Z. mays* (NM_001175656.1), *H. vulgare* (AK360537.1), *A. tauschii* (XM_020317496.1), *O. sativa* (Japonica) (KX430016.1), *B. distachyon* (XM_003560941.3), *S. italic* (XM_004964598.3) and *A. thaliana* (NM_124270.4). Block colors show the conserved regions of the gene sequences. The consensus nucleotide sequences amongst the 8 amino acid sequences is indicated on the 9th line.
4.2 Phylogenetic analysis of the HCT proteins

BLASTP search using default settings identified a high similarity between the *Sorghum bicolor* HCT protein with 6 other HCT proteins from the same family but a distant similarity from HCT protein from a different family. Subsequently, a phylogenetic tree was constructed based on amino acid sequences of these 7 proteins from grass family (Figure 4.2).

The HCT proteins from both *Sorghum bicolor* and *Zea mays* were grouped on the same clade (92% bootstraps support) indicating a closer phylogenetic relationship. HCT protein from *Arabidopsis thaliana* was the farthest placed from *Sorghum bicolor* HCT, indicating the distant phylogenetic relation due to the divergence between the protein domains. *Sorghum bicolor* HCT protein had 62% bootstraps support to the other 6 protein orthologs from the grass family and a distant 38 % to *Arabidopsis thaliana* HCT protein.
4.3 Cloning of *HCT* gene from sorghum cultivar N-13

This study successfully assembled a pJET 1.2/*HCT* gene construct by ligating *HCT* gene from sorghum cultivar into pJET 1.2 vector (Figure 4.3c). PCR analysis of the cDNA template from sorghum revealed an *HCT* gene band size of 1489 bps (Figure 4.3a). The PCR products were of expected size based on the sequences obtained from the NCBI. The gene presence in the vector was confirmed by PCR using *HCT* gene-specific primers (Figure 4.3b).
Figure 4.3: Cloning of *HCT* gene into pJET1.2 vector. (a) *HCT* gene amplified from sorghum cultivar N-13 DNA. Expected size of the band is indicated. Lane M, 1kb DNA ladder; Lane 1, PCR negative control; Lane 2, Amplification of *HCT* gene. (b) PCR confirmation of the presence of cloned gene in pJET 1.2 vector. Lane M, 1kb DNA ladder; Lane 2, amplification of control(*HCT*); Lane 2 to 5 amplification of colonies putatively transformed with pJET1.2/*HCT* vector. (c) Map of the pJET 1.2/*HCT* gene construct.
4.4 Generation of maize-sorghum hybrids

*Striga* susceptible maize inbred line EO4 was pollinated with *Striga* resistant sorghum cultivar N-13 at the silking stage. Only 9 out of 540 (1.67%) maize cobs formed kernels with the highest number of 20 kernels per cob. This was indicative of partial fertility (Figure 4.4).

![Pollinated maize cobs](image)

**Figure 4.4:** The pollinated maize cobs after 32 days post pollination with sorghum pollen. (a) Maize cob with 5 kernels (b) Maize cob with 10 kernels (c) Maize cob with 20 kernels.

Hybridization rate = \( \frac{Number\ of\ maize\ cobs\ that\ formed\ kernels}{Number\ of\ maize\ pollinated\ with\ sorghum\ pollen} \) * 100

Hybridization rate = \( \frac{9}{540} \) * 100 = 1.67%

4.5 Embryo rescue and regeneration of the hybrid

The hybrid embryos germinated and well–developed radicle and plumule formed on the fourth day (Figure 4.5a). On the 14th day, the hybrids had developed into plantlets with a well-established root system (Figure 4.5b). The
hybrid plantlets took a total of 7 days to acclimatize under glasshouse conditions (Figure 4.5c & 4.5d). The plantlets successfully matured in potted soil at the research field while others were put into rhizotron under glasshouse conditions for 25 days (Figure 4.5f).

![Figure 4.5: Embryo rescue and regeneration of maize sorghum hybrid. (a) 4 day old rescued putative hybrid embryo (b) 14 day old putative hybrid plantlet (c & d) Hardening and acclimatization of the maize-sorghum hybrid (e) Maize-sorghum hybrid in potted soil. (f) Maize-sorghum hybrids in the rhizotrons.]

4.6 Hybridization response to 2, 4-Dichlorophenoxyacetic acid regime

Following pollination, 50mg/l 2, 4 Dichlorophenoxyacetic acid (2, 4-D), best performing concentration formed the highest number of hybrid kernels with a mean number of 13.33±3.3. The second best performing 2, 4-D was 40mg/l with a mean number of 4.3 ± 2.9 formed kernels. The least performing concentrations were 10mg/l, 20mg/l, and 30mg/l with a mean of 0.3±0.33,
1.0±1.0 and 1.6 ±1.67 respectively. No embryos formed under sterile distilled water (negative control) supplementation (Figure 4.6). The performance of 50mg/l 2, 4-D auxin was significantly different from the other concentrations. The fertilized maize cob with the highest count of kernels had only 20 kernels formed at 50mg/l 2, 4-D supplementation while the least count of the kernel on fertilized maize cob was 1 with 10mg/l 2,4-D supplementation (Table 4.2).

**Figure 4.6:** Different concentrations of 2, 4-D to promote intergeneric hybridization. The vertical bars represent standard error of the means. The different letters represent mean separation at p≤0.05.
Table 4.2: Number of formed kernels and fertilized maize cobs after applying different concentrations of 2, 4-D

<table>
<thead>
<tr>
<th>2, 4-D Concentration</th>
<th>Number of maize cobs that formed kernels</th>
<th>Number of kernels that formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mg/l</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>40mg/l</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>30mg/l</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>20mg/l</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>10mg/l</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

4.7 Screening for CEN38 marker in the maize sorghum hybrid

A total of 41 hybrids were positive for the CEN38 marker. CEN38 appeared as a repetitive marker whose size ranged from 280 bps to 2000 bps in sorghum cultivar N-13. There was no amplified PCR product on maize inbred line EO4. The hybrids produced between two, three and four bands of the marker indicating how the marker appears as a repetitive element in the *Sorghum bicolor* genome (Figure 4.7a). Only 32 hybrid lines produced a single band of 280 bps (Figure 4.7b).
**Figure 4.7:** PCR confirmation of the presence of the CEN38 marker in the hybrids. (A) Lane M, 1kb DNA ladder; Lane 1, maize inbred line EO4 (Negative control); Lane 2, sorghum cultivar N-13 (Positive control); Lanes 3 to 10, different maize-sorghum hybrid lines. (B) Lane M, 100bp ladder; Lane 1, maize inbred line EO4; Lane 2, sorghum cultivar N-13; Lane 3 to 9 different maize sorghum hybrid lines.

### 4.8 Generation of F₂ hybrids

The maize-sorghum F₁ hybrids were self-pollinated to generate the F₂ progeny. The fertility of the hybrid cobs was determined by comparison of the kernel set-up. The F₂ hybrid cobs were more filled with the kernels compared to the F₁ hybrids. This was indicative of restoration of fertility in the F₂ hybrids (Figure 4.8).
Figure 4. 8: Generation of the F₂ hybrids by self-pollination of the F₁ hybrids. N-13 is the sorghum cultivar (pollen donor), EO4 is the maize inbred line (the pollen acceptor).

F₃ hybrids were generated by self-pollination of the F₂ hybrids. The F₃ hybrid cobs were more kernel filled in comparison to the F₁ and F₂ hybrids. This was indicative of fertility recovery by self-pollination (Figure 4.9).
Figure 4.9: Generation of the F$_3$ hybrids by self-pollination of the F$_2$ hybrids. N-13 is the sorghum cultivar (Pollen donor), EO4 is the maize inbred line (Pollen acceptor).

4.9 Hybrid viability

Some of the F$_1$ hybrids formed were not viable as they died during the tissue culture processes. Precisely, 16 hybrid plantlets died during the germination period, hardening and acclimatization. However, the remaining 46 plantlets were healthy and grew to maturity in potted soil. The survival rate of the formed hybrids was 74.19% and the death rate was 25.81% (Table 4.3). The inviability was observed only in the first filial generation only. The subsequent F$_2$ and F$_3$ hybrids survived at 100% to maturity.
Table 4.3: The hybrid viability percentages.

<table>
<thead>
<tr>
<th>Number of embryos that survived (NES)</th>
<th>Number of embryos that died (NED)</th>
<th>Total number of embryos that formed (NEF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
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</tr>
<tr>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
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</tr>
<tr>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Total=46</td>
<td>Total=16</td>
<td>Total =62</td>
</tr>
</tbody>
</table>

\[
\text{NES} \times 100\% = 74.19\% \\
\text{NED} \times 100\% = 25.81\% \\
\text{NEF} \times 100\% \\
\text{NEF}
\]

4.10 The general phenotype of maize-sorghum hybrids.

Maize-sorghum hybrids were grown to maturity to compare the phenotype of the hybrid and the parental lines. The F₁, F₂ and F₃ hybrids exhibited a phenotype similar to the maternal parent (maize) regarding branching, rooting, flowering and seed formation. This was an indication that hybridization did not affect the overall phenotype of the hybrid (Figure 4.10).
Figure 4.10: Phenotypes of the maize-sorghum hybrid. (a) Maize inbred line EO4 (b) Sorghum cultivar N-13 (c) Maize-sorghum hybrid.

Eight out of 9 F₂ hybrids formed kernels on the cobs and silk could be seen emerging from the ear (Figure 4.11a). The tassels were well-developed with pollen formation and were morphologically similar to the maternal parent, maize (Figure 4.11b). However, 1 of the F₂ hybrids exhibited signs of hybrid breakdown. The F₂ hybrid formed pollen, kernels and silk on the tassel resembling the sorghum (Figure 4.11c). The silk did not emerge from the hybrid ear and therefore, no kernels were formed on the cob (Figure 4.11d).
4.11 Germination of *Striga* seeds

*Striga* seeds were successfully germinated following exposure to a germination stimulant (GR24) for 18 hours. Germinated seeds had radicles sprouting out of the seeds (Figure 4.12). *Striga* seeds having over 70% germination frequency were used to infect hybrids that had a well-developed root system.

*Figure 4.11:* Breakdown of the F2 hybrids. (a & b) F2 hybrid having normal maize reproduction organs (c & d) F2 hybrid exhibiting reproduction breakdown.

*Figure 4.12:* *In vitro Striga* germination after induction using synthetic GR24.
4.12 *Striga* attachment on the hybrid and parents roots

At 21st day post *Striga* infection, the number of *Striga* attachments on the sorghum, maize and hybrids varied. Sorghum cultivar N-13 had a low number of *Striga hermonthica* attachments (Figure 4.13a). Maize inbred line E04 had the highest number of *Striga* attachments that were well nourished and developed (Figure 4.13d). Maize-sorghum F₁ and F₂ hybrid had the least *Striga* attachments that were poorly developed (Figure 4.13 b and c).

![Attached *Striga* plantlets growing on host plants. *Striga hermonthica* growing on the host roots on the 21st day. (a) Sorghum cultivar N-13 (b & c) Maize-sorghum F₁ and F₂ hybrid (d) Maize inbred line E04. The red arrows show *Striga hermonthica* attachments on the host roots.](image)

4.13 Mean number of attachment of *Striga hermonthica* on the host roots

The F₁ and F₂ hybrids had the lowest number of attached *Striga* per plant with a mean of 31.0 ± 4.98 and 32.33 ± 3.46 respectively, and there was no significant difference in *Striga* attachments between the F₁ and F₂ hybrids. *Striga* susceptible maize inbred line E04 had the highest number of attached *Striga* per plant with a mean of 105.86 ± 1.27 while the *Striga* resistant
sorghum cultivar N-13 had a mean of 54.36±2.04 attached *Striga* per plant which was significantly higher than the hybrids and lower than the susceptible maize (Figure 4.14).

![Bar chart showing mean number of Striga attachments on host roots.](image)

**Figure 4.14:** Mean number of *Striga* attachments on host roots. Vertical bars indicate the standard error of the means. The different letters represent mean separations at p≤0.05.

**4.14 Mean length of *Striga hermonthica* attached on the host roots**

The attached *Striga* on the maize inbred line EO4 roots had the highest mean length of 0.76 ± 0.01cm. The observed mean length of *Striga* attached to sorghum cultivar N-13 roots was 0.35±0.02 cm while the mean length of the *Striga* attached on both F₁ and F₂ hybrids was 0.34± 0.01 cm and 0.32± 0.02 cm respectively. There were no significant differences between the mean length of *Striga* attached on the hybrids and sorghum cultivar N-13 roots (Figure 4.15).
**Figure 4.15:** Mean length of *Striga* attached on host roots. The vertical bars represent the standard error of the means. The different letters represent mean separations at $p \leq 0.05$.

### 4.15 Mean *Striga hermonthica* biomass on the host roots

*Striga* resistant sorghum cultivar N-13 had a mean *Striga* biomass of 0.017±0.0002 grams while *Striga* susceptible maize inbred line E04 had a mean *Striga* biomass of 0.032±0.0004 grams. The F$_1$ and F$_2$ hybrids had the least dry *Striga* mean biomass of 0.0052±0.001 g and 0.0048±0.001 grams. There was no significant difference between the *Striga* dry biomass harvested from F$_1$ hybrids and F$_2$ hybrids (Figure 4.16).
Figure 4.16: Mean dry biomass of *Striga* on host roots. The vertical bars represent standard error of the mean. Different letters represent mean separations at p≤0.05.

4.16 Microscopic screening of *Striga* resistance

*Striga* attached host root tissues were collected three days post infection and screened microscopically. *Striga hermonthica* haustorium penetrated the hybrid root cell epidermis and cortex but it was inhibited from further penetration past the endodermis, and hence, the subsequent connection was not established (Figure 4.17a). On the contrary, *Striga hermonthica* haustorium penetrated the sorghum cultivar N-13 root cell and established a xylem-xylem connection with the sorghum xylem (Figure 4.17b). On the Striga susceptible maize inbred line root cell, Striga established xylem-xylem connection and the parasite formed a well developed xylem tissue (Figure 4.17c).
**Figure 4.17:** Microscopic screening of *Striga* resistance on day 3 post-infection. Cross sections of root cells of (a) hybrid (b) sorghum cultivar N-13 (c) maize inbred line E04. P=Parasite H=host Px=Parasite xylem Hc=Host cortex Hx-Px=Host parasite xylem-xylem connection Hy=Hyaline body. The red arrow indicates the xylem-xylem connection between the *Striga* xylem and sorghum xylem.

Similarly, *Striga* penetration into the host cell was determined microscopically on day 9 post-infection. *Striga hermonthica* made a xylem connection to hybrid root cell xylem on the 9th day but its structures were poorly developed, and the hyaline body entry was substantially restricted (Figure 4.18a). On the contrary, *Striga hermonthica* made a xylem connection to the sorghum cultivar N-13 root cell on day 3 and day 9, it fully colonized the root cell. Its haustorium enlarged and had a well-developed structure with endophyte, xylem and hyaline body penetrating and occupying the sorghum root cell (Figure 4.18b). On day 9, *Striga* parasitism on the *Striga* susceptible maize inbred line E04 was well-maintained and the parasite had fully colonised the
host vascular system and had well-established structures with endophyte, xylem and hyaline body occupying the host stele (Figure 4.18c).

**Figure 4.18:** Microscopic screening of *Striga* resistance on day 9 post infection. (a) Hybrid (b) Sorghum cultivar N-13 (c) Maize inbred line E04. P= Parasite H=host  Px= Parasite xylem Hx= Host xylem Hx-Px=Host parasite xylem-xylem connection En=endophyte. The red arrows indicate the overall xylem connection between the parasite and the host.

**4.17 Lignin deposition of the hybrid root cells**

The sorghum, maize parental lines and hybrid root cells did not stain at the vegetative stage (Figure 4.19) while all the root cells stained pink at the flowering stage (Figure 4.20). Maize inbred line E04 had a faded pink stain appearing only at the endodermis region (Figure 4.20c). The hybrid had the highest lignin distribution levels as shown by the high concentration of pink colour from the pith to the endodermis region (Figure 4.20b). Lignin staining of sorghum parental line extended from the stele to the endodermis region indicating a larger lignin distribution compared to the maize inbred line (Figure 4.20a).
Figure 4.19: Lignin staining of the root cell walls at vegetative stage (1 month old). (a) Maize inbred line E04 (b) Maize-sorghum F1 hybrid (c) Sorghum cultivar N-13.

Figure 4.20: Lignin staining of the root cell walls at flowering stage (3 months old). (a) Sorghum (b) Maize-sorghum hybrid (c) Maize inbred line E04. The arrows indicate the extent of lignin distribution. S=Stele, E=Endodermis, P=Pericycle.
CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Genomic relationship between sorghum and maize

Crop improvement programs utilise genetic variation within the species to develop new varieties with desirable traits such as disease and insect resistance, as well as acid soil tolerance. Interspecific and intergeneric hybridization is a useful technique in transferring desirable traits from wild species to the cultivated plants. The main goal of this research was to develop a *Striga* resistant maize-sorghum hybrid based on enhanced lignification of sorghum cultivar N-13’s systemic acquired resistance gene (*Hydroxycinnamoyl transferase*).

In the present research, hybridization of maize and sorghum was evaluated by reconstructing phylogenetic relationship of *S. bicolor* *HCT* gene and its orthologs in the grass family. Sorghum cultivar N-13 has high lignin deposition around the secondary cell wall of its roots. This provides mechanical resistance to *Striga* penetration into its root cell. Furthermore, overexpression of *HCT* gene and other lignin biosynthesis genes under stress conditions has been shown to occur as a defence mechanism. For example, *HCT* gene has been shown to increase by 3.9-fold when flax plants were subjected to *Fusarium oxyporium* (Wojtasik *et al.*, 2016).
Although sorghum and maize diverged about c.16.5 Mya (Gaut et al., 2000) searches of S. bicolor HCT protein using BLASTP and multiple alignments using 6 other HCT orthologs revealed a 92% identity between sorghum and maize HCT proteins. A 62% identity was observed when S. bicolor HCT protein was compared to its orthologs from the grass family.

*HCT* gene is the target gene for guacryl (G) and syringyl (S) in lignin biosynthesis (Whetten & Sederoff, 1995). The biological functions of all these HCT protein orthologs are linked to lignin biosynthesis, and the presence of the conserved regions in the 7 amino acid sequences must be linked to their function (Wagner et al., 2007). The significant homology between sorghum and maize HCT proteins was putative evidence for a successful intergeneric hybridization between the two crops. Studies have shown that the probability of successfully hybridizing different crops increases when the species are phylogenetically more closely related (Hodnett et al., 2010).

As a proof of concept, the current research successfully characterized and cloned a sorghum cultivar N-13 *HCT* gene, a key lignin biosynthesis gene, into a vector. Such a construct provides a tool for future genetic improvement of *Striga* susceptible maize using genetic modification. Furthermore, the pJET 1.2/HCT vector can be used to increase the amount of genetic variation available to plant breeders for *Striga* resistance crop improvement.
5.1.2 Intergeneric hybridization of *Sorghum bicolor* and *Zea Mays*

Maize improvement strategies by plant breeders have focused on yield, biotic and abiotic stress resistance, by using germplasm from within the species. Where these traits are lacking, intergeneric hybridization can be used to increase the amount of genetic variation available to plant breeders for improvement.

This study successfully transferred *Striga* systemic acquired resistance from sorghum cultivar N-13 to maize inbred line E04 by developing a maize-sorghum hybrid. This study showed that it is feasible to cross *Zea mays* with *Sorghum bicolor* to obtain a hybrid. The hybrid had a phenotype similar to the maize, the maternal parent than sorghum, the paternal parent. The maternal parent had a significant influence on hybrid seed formation, germination, and flowering. The paternal parent did not influence hybrid germination and there was no maternal × paternal interaction phenotypically. The One-Way ANOVA indicated a significant effect of paternal parent on hybrid *Striga* resistance.

In the present study, the major bottleneck entailed eliminating hybridization barriers and improving the fertilization rates. Crossing sorghum with maize was a major challenge because of the inherent genomic features of the parents. Firstly, only 1.67% of total pollinated maize plants yielded embryos. This is due to the inter-generic differences between the two parental taxa (JinFeng & Adelberg, 2000). These difficulties have been reported earlier when such a cross was done and revealed that sorghum pollen tube could not reach the
maize micropyle and, therefore, no hybrid embryos were obtained (Heslop-Harrison et al., 1985).

Secondly, intergeneric reproductive barriers contributed towards the failure of formation of embryos. This was overcome by spraying different concentrations of the 2, 4-D hormone on the silk of maize pollinated with sorghum pollen, 24 hours after pollination. Varying concentrations of 2, 4-D of 10mg/l, 20mg/l, 30mg/l, 40mg/l and 50mg/l were used, and the maize cobs formed varying number of kernels. 50mg/l 2, 4-D was the best auxin concentration since the highest number of hybrid embryos were formed at this concentration (mean of 13.33±3.3). Increasing the auxin concentration past 50mg/l did not promote kernel formation. The pollinated maize silk sprayed with sterile distilled water 24 hours post-pollination did not form any kernels, and this proved that 2, 4-D auxin was essential for increasing the number of pollen tubes reaching the micropyle in intergeneric hybridization.

Previous studies reported the significance of spraying 2, 4-D auxin as observed in intergeneric hybridization between *Triticum aestivum* and *Zea mays*, whereby the hormone promoted an increase in the number of pollen tubes reaching the micropyle to promote fertilization (Wędzony & Lammeren, 1996). Moreover, successful reports of 2, 4-D overcoming interspecific barriers were also reported in a cross between wild and cultivated *Solanum tuberosum* interspecific hybridization (Augustin et al., 2012).
Thirdly, there was hybrid embryo abortion. Embryo rescue technique was used to mitigate this impediment. The rescued embryos grew to maturity and had a 74.19% survival rate. Embryo rescue has been shown to overcome reproduction barrier in an interspecific hybridization within the *Leucadendron* genus (Liu *et al.*, 2005). In another study, an interspecific cross between wild and cultivated *Vigna unguiculata* was achieved by embryo rescue to overcome embryo abortion (Fatokun, 1991). Furthermore, embryo rescue has been used to overcome reproduction barrier in intergeneric hybridization between *chrysanthemum* and *Ajania przewalskii* (Deng *et al.*, 2011).

Although crossing sorghum and maize with the supplementation of 2, 4-D auxin resulted to successful fertilization, the F₁ hybrids had very few kernels on the cobs. For example, some cobs had one kernel per maize cob while others had 20 kernels per maize cob. This was indicative of partial fertility in the maize-sorghum hybrids. The major cause of partial fertility resulted from genetic incompatibilities due to intergeneric hybridization between *Zea mays* and *Sorghum bicolor*, which both belong to the Poaceae family but different genera.

It has been suggested that crossing intergeneric crosses causes inter-locus incompatibilities leading to hybrid partial fertility (Sweigart *et al.*, 2006). This is because an increase in inter-locus incompatibilities between diverging taxa leads to an increase in hybrid sterility. Also, hybrid sterility increases as the genetic distance between the two parents increase especially if the divergence is more than c. 5 Mya (Levin, 2012). Previous studies have reported partial
fertility on intergeneric hybrid resulting from a cross of *Dubautia* species and *Wilkesia gymnoxiphium* that had 28% and 44% fertility (Carr & Kyhos, 1986). Correspondingly, hybridization between genera *Argyroxiphium sandwicense* and *Dubautia menziesii* that diverged c.5 Mya resulted in 11% fertility (Levin, 2012).

### 5.1.3 Molecular characterization of the hybrids

The presence of CEN38 marker in the hybrids confirmed that the hybrids had introgressed the sorghum genome into the maize genome. The hybrids displayed varying band sizes whereby some appeared as an array of tandem repeats ranging from 280 bps to 2000 bps while others had only single bands of 280 bps indicating the level of sorghum genome introgression into the maize genome. The tandem array of different band sizes in the hybrids mimicking the array of bands observed on sorghum indicated the degree of sorghum genome integration in the maize genome. Previous studies have reported comparable observations when *Sorghum bicolor* was screened for CEN38 marker (Anderson, 2005).

The use of CEN38 marker as reliable marker in *sorghum bicolor* crosses has been observed in interspecific hybridization between *Sorghum bicolor* and *Sorghum macrospermum* (Miller *et al.*, 1998). The CEN38 marker did not amplify in maize because it does not exist in the maize genome (Wang & Bennetzen, 2012).
5.1.4 Determination of hybrid viability

A minority (25.81%) of F₁ hybrids were not viable. Some embryos failed to germinate, while others died after the embryo had germinated and formed a seedling and the rest died during acclimatization stage. However, 74.19% survival rate of the F₁ hybrids was observed. This observation can be attributed to inter-loci incompatibilities or reduced chromosomal recombination and negative epistasis occurring in the hybrids (Sweigart et al., 2006). The hybrids from the F₂ and F₃ generation developed to maturity with 100 % viability in potted soil. Similar observations have been reported in an interspecific hybrid between Triticum durum and Aegilops umbellulata that formed seeds that could not germinate (Hadzhiivanova et al., 2012). Moreover, hybrid inviability was reported in intergeneric hybridization between Zea mays and Tripsacum dactyloides in which 80% of the F₁ hybrids did not form viable seeds (Molina et al., 2006).

5.1.5 Hybrid fertility

To determine hybrid fertility, maize-sorghum F₁ generation was self-pollinated to generate the F₂ hybrids and the F₂ hybrids were self-pollinated to generate the F₃ hybrids. This was an indication of pollen grain fertility because the hybrids pollen was able to self-pollinate the hybrid silk and generate the subsequent filial generation. The F₂ hybrids had more kernels in comparison to the F₁ hybrids. Furthermore, the F₃ hybrids had fully recovered fertility by having completely kernel filled up cobs. This observation corresponds with a
study of interspecific hybridization between *Triticum durum* and *Aegilops umbellulata* where the F₁ hybrids had few seeds (Hadzhiivanova *et al.*, 2012).

The recovery of the maize sorghum hybrid fertility can be attributed to increasing chromosomal compatibility as a result of self-pollination from one generation to another. However, one F₂ hybrid line was exceptional from the rest by developing a seed-set on the tassel rather than on the hybrid cob and the silk emerged from the tassel rather than the ear. This might be due to a hybrid breakdown or maybe due to the hybrid mimicking the seed set of its paternal parent, sorghum. Additionally, this observation might be due to the dominance of the sorghum flowering genes over the maize flowering genes, occurring due to incompatibilities in the interaction of the genes. Hybridization can result in silencing of some genes in a hybrid (Baack *et al.*, 2015). In a previous study, the F₂ hybrids of *Indica* and *Japonica* cross exhibited hybrid breakdown as a result of complimentary recessive sterility genes between the two species in the hybrid (Li *et al.*, 1997).

**5.1.6 Screening for *Striga* resistance**

Successful hybrids were subsequently screened for *Striga* resistance. The hybrids were categorised as *Striga* resistant if they exhibited the low attached *Striga* length, biomass and number relative to sorghum cultivar N-13. The hybrids had the least attached *Striga* length, biomass and number significantly different from both parental taxa. Moreover, cross-sections of *Striga* infected hybrid roots revealed a restricted *Striga* access to the hybrid stele at day 3 post-infection and limited xylem-to-xylem *Striga*-hybrid connection at 9 days
post-infection when compared to sorghum and maize parental lines. This is attributed to the mechanical resistance towards Striga penetration. Following haustorial penetration of the host root cell cortex and pericycle, extra thickening occurs around the endodermal and pericycle cells due to enhanced lignification. The enhanced lignification inhibited the number of parasite haustorium penetrating to the host root cell vasculature. Similar observations have been reported in Striga infected sorghum cultivar N-13 in which the cultivar developed extra thickening on the pericycle to inhibit Striga haustorium penetration into the vascular tissue (Maiti et al., 1984).

In this study, the hybrids exhibited a higher Striga resistance in comparison to sorghum cultivar N-13 parental lines. A superior Striga resistance meant that the hybrid secondary metabolism had been greatly altered leading to the observed Striga resistance. Enhanced lignin deposition was confirmed by cytological staining. A 1% Phloroglucinol lignin staining revealed that the hybrids had a higher level of lignin deposition on their secondary root cell wall compared to the sorghum cultivar N-13 and maize inbred line E04. This observation corroborates previous studies that reported the superiority of hybrids than the parental taxa to biotic and abiotic conditions. For example, Arabidopsis accessions hybrids exhibited a higher level of resistance towards bacterial pathogen Pseudomonas syringae due to an elevated level of salicylic acid than the parents, thereby increasing defence against the pathogen (Yang et al., 2015). In addition, studies have shown that inter-generic hybridization results in alteration of secondary metabolites (Orians, 2000).
Furthermore, similar resistance superiority was observed in a hybrid between *Nicotiana glutinosa* and *Nicotiana debneyi* that had superior resistance to tobacco mosaic virus and other bacterial and fungal infections more than the parental species because it produced a higher level of chitinase and peroxidase enzyme levels (Goy *et al.*, 1992).

### 5.2 Conclusions

This study derives three conclusions. Firstly, a lignin biosynthesis gene, *HCT* from sorghum cultivar N-13 was isolated and cloned into a vector. *Sorghum bicolor* HCT protein has a very high homology to *Zea mays* HCT protein that increased the feasibility of successful transfer of SAR from sorghum (N-13) to maize (E04). Therefore, this gene construct can be transformed into susceptible host plants to confer *Striga spp* resistance.

Secondly, hybridization between sorghum and maize was only feasible when the crossing was supplemented with (2, 4-D auxin) hormone. The best 2, 4-D concentration was 50mg/l. The hybrids exhibited pollen fertility and were able to carry out self-pollination to generate consecutive filial generations.

Thirdly, sorghum cultivar N-13 and maize inbred line EO4 hybrid exhibited increased mechanical resistance to *Striga hermonthica* than the well-known sorghum cultivar N-13. This was due to an increased lignin deposition on the hybrid root secondary wall and therefore, inhibiting the xylem-xylem connection by *Striga* to the hybrid.
5.3 Recommendations

5.3.1 Recommendations from the study

i. Based on the overexpression of \( HCT \) gene in plants during biotic and abiotic constraints, sorghum cultivar N-13 \( HCT \) gene can be introduced into \textit{Striga} susceptible host plants by recombinant DNA technology.

ii. Based on the successful development of a maize-sorghum hybrid, breeders can use hybridization approach to transfer other desirable traits from sorghum to maize.

iii. Due to the high level of \textit{Striga} resistance observed in the maize-sorghum hybrids, farmers in \textit{Striga} prone regions in Kenya can incorporate maize-sorghum hybrids in their farming systems.

5.3.2 Recommendations for future research

1. Organoleptic, toxicity and fitness evaluation of the maize-sorghum hybrids.

2. Chromosomal staining to determine genomic sites of integration and proportions of the maize-sorghum hybrids.

3. Transformation of the sorghum cultivar \( HCT \) gene into \textit{Striga} susceptible host plants.

4. \textit{Striga} resistance screening of the consecutive hybrid filial generations.
REFERENCES


APPENDICES

Appendix 1: *Sorghum bicolor* Hydroxycinnamoyltransferase (HCT) gene, mRNA complete cds

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## Appendix 2: Distance and homology matrices of 8 HCT protein orthologs

### Distance matrix of 8 sequences

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### Homology matrix of 8 sequences

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Appendix 3: Distance phylogenetic tree between the 8 aligned HCT proteins
Appendix 4: Response of hybrid formation to different concentrations of 2, 4-D auxin.

The ANOVA Procedure

Class Level Information

Class  Levels  Values
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Number of Observations Used  18
11:21 Thursday, May 25, 2017  2

The ANOVA Procedure

Dependent Variable: No_of_maize_cobs_with_kernels_  No of maize cobs with kernels_

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<td>142.6666667</td>
<td>11.8888889</td>
<td></td>
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</tr>
<tr>
<td>Corrected Total</td>
<td>17</td>
<td>530.4444444</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

R-Square  Coeff Var  Root MSE  No_of_maize_cobs_with_kernels_  Mean
0.731043  100.1040  3.448027  No_of_maize_cobs_with_kernels_  Mean

The ANOVA Procedure

Welch’s ANOVA for No_of_maize_cobs_with_kernels_

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Anova SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>_<em><strong>4_D_Concentration</strong></em></td>
<td>5</td>
<td>387.7777778</td>
<td>77.5555556</td>
<td>6.52</td>
<td>0.0037</td>
</tr>
<tr>
<td>Error</td>
<td>4.0000</td>
<td>2.96</td>
<td>0.1503</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>4.2825</td>
<td>11:21 Thursday, May 25, 2017  3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The ANOVA Procedure

Tukey’s Studentized Range (HSD) Test for No_of_maize_cobs_with_kernels_

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.
Means with the same letter are not significantly different.

<table>
<thead>
<tr>
<th>Tukey Grouping</th>
<th>Mean</th>
<th>N</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>13.333</td>
<td>3</td>
<td>50mg/l</td>
</tr>
<tr>
<td>A</td>
<td>4.333</td>
<td>3</td>
<td>40mg/l</td>
</tr>
<tr>
<td>B</td>
<td>1.667</td>
<td>3</td>
<td>30mg/l</td>
</tr>
<tr>
<td>B</td>
<td>1.000</td>
<td>3</td>
<td>20mg/l</td>
</tr>
<tr>
<td>B</td>
<td>0.333</td>
<td>3</td>
<td>10mg/l</td>
</tr>
<tr>
<td>B</td>
<td>0.000</td>
<td>3</td>
<td>0mg/l</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NO_OF_MAIZE_2, 4-D CONCENTRATION_ NO_OF_MAIZE_COBS_WITH KERNELS_</th>
<th>Std. Error of NO_OF_MAIZE_COBS_WITH KERNELS_</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>0.3333</td>
<td>0.3333</td>
</tr>
<tr>
<td>1.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>1.6667</td>
<td>1.6667</td>
</tr>
<tr>
<td>4.3333</td>
<td>2.96273</td>
</tr>
<tr>
<td>13.3333</td>
<td>3.3333</td>
</tr>
</tbody>
</table>
Appendix 5: ANOVA analysis of *Striga* resistance data from soil free assays

The ANOVA Procedure

Class Level Information

<table>
<thead>
<tr>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
<td>4</td>
<td>E04 HF1 HF2 N13</td>
</tr>
</tbody>
</table>

Number of Observations Read: 12
Number of Observations Used: 12

19:28 Thursday, March 22, 2017

**The ANOVA Procedure**

**Dependent Variable: Attachments**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>11023.24917</td>
<td>3674.41539</td>
<td>114.95</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>255.72000</td>
<td>31.96500</td>
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<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>11</td>
<td>11278.96917</td>
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</tbody>
</table>

R-Square Coeff Var Root MSE Attachments Mean
0.977328 10.11557 5.653760 55.89167

**The ANOVA Procedure**

**Dependent Variable: Length**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>0.39439616</td>
<td>0.13146539</td>
<td>177.09</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>0.00593895</td>
<td>0.00074237</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>11</td>
<td>0.40833512</td>
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<td></td>
</tr>
</tbody>
</table>

R-Square Coeff Var Root MSE Length Mean
0.985165 6.093776 0.027266 0.447119

**The ANOVA Procedure**

**Dependent Variable: Weight**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>0.39439616</td>
<td>0.13146539</td>
<td>177.09</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

19:28 Thursday, March 22, 2017

**The ANOVA Procedure**

**Dependent Variable: Weight**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>0.39439616</td>
<td>0.13146539</td>
<td>177.09</td>
<td>&lt;.0001</td>
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</tbody>
</table>

19:28 Thursday, March 22, 2017
<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>0.00155503</td>
<td>0.00051701</td>
<td>334.44</td>
<td>&lt;.0001</td>
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<tr>
<td>Error</td>
<td>8</td>
<td>0.00002237</td>
<td>0.00000155</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>11</td>
<td>0.00156340</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

R-Square: 0.992880
Root MSE: 8.241897
Mean: 0.0801243

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Anova SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
<td>3</td>
<td>0.00155103</td>
<td>0.00051701</td>
<td>334.44</td>
<td>&lt;.0001</td>
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<tr>
<td>Error</td>
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<td>0.00002237</td>
<td>0.00000155</td>
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</tbody>
</table>

The ANOVA Procedure

Welch's ANOVA for Attachments:

<table>
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<th>Source</th>
<th>DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
<td>3</td>
<td>211.19</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>4.0555</td>
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</tr>
</tbody>
</table>

Welch's ANOVA for Length:

<table>
<thead>
<tr>
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<th>DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
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<td>212.03</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
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</tr>
</tbody>
</table>

Welch's ANOVA for Weight:

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
<td>3</td>
<td>317.46</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>3.9693</td>
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</table>

The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for Attachments

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>N</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Mean</th>
<th>Std Dev</th>
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</thead>
<tbody>
<tr>
<td>E04</td>
<td>3</td>
<td>105.866667</td>
<td>2.20075745</td>
<td>0.76070500</td>
<td>0.02123564</td>
<td>0.03260600</td>
<td>0.00084468</td>
</tr>
<tr>
<td>H01</td>
<td>3</td>
<td>31.000000</td>
<td>8.02766184</td>
<td>0.34677567</td>
<td>0.01552567</td>
<td>0.00520600</td>
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<tr>
<td>H02</td>
<td>3</td>
<td>32.333333</td>
<td>6.08444280</td>
<td>0.32776200</td>
<td>0.03539555</td>
<td>0.00481023</td>
<td>0.00100826</td>
</tr>
<tr>
<td>N03</td>
<td>3</td>
<td>54.366667</td>
<td>3.53883220</td>
<td>0.35523433</td>
<td>0.03280770</td>
<td>0.01771700</td>
<td>0.00045323</td>
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</tbody>
</table>

The ANOVA Procedure

Alpha: 0.05
Error Degrees of Freedom: 8
Error Mean Square: 31.965
Critical Value of Studentized Range: 4.02880
Minimum Significant Difference 14.783

Means with the same letter are not significantly different.

Tukey Grouping  Mean  N  Experiments
A  105.867  3  E04
B  54.367  3  M13
C  32.333  3  HF2
C  31.000  3  HF1

The ANOVA Procedure
Tukey’s Studentized Range (HSR) Test for Length

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha 0.05
Error Degrees of Freedom 8
Error Mean Square 0.000742
Critical Value of Studentized Range 4.5200
Minimum Significant Difference 0.072

Means with the same letter are not significantly different.

Tukey Grouping  Mean  N  Experiments
A  0.76071  3  E04
B  0.35323  3  M13
B  0.34678  3  HF1
B  0.32776  3  HF2

The ANOVA Procedure
Tukey’s Studentized Range (HSR) Test for Weight

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha 0.05
Error Degrees of Freedom 8
Error Mean Square 1.546E-6
Critical Value of Studentized Range 4.5200
Minimum Significant Difference 0.0033

Means with the same letter are not significantly different.

Tukey Grouping  Mean  N  Experiments
A  0.032606  3  E04
<table>
<thead>
<tr>
<th>Experiments</th>
<th>Mean of ATTACHMENTS</th>
<th>Std. Error of ATTACHMENTS</th>
<th>Mean of LENGTH</th>
<th>Std. Error of LENGTH</th>
<th>Mean of WEIGHT</th>
<th>Std. Error of WEIGHT</th>
<th>Mean of G</th>
<th>Std. Error of G</th>
</tr>
</thead>
<tbody>
<tr>
<td>E04</td>
<td>185.857</td>
<td>1.27061</td>
<td>0.76671</td>
<td>0.082263</td>
<td>0.015886</td>
<td>0.005441499</td>
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<tr>
<td>HF1</td>
<td>21.000</td>
<td>4.01330</td>
<td>0.34678</td>
<td>0.009663</td>
<td>0.005200</td>
<td>0.000815434</td>
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<td></td>
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<tr>
<td>HF2</td>
<td>32.333</td>
<td>3.46667</td>
<td>0.32776</td>
<td>0.020436</td>
<td>0.004819</td>
<td>0.001844000</td>
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<tr>
<td>N13</td>
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<td>0.35323</td>
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<td>0.017717</td>
<td>0.000261672</td>
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### Appendix 6: Plasmid DNA extraction buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Constituents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lysis</td>
<td>NaOH</td>
<td>8g/l</td>
</tr>
<tr>
<td></td>
<td>SDS</td>
<td>100ml/l</td>
</tr>
<tr>
<td>2. Re-suspension</td>
<td>Tris</td>
<td>6.1/l</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>3.7g/l</td>
</tr>
<tr>
<td></td>
<td>RNase</td>
<td>100mg/l</td>
</tr>
<tr>
<td>3. Neutralization</td>
<td>KCH3COO</td>
<td>294g/l</td>
</tr>
</tbody>
</table>