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PERFORMANCE OF SORGHUM HYBRID LINES WITH TANNIN GENE FROM CROSSES BETWEEN GADAM AND TANNIN HARD COAT SORGHUM VARIETIES

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DECLARATION

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

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DEDICATION

This work is dedicated to God for giving me the wisdom and to my parents for their support during my study period.

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LIST OF ABBREVIATIONS AND ACRONYMS

AOAC - Association of Official Analytical Chemists

ANOVA - Analysis of Variance

BPH - Better parent heterosis

CAN - Calcium Ammonium Nitrate

cDNA - Complementary Deoxyribonucleic Acid

CRD - Completely Randomized Design

CE - Catechin equivalent

CMS - Cytoplasmic male sterility

DNA - Deoxyribonucleic Acid

DD - Differential display

DEPC - Diethylpyrocarbonate

EABL - East African Breweries Limited

GS - Growth stage

HSD - Tukey's Studentized Range

HCl - Hydrochloric acid

IBPGR - International Board for Plant Genetic Resources

ICRISAT - International Crops Research Institute for the Semi-Arid Tropics

KALRO - Kenya Agricultural and Livestock Research Organization

MPH - Mid parent heterosis

Oligo (dT) - Oligo deoxythymine

OMA - Official Methods of Analysis

PCR - Polymerase Chain Reaction

PTGMS - Photo-thermo-sensitive genic-male sterility

QTL	-	Quantitative trait loci
RCBD	-	Randomized Complete Block Design
RNA	-	Ribonucleic Acid
RT	-	Reverse Transcription
SSH	-	Suppressive Subtractive Hybridization
SAGE	-	Serial Analysis of Genes Expression
TSP	-	Triple Super Phosphate
UV	-	Ultra Violet
V	-	Voltage

LIST OF SYMBOLS

S - South

E - East

m - meters

cm - centimeters

mm - millimeters

ml - milliliter

Kg - Kilogram

g - grams

mg - milligram

nm - nanometre

w/v - Weight by volume

% - Percentage

Kb - kilobase pairs

^{o}C - Degree Celsius

µg - Microgram

µL - Microliter

ha - hectares

F_1 - First Filial Generation

ppm - parts per million

ABSTRACT

Sorghum is a major staple food source in many African countries. In Kenya, it is cultivated by small-scale farmers. Gadam sorghum is the common variety mainly grown for brewing, manufacture of animal feeds and sorghum cakes. However, its yield is about 0.8 t ha-^{1} which is low compared to international standards. This is exacerbated by birds' that tend to have a preference for it. The use of birds' resistant sorghum varieties containing higher amounts of tannin than Gadam is a strategy to minimize the damage. However, tannin is an anti-nutritional factor that binds proteins together and inhibits many enzymes in *in vitro* assays reducing their digestibility and efficiency of utilization. Production of hybrids is one way of improving yield through heterosis. The objective of this study was to evaluate the performance of sorghum through hybridization of Gadam sorghum and hard coat tannin sorghum varieties. The study was conducted at the University of Embu research farm. The experiment was laid out in a Randomized Complete Block Design (RCBD) with three replicates. Four (4) sorghum varieties namely, Serena, Gadam, Seredo and Kari/Mtama-1 (used as a control due to its low tannin content) were sourced from the KALRO seed unit at Katumani. Sowing of sorghum varieties was staggered over three weeks to synchronize heading time to enable crossing. Development of F_1 hybrid lines was done by reciprocal crossing of Gadam and other varieties, using manual emasculation method. Crosses and their reciprocals were used as plant materials while parents were the controls. Data taken was; compatibility between the parents, changes in levels of expression of the tannin gene, grain nutritional levels, heterosis and yield traits compared to their parents. Collected data was subjected to a one-way analysis of variance (ANOVA) using R statistical software. Mean separation was done using Tukey's Studentized Range (HSD) at 95% confidence level. The cross Gadam x Serena, Serena x Gadam and the parent Gadam exhibited moderate mean plant height values of 99.5 cm, 120.5 cm and 103.3 cm respectively. The cross Gadam x Serena recorded a desirable negative mid-parent heterosis of -19.89 and -16.16 for plant height and days to maturity respectively. All F_1 hybrids recorded positive mid parent heterosis for the weight of full panicle, weight of a thousand seeds, number of tillers per plant, number of reproductive tillers and panicle length indicating possible yield improvement of Gadam sorghum through hybridization. The crosses Gadam x Seredo, Seredo x Gadam, Gadam x Serena and Kari/Mtama-1 x Gadam recorded significantly lower grain filling percentages compared to their parents. Both RNA levels and tannin content were observed to be at the maximum at soft dough stage and declined in subsequent stages indicating synchrony between RNA levels and tannin levels. Parents, crosses and reciprocals also differed significantly ($p<0.001$) for crude protein, fat, crude fibre, ash, carbohydrates, moisture and tannin content with values ranging from 5.323% to 10.390%, 1.691% to 2.299%, 2.230% to 3.520%, 1.215% to 1.360%, 76.790% to 85.677% , 5.433% to 9.667% and 0.034 mg/g to 1.763 mg/g respectively. In conclusion, all the F_1 hybrids have positive mid parent heterosis for number of reproductive tillers, number of tillers per plant, panicle length, weight of full panicle, and a thousand seed weight. There is maternal influence on days to heading, flowering and maturity, and a thousand grain weight. The lower grain filling percentage recorded in the F_1 hybrids indicates that the parental lines used have a narrow wide compatibility gene. The decline in *Tan1* gene after the soft dough stage shows that *Tan1* gene expression in sorghum is determined by the level of seed maturity. The F_1s showed marginal improvement in crude protein, crude fibre and carbohydrates but need to be evaluated further to determine the influence of hybridization on total nutrition. Also, heterosis can be utilized to improve the growth and yield of sorghum.

CHAPTER ONE
INTRODUCTION

1.1 Background information

Sorghum (*Sorghum bicolor* (L.) Moench) is a cereal crop in the *Gramineae* family of the genus *Sorghum* (Mofokeng & Shargie, 2016). It is the main food source for many people in the world with a high nutrition composition (Aruna & Visarada, 2019). It's ranked fifth globally in terms of importance following barley, maize, rice and wheat (FAOSTAT, 2017). In Sub – Saharan Africa, sorghum is ranked third after wheat and maize (Zidenga, 2015). Major sorghum growing countries in Africa include Nigeria, Sudan and Tanzania (Ratnavathi, 2016; Abraha *et al*., 2017; Aruna & Visarada, 2019). In Kenya, the major sorghum growing regions include Western, Nyanza, Northern Rift valley, Coast and Central regions (Ngugi & Maswili, 2011). The major cultivated species of sorghum is *S. bicolor (*Assar *et al*., 2013). The average yield of sorghum in Kenya is 0.8 tons per hectare (FAOSTAT, 2019; Kilambya &Witwer, 2013) which is low compared to the global average yield of 1.4 tons per hectare (FAOSTAT, 2019).

The main uses of sorghum in Kenya and some other parts of the world are production of porridge, unleavened bread, couscous, wallboards, dextrose, and malted beverages such as beer (Johnson *et al*., 2016; Kilambya & Witwer, 2019). Also, it is used in the manufacturer of pharmaceuticals used to manage coeliac disease (Arendt *et al*., 2010; Kasarda, 2010). The sweet sorghum varieties form a major source of food for livestock and their foliage and stems have been utilized in making the green chop, silage, hay and pasture both for livestock feeding (Makori, 2013). Also, it is an excellent feedstock for renewable energy used in the production of biofuel, sugar-to-ethanol, starch-to-ethanol and lignocellulosic or cellulosic-to-biogas production (Prakasham *et al*., 2014; Mathur *et al*., 2017).

Commonly cultivated varieties in Kenya are Gadam, Kari/Mtama–1, Serena, Seredo, Kari/Mtama–3 and Kimbeere (Timu *et al*., 2014). Gadam is a dwarf early maturing variety with white seeds (Olmstead & Rhode, 2014) and with a yield of 3,100 Kg/ha (Karanja *et al*., 2014). It tolerates little rainfall hence suitable for cultivation in marginal areas in the lower eastern and upper eastern Kenya (Mwadalu & Mwangi, 2013). Gadam is unique in that, it is commercially used for brewing beer because of

its good malting quality (Kilambya & Witwer, 2013; Kamau & Kavoi, 2015). Other uses of Gadam include the manufacture of animal feeds and sorghum cakes (Olmstead & Rhode, 2014; Kamau & Kavoi, 2015).

The challenge is that the yield of Gadam sorghum is low due to birds' infestation especially by *Quelea quelea* lowering the yield per hectare (Mey *et al*., 2012; Mutisya *et al*., 2016). This problem can be solved by growing varieties such as Serena, Seredo, and Kimbeere which have brown and red seed coats and are pigmented with tannin. The tannin in these types of sorghum varieties protects them from birds' infestation. However, it remains active in the mature grain, giving it an acidic flavour reducing its nutritional value and food quality for human consumption (Duodu *et al*., 2003). They bind proteins and inhibit many enzymes in *in vitro* assay (Gilani *et al*., 2012). Sorghum breeders have focused mainly on breeding for cold-tolerant, reduced maturity period, high food quality, salt tolerance, and insect resistance (Burow *et al*., 2011; Calone *et al*., 2020). In Kenya, new sorghum varieties have been released however, their yield is still low due to birds infestation and overreliance on local varieties whose productivity is low (Karari, 2006).

Hybrids have recorded higher yields compared to pure lines (Xie *et al*., 2019). This is possible by crossing compatible cultivars that display hybrid vigour at F_1. Compatibility is the highest degree of fitness between the male and female gametes that results in fertilization in the flowering plants (Hettyey *et al*., 2010). Breeding among compatible crop species has led to the production of both forage and grain sorghum varieties (Bean *et al*., 2013; Grossenbacher *et al*., 2017). *Sorghum bicolor ssp arundinaceum* is an important germplasm source for traits such as increased grain size, photoperiod insensitivity, less seed shattering, post and pre-anthesis drought tolerance, and dwarfness (Jordan *et al*., 2011). These traits have led to improved yields in sorghum hybrids. The compatibility of sorghum with its wild relatives including sudangrass, shatter cane, and Johnson grass has been reported too, which widens the scope of breeding (Wang *et al*., 2016).

Hybrid technology has increased sorghum yield per acre to over 50% and 47% in China and India respectively (Milomirka *et al*., 2014; Ashok *et al*., 2019). This has been realized through exploitation of heterosis which is expressed in F_1 grain sorghum hybrids (Kante *et al*., 2019; Smale *et al*., 2018). Heterosis is the ability of F_1 hybrids to perform better compared to their respective parents (Aruna & Cheruku, 2019). However, heterosis is limited by incompatibility in the parents which brings about sterility in F_1 hybrids. This is prevented by the introgression of the wide compatibility gene into the parents. In sorghum, heterosis is expressed in form of early anthesis, earlier maturity, increased height, larger panicle length, and high panicle weight in the hybrids (Crozier *et al*., 2020). Sorghum breeders have focused mainly on breeding for cold-tolerant, reduced maturity period, high food quality, salt tolerance, and insect resistance (Burow *et al*., 2011; Calone *et al*., 2020). Although new sorghum varieties have been released in Kenya, their yield is still low compared to international levels (Timu *et al*., 2014).

Many sorghum hybrids have been developed to meet various needs (Edgerton, 2009). There are those which have been improved specifically for fodder, ethanol and grains. Several seed-producing companies have released various sorghum hybrids for use in other countries (Tripp & Rohrbach, 2001). The utilization of hybrid seeds is highest in the USA which also happens to be leading in production (Steduto *et al*., 2012). The use of hybrid sorghum has also been reported in Ethiopia where the productivity of 6.2tha-1 was realized (Patil, 2007). In both Nigeria and Niger, hybrid sorghum outperformed the local varieties (House *et al*., 1997). In Sudan (former), Ejeta (1986) reported that hybrid sorghum out performed local varieties by between 50 to 85% under field conditions and between 300 to 400% under irrigation (Ejeta, 1986). In West Africa, Smale *et al*. (2018) reported a yield advantage of up to 17 – 47% in hybrids compared to pure bred lines. There is a high correlation between the utilization of sorghum hybrid seeds and productivity. These studies suggest that the utilization of sorghum hybrid seeds can increase sorghum production. None-use of hybrid sorghum in Kenya has made yields remain low due to non-exploitation of heterosis using hybrid seeds (Rattunde *et al*., 2013).

This research conceptualizes that hybrids between white Gadam sorghum and brown tannin sorghum have increased yield due to heterosis. Additionally, tannin is genetically controlled by additive alleles (Wu *et al*., 2012; Hill, 2015). Hybrid inherits only one of the tannin gene pairs hence lower genetic dosage. Since tannin is under quantitative trait loci (QTL), the level of tannin in hybrids is hypothesized to be low compared to parents. This study provides an opportunity to understand levels of tannin gene expression as sorghum matures. Tannin levels are expected to be modified downwards due to additive gene effects. The limited modified tannin level is meant to give some natural protection of seeds against birds' damage (Xie *et al*., 2019) hence reduction in losses. An increase in yield due to hybrid vigour compensates for food value lost due to limited tannin above that of Gadam.

1.2 Problem statement

The yield of Gadam sorghum has remained low over the years compared to international levels. Hybrids between Gadam and Seredo or Serena are expected to increase yield due to heterosis however, varieties best compatible with Gadam in hybrid seed production programs have not been evaluated in Kenya. The compatibility between Gadam and hard coat sorghum varieties needs to be assessed to inform lines suitable in hybrid seed production programs. Besides, hybrid lines yield performance has yet to be evaluated under local conditions in Embu and Kenya at large. Also, there is no available information on tannin gene expression behavior and nutritional levels in the hybrid lines in Kenya. In Gadam sorghum, the bird infestation has been accelerated by low tannin levels (Mutisya *et al*., 2016). The level of tannin gene expression and hence tannin content in hybrid sorghum lines need to be tested. This will inform nutrition changes in sorghum hybrid lines that affect food security.

1.3 Justification

The yield of hybrid sorghum is potentially high due to hybrid vigour. In a previous study by Sheunda (2019), sorghum hybridization has been reported to increase yield by 35%, thus local yield that stands at 0.8 tons can potentially be boosted to 1.08 tons per hectare with no increase in land area. The four sorghum varieties Gadam, Serena, seredo and Kari/Mtama-1(control) were important for this study because they are landraces that are commonly grown by farmers. Gadam sorghum was the key variety because of its low tannin content and good malting qualities. Tannin has been reported to be under QTL gene control in an additive manner (Hill, 2015) thus, hybridization

can reduce the tannin gene effect due to lower gene dosage in hybrid line seeds. Reduced tannin in hybrid seeds below that of hard coat sorghum varieties consequently increases their food value. In addition, a study on the mechanism of tannin gene control enables determining the stage of the hybrid lines seed maturity when tannin starts to decline and thus affecting food security and nutrition. Successful hybridization program further increases sorghum yield due to heterosis. The knowledge from this study will enable more understanding of the influence of hybridization on tannin in sorghum. Besides, the observed varietal compatibilities with Gadam will aid in sorghum hybrid lines production program. This study has provided a deeper understanding of hybridization of Gadam with tannin hard coat varieties for increased food security.

1.4 Null hypotheses

i. There is no significant difference in yield between the hybrid lines from Gadam and hard coat tannin sorghum compared to their parents.
ii. Gadam and hard coat tannin sorghum are not compatible in hybrid lines production.
iii. The level of expression of the gene controlling tannin does not change as hybrid sorghum seeds mature.
iv. There is no significant difference in nutritional levels and tannin content between the hybrid sorghum lines and their parents.

1.5 Research objectives

1.5.1 General objective

To evaluate the performance of sorghum through hybridization of Gadam sorghum and hard coat tannin sorghum varieties

1.5.2 Specific objectives

i. To evaluate the yield traits of hybrid sorghum lines between Gadam and hard coat tannin sorghum for selection of best performers compared to their parents.
ii. To determine the compatibility between Gadam and hard coat tannin sorghum in hybrid lines production.
iii. To determine changes in tannin gene expression within the growth cycle of hybrid lines from crosses between Gadam and hard coat tannin sorghum.

iv. To assess the nutritional levels and tannin content of hybrid lines between Gadam and hard coat tannin sorghum compared to their parents.

CHAPTER TWO
LITERATURE REVIEW

2.1 Reproductive biology of sorghum

Sorghum (*Sorghum bicolor* (L.) Moench) is a cereal species belonging to *Gramineae* family (Abraha *et al*., 2017). Knowledge of the sorghum floral biology and its pollination control mechanism is very important in designing effective breeding methods and suitable breeding strategies for its genetic improvement (Aruna *et al*., 2018). Sorghum has an inflorescence which is a determinate panicle that may be open or compact (Acquaah, 2012). Panicle initiation occurs between 30 – 40 days after germination (Patil, 2016). Both environment and genotype influence the duration of transformation from the vegetative primordial stage to the reproductive stage (Rao *et al*., 2015). Sorghum panicles have several or only a few spikelets (Visarada & Aruna, 2019). Spikelet has the flowers and occurs in two pairs, one pair is male – sterile and pedicelled while the other pair is bisexual, sessile and fertile (Kaur & Soodan, 2017). Sessile spikelet has a perfect flower comprising of three stamens, two lodicules, an ovary with two prolonged styles with plumose stigmas, a palea and a lemma (Acquaah, 2012). Pedicelled spikelets have only anthers but occasionally, have empty glumes and dysfunctional ovary (Kaur & Soodan, 2017). Pedicelled spikelets may be longer, smaller than the sessile spikelet or sometimes they may be of the same size (Patil, 2016).

Sorghum is a short–day plant whose flowering is accelerated by short periods of the day accompanied by long nights (Ratnavathi & Patil, 2013). Blooming in sorghum starts from the boot immediately after panicle emergence (Rao *et al*., 2015). Sorghum flowers best at temperatures between 21^{o} C to 35^{o} C (Mashao & Prinsloo, 2014). The pollen may remain viable for about thirty minutes while stigma receptibility may last for five to sixteen days after anthesis in a flower that is unpollinated depending on the environmental conditions of a place (Aruna *et al.*, 2018).

2.2 Importance of sorghum

Sorghum is an important source of food for human beings in many Asian and African countries (Dahlberg *et al*., 2011). The grain is used in making various food products including rice-like products, boiled porridge, couscous, snacks and unleavened bread (Dahlberg *et al*., 2011; Kumar *et al*., 2011). Sorghum is a gluten-free crop that is highly

recommended for coeliac patients (Staggenborg *et al*., 2016). The sweet sorghum varieties form a major source of food for livestock and their foliage and stems have been utilized in making the green chop, silage, hay and pasture both for livestock feeding (Makori, 2013). The Sorghum grain is a source of raw material in commercial food industry in making products such as potable alcohol, beer, starch, gruels, malt, adhesive core binders for ore refining, metal casting and packaging materials such as grits (Shoemaker & Bransby, 2010; Ochieng *et al*., 2011; Ogeto *et al*., 2012; Srinivasa *et al*., 2014). In Kenya, Gadam sorghum is commercially used for brewing beer because of its good malting quality (Kilambya & Witwer, 2013; Kamau & Kavoi, 2015). Sorghum beer obtained from Gadam sorghum has earned bigger sales for East African Breweries Limited (EABL) (Orr *et al*., 2013). Sweet sorghum is an excellent feedstock for renewable energy used in production of biofuel, sugar-to-ethanol, starch-to-ethanol and lignocellulosic or cellulosic-to-biogas production (Mathur *et al*., 2017; Prakasham *et al*., 2014). Bagasse which is obtained after extracting the juice is used for the manufacture of paper and pulp (Whitfield *et al*., 2012). Sorghum fibres have served as a source of raw material in making broomcorn, wallboards, solvents and fences (Makori, 2013).

2.3 Sorghum yield performance

Global yield for sorghum over the four years 2015 – 2018, averaged 1.44 tons per hectare (FAOSTAT, 2019). In Africa and East Africa, the yield for sorghum over the same period averaged 0.98 and 1.41 tons per hectare respectively (FAOSTAT, 2019). Kenya produced an average yield of 0.82 tons per hectare within the same period (Kilambya & Witwer, 2013). This is low despite the introduction of new improved varieties which have the potential to yield higher in the country (Muui *et al*., 2013). Kenya produces only 0.8% of the total production of sorghum in Africa despite the potential of the crop for improving household food security especially in marginal regions of the tropics (Ochieng *et al*., 2011).

Common sorghum varieties cultivated in Kenya include Gadam, Kari/Mtama-1, Serena and Seredo (Timu *et al*., 2014b). Gadam sorghum is a semi-dwarf early maturing variety with white sweet grains (Kagwiria, 2012). The crop matures in two and a half to three months depending on the rainfall amount and altitude of the area (Bosire, 2019). The average yield of Gadam per hectare is 3.15 tons (Olmstead &

Rhode, 2014). Because of its sweet grains, the crop is highly susceptible to bird damage (Este *et al*., 2019). However, the crop is highly tolerant to drought hence suitable for cultivation in marginal regions of Kenya including Makueni, Kitui, Tharaka, Mbeere, Mwingi, Kilifi, Machakos, Moyale, Tana river, Kajiado and Marsabit districts (Karanja *et al*., 2006). The variety is good for home consumption and commercialization (Orr *et al*., 2013). It is used in the brewing industry to make malted beverages such as beer due to high malting quality (Orr *et al*., 2013). Kari/Mtama-1 is a tall variety with large cream white grains (Lado & Muthomi, 2020). It takes three and a half to four months to reach physiological maturity and is capable of yielding 3.8 tons per hectare (Karanja *et al*., 2014). Kari/Mtama-1 is highly palatable to birds due to its sweet grains which lack tannins (Karanja *et al*., 2006). The variety is cultivated in lower eastern and upper eastern Kenya (Karanja *et al*., 2014). Serena is a medium maturing variety with brown grains which takes three to three and a half months to mature (Mwadalu & Mwangi, 2013). The crop yields 2.25 tons per hectare and it is capable of resisting birds' damage because of tannins in the grains (Monyo *et al*., 2004). It is cultivated in the Western and eastern regions of Kenya. Seredo is a medium maturing variety with dark brown grains that is cultivated in lower eastern and western Kenya (Mwadalu & Mwangi, 2013). It takes three months to reach physiological maturity and is capable of yielding 2.7 tons per hectare (Karanja *et al*., 2014). The crop has high tannin content in the grains which makes it tolerate birds attack (Este *et al*., 2019).

2.4 Sorghum production constraints

Sorghum production is faced with biotic, socio-economic and abiotic constraints such as pest and disease infestations, aluminium toxicity and birds infestation (Mengistu *et al*., 2018). Bird damage is the major biotic limitation to sorghum production in the world (Mofokeng & Shargie, 2016). *Passer domesticus*, *Psittaciformes*, *Corvus brachyrhynchos*, *Patagioenia spicazuro*, *Quelea quelea*, *Volatina jacarina* and *Aratingaophthama* are the major bird species that have been reported to cause huge losses in sorghum fields (Melo & Cheschini, 2012). Red-billed quelea (*Quelea quelea*) is the most dangerous and most common sorghum pests in Kenya (Mofokeng & Shargie, 2016). White sorghum varieties with tannin levels of 0.03% C.E - 0.81% C.E (Omondi *et al*., 2012) are more susceptible to bird damage because of their low tannin contents (Mashao & Prinsloo, 2014). Birds are capable of causing huge losses and

farmers can realize a 100% yield loss (Kagwiria *et al*., 2019). Strategies to increase yield include controlling bird damage in grain sorghum mainly through the use of repellants, bird scaring, chemical control, bagging panicles, plant characteristics, building anti-bird nets, lethal methods, non-lethal methods and the use of host plant resistance (Hiron *et al*., 2014; Mofokeng & Shargie, 2016). Most of these strategies however, require material investment and immense manpower particularly building anti-bird nets and panicle bagging (Xie *et al*., 2019). Chemical control is very expensive thus not every farmer can afford them (Hiron *et al*., 2014).

2.5 Compatibility in sorghum breeding

Both inter and intra-specific hybridization are common in the genus *Sorghum* (Barro-Kondombo *et al*., 2010). Cross compatibility between *Eu-sorghum* species has long enhanced sorghum breeding with selection for desirable traits (Ohadi *et al*., 2017). *Sorghum bicolor ssp arundinaceum* has been used as an important source of germplasm for improving the grain yield of hybrid grain sorghum cultivars. Crosses between sorghum and sudangrass have led to the production of forage and grain varieties (Bean *et al*., 2013). One avenue to achieve this breeding target is by breeding crop cultivars that are more compatible (Grossenbacher *et al*., 2017). Cross-compatible cultivars with synchronous flowering are important factors in ensuring fertilization in sorghum (Teshome, 2013). The rate of gene flow can be limited by the presence of genetic barriers such as pollen-pistil incompatibility among sorghum genotypes (Ohadi *et al*., 2017). To produce viable seeds, the pollen grain must adhere to the recipient stigma, germinate and grows into a pollen tube that later successfully delivers sperm which fertilizes the central cell and the ovule (Wright *et al*., 2013). If the pollen is non-compatible, the pistil will reject the pollen tube leading to non-fertilization of the ovule (Leducq *et al*., 2010; Young *et al*., 2012). Sorghum has been reported to be compatible with its wild relatives including sudangrass, shattercane and johnsongrass (Wang *et al*., 2016).

Inhibitory gene, *Iap* has been reported to lead to pollen-pistil incompatibility in crosses between maize x sorghum and *S. bicolor* x Australian sorghum species (Visarada & Venkateswaran, 2018). However, the presence of *iap* gene in its recessive form has increased the likelihood of interspecific hybridization in sorghum lines (Wang *et al*., 2016). This has facilitated the quick transfer of desirable genes in the sorghum breeding program In rice, crosses between *indica* and j*aponica* rice subspecies have

frequently resulted in hybrid sterility (Kumar *et al*., 2016). However, this fertility barrier can be broken by crossing wide compatibility varieties to both japonica and i*ndica* rice varieties (Wang *et al*., 2005). Sterility in *indica-japonica* hybrids was reported to be controlled by a locus *f5* whereas a neutral allele, *f5*-Du from Dular variety was reported to increase hybrids fertility when crossed both to *japonica* and *indica* rice varieties (Wang *et al*., 2005).

2.6 Genetic control of tannin in sorghum and the level of seed coat hardness

Sorghum can be grouped into five types namely; yellow, brown, white, red and black sorghum based on phenolic levels, genotype and colour (Xiong *et al*., 2019). The *R* and *Y* genes in sorghum control its pericarp colour (Waniska, 2012). A red (*R _Y_*), lemon yellow (*rrY_*) or a colourless or white (*rryy* or *R _ yy*) colour may be produced when these genes occur in combination (Rooney *et al*., 2014). The pericarp brightness especially that of the red sorghum is increased by the intensifier (*I*) gene (Rooney, 2010). The pericarp thickness is conditioned by the *Z* gene for instance a homozygous recessive (*zz*) gene is responsible for the thick mesocarp in sorghum (Miller *et al*., 2012). Endosperm and testa colour may be chalky in appearance as a result of small starch granules that are contained in a thick pericarp (Rooney *et al*., 2014). Dominant B_1 _ and B_2_ genes lead to a pigmented testa in sorghum (Waniska, 2012). The existence of one set of B_1_B_2_ gene in a homozygous recessive state for instance, $b_1b_1B_2B_2$ leads to unpigmented testa (Earp & Rooney, 2010).

Sorghum with dominant spreader gene (*S* _; type III) has condensed tannin hence the name tannin or brown sorghum (Hahn & Rooney., 2011). Sorghum with a recessive spreader gene (*ss*; type II) has a purple or brown caryopsis with a testa that is pigmented (B_1 _ B_2) (Wu *et al*., 2012). Type I sorghum has no tannins whereas type II sorghum has fewer tannins as compared to type III sorghum that has more tannins (Dykes *et al*., 2009). Seed coat colour has been correlated to the level of hardness seed coat among sorghum varieties. In a previous study by Karami *et al*. (2017), a black seeded genotype, A82, gave a considerably higher average value of seed hardness as compared to the white seeded sorghum genotypes. Mwithiga & Sifuna (2006) reported a rupture strength of 48.66, 59.64 and 90.84 N in Serena, Seredo and Kari/Mtama-1 respectively.

2.7 Hybrid program in sorghum production

Utilization of heterosis helps to exploit the vigour with the existing genetic variability of the parents for yield maximization (Jain & Patel, 2013). The choice of parents to use in hybridization and their genetic variability that is present greatly determines the success in developing superior hybrids (Makanda *et al*., 2010). Sorghum production can be increased mainly through systematic improvement of varieties and heterotic exploitation on a commercial scale (Jadhav & Deshmukh, 2017). The major objective of sorghum breeding program is to breed varieties that yield higher than the parents with a desirable combination of traits (Mwenda *et al*., 2019). Heterosis for traits such as yield depends on the cumulative effect of heterosis for component traits (Jain & Patel, 2013). Farmers in developed countries have grown hybrid sorghum varieties since the late 1950s following the discovery of cytoplasmic male sterility system (Kumar *et al*., 2011). This has allowed cost-effective production of hybrid sorghum and is increasingly being adopted in the developing world. The use of hybrid technology in sorghum production has resulted in an increased grain and forage yield of sorghum by 17 – 47% (Assefa & Staggenborg, 2010; Smale *et al*., 2018). Additionally, hybrid lines have been reported to have increased yield stability unlike the inbred lines in sorghum due to heterosis (Mindaye *et al*., 2016). Ashok *et al*. (2019) reported an increase in sorghum productivity by over 50%, 47% and 40% in China, India and United States respectively between 1960 and 1990. This is a significant breeding advantage in sorghum when compared to the prehybrid era of the early 1960s when China, India and the United States could realize 0.6, 0.49 and 2.8 tons per hectare respectively (Ashok *et al*., 2019).

2.8 Reciprocal crossing in Sorghum

The reciprocal cross is the process of making crosses between two parents whereby each parent serves as a female and also as a male in a cross to generate two reciprocal crosses (Gai & He, 2013). Hybrid yield can significantly be influenced by reciprocal effects (Yao *et al*., 2013). According to Mahgoub (2011), including reciprocal crosses in the analysis will help to compute for both reciprocal effects and combining abilities. This will help in predicting the best parental combination that can lead to the realization of the full heterotic expression of the hybrid (Brou *et al*., 2018) by estimating the genetic characters that are very important to the selection of parental lines by a breeder (Paterniani, 2012). Nardino *et al*. (2016) described general

combining ability to be the main effects while specific combining ability to be an interaction. Knowledge of interaction among traits is important in estimating genetic gain from both non-additive and additive genetic variances to develop cultivars with good agronomic trait performance (Hallauer, 2013). Reciprocal interspecific hybridization has been conducted between *C. citriodora* subspecies *citriodora* and *C. torelliana* to develop new genetic combinations with desirable genotypes (Dickinson *et al*., 2013). The direction of the cross greatly influences the genetic interaction affecting hybrid viability, reproductive isolation and heritability of traits within the hybrid progeny (Rix *et al*., 2012).

2.9 Methods of emasculation in hybrid seed production program

Sorghum is a self-pollinating crop (Raimi *et al*., 2012). The main method of emasculating the female flowers to enable cross-breeding without self-pollination include the use of genetic male sterility, mechanical emasculation, genetic transformation and the use of chemical agents (Yahaya *et al*., 2020). Mechanical emasculation which involves anthers removal followed by pollination has predominantly been used in hybrid seed production in crops such as rice, wheat and sorghum (Veerappan *et al*., 2014). Mechanical techniques include hot water treatment, hand emasculation, plastic bag method, anther aspiration and alcohol emasculation (Yahaya *et al*., 2020). A hybridization rate of more than 50% (in sorghum and finger millet) and 48.2% in rice has been reported while using hand emasculation (Shailaja *et al*., 2010). Hand emasculation is a useful technique only where small seed quantities are required, where labour is cheap and also where the value of the seed is high (Acquaah, 2012). Hot water treatment involves the use of hot water to kill anthers (Otsuka *et al*., 2010). Temperature and time specifications for specific crops must be observed keenly to avoid damaging the ovaries. An increase in temperature from 4^{o}C to 47^{o}C has been reported to kill the ovaries in rice (Jan, 2018). Anther aspiration involves the use of vacuum-suction to physically remove anthers from the flowers (Yahaya *et al*., 2020). A hybridization rate of about 65% has been reported in rice when using the plastic bag method (Altosaar & Greenham, 2013). However, the major drawback of this method is that, the method allows certain amounts of self-pollination to occur (Shailaja *et al*., 2010).

The use of chemical agents and cytoplasmic and genetic male-sterility systems have been utilized in large-scale hybrid seed production. Chemical agents (hormonal hybridization, ethephon, gibberellins, synthetic detergents and ethyloxanilates) have been utilized in hybrid breeding programs in soybean, wheat, sorghum and rice (Cheng *et al*., 2013). The main disadvantages of using chemical agents include less effectiveness due to interaction with the environment and genotype, lower doses that leads to temporary male sterility or high doses causing not only male sterility, but also female sterility, difficulties in field applications due to environmental factors such as rain and wind and also as a result of precise stage of plant development and toxicity effects on the F_1 seed or female parent (Adhikari, 2012; Fu *et al*., 2014; Colombo & Galmarini, 2017; Tinna, 2019). In addition, the method has been reported to cause unwanted morphological changes, self-pollination of the female progenitor expressed mainly as a substandard seed quality, less seed production in female parents treated with chemical agents compared to the female parents with cytoplasmic male sterility, as a result of overdoses and also, it is costly (Parodi & Gaju, 2009).

Environment sensitive male sterility (EGMS) lines have been developed and used in hybrid rice production in China (Chen *et al*., 2010). EGMS lines include photo-sensitive genic male sterility (PGMS) and thermo-sensitive genic male sterile (TGMS) lines (Nthakanio & Njau, 2019). PGMS rice lines are completely sterile when grown under 14 hours daylight length growth conditions and are fertile when grown under less than 14 hours daylight length conditions (Ireri *et al*., 2013; Nthakanio & Qingzhong, 2013). On the other hand, TGMS rice lines are sterile when grown under high temperatures and are fertile when grown under low temperature conditions (Ireri *et al*., 2013; Reddy, 2007).

Cytoplasmic male sterility (CMS) has been used as an alternative method to chemical agents. It leads to complete sterility of male gametes (non-functional pollen) in female parents hence no danger of self-pollination in dioecious crops (Luo *et al*., 2006). It has also been reported to be less costly and to lead to genetically pure seeds compared to chemical agents (Swamy *et al*., 2017). Furthermore, CMS systems encourage the use of hybrid technology to dramatically generate large quantities of superior F_1 plants that exhibit superiority over their parents in terms of stress tolerance, yield and adaptability (Sultana & Saxena, 2017). Over 150 plant species have been reported to

have cytoplasmic male sterility (CMS) which occurs either spontaneously or can be created through experimental means such as wide or inter-specific hybridization, genetic engineering, induced mutations and protoplasmic fusion (Bohra *et al*., 2016). In sorghum, the first CMS is presented by the milo CMS (A1) which is placed into the nuclear background of kafir (Jordan *et al*., 2010). In sorghum hybrid seed production, a number of *Rf* genes, *Rf1 – Rf5* have been reported in F_1 hybrids (Elkonin *et al*., 2015; Kiyosawa *et al*., 2020). Jordan *et al*. (2010) reported two major non-allelic fertility restorer genes, *Rf* in A1-CMS which are influenced by partial or modifier fertility loci. CMS technique has been applied in sorghum in which the hybrids have been reported to exhibit a grain yield heterosis of 30% - 40% (Yahaya *et al*., 2020).

2.10 Tracking tannin gene expression in sorghum

Gene tracking helps in the detection and explanation of particular genes signal transduction, defense mechanism, physiological events, primary and secondary metabolism and stress response hence gene function (Libault *et al*., 2010; Wang *et al*., 2010). Sorghum tannin content highly depends on the genotype and not the environment (Guixiang *et al*., 2010; Mkandawire *et al*., 2013). The main methods used in gene tracking are differential display (DD), suppressive subtractive hybridization (SSH) and serial analysis of genes expression (SAGE).

2.10.1 Differential Display (DD)

This is a simple and sensitive technique used to identify differentially expressed genes at different time periods under different environmental conditions (Jamil *et al*., 2011). The method involves reverse transcription using 3’ oligo (dT) anchored primers followed by polymerase chain reaction using arbitrary primers (Casassola *et al*., 2013). This is followed by the separation of amplified products on a gel followed by visualization, extraction and sequencing of differentially expressed control and sample bands (Park *et al*., 2010). The method has been used to analyze salt inducible genes in various crops including wheat, barley and rice (Jamil *et al*., 2011).

2.10.2 Suppressive Subtractive Hybridization (SSH)

In this technique, differentially expressed genes among different samples are separated using hybridization (Henriquez & Daayf, 2010). First, the synthesis of first-strand cDNA from the sample from which differentially expressed genes are to be isolated is done followed by hybridization with the first-strand cDNA from the control sample

(Guo *et al*., 2013; Padmanabhan & Sahi, 2011). This method has been used to identify 24 differentially expressed genes involved in the interaction between *F. graminearum* pathogen and wheat crop, of which 16 genes showed homology with wheat genes and 8 with pathogen genes (Casassola *et al*., 2013).

2.10.3 Serial Analysis of Genes Expression (SAGE)

This technique was developed to quantify global gene expression and is based on small transcript-specific sequences (Jamil *et al*., 2011). First, reverse transcription using biotin 3' oligo dT primers is done followed by cleaving using restriction enzymes (Hrdlickova *et al*., 2017). This is followed by attaching and linking generated fragments to adapters and amplifying them using polymerase chain reaction (Casassola *et al*., 2013). The fragments generated will then be cloned and sequenced for the analysis to check differentially gene expression (Lee *et al*., 2010).

2.11 Nutritional evaluation in sorghum and its anti-nutritional factors

The sorghum embryo is abundant in minerals, proteins, vitamin B complex, lipids and fat-soluble vitamins thus removing the outer pericarp will lead to high protein content with a decrease in the amounts of lipids, minerals and cellulose of the grain (Dicko *et al*., 2006; Etuk *et al*., 2012). The endosperm is composed of B – complex vitamins, starch and minerals whose contents vary across regions of cultivation (Shegro *et al*., 2012; Morais *et al*., 2017; Mwenda *et al*., 2019).

2.11.1 Determination of carbohydrates, ash and moisture content

Starch (32.1 – 72.5 g/100g) in sorghum comprises of amylose (3.5 – 19.0%) and amylopectin (81.0 – 96.5%) (Singh *et al*., 2010; Udachan *et al*., 2012). Sorghum starch digestibility is low compared to other cereal crops and this is attributed to strong associations between proteins, tannins and starch granules (Barros *et al*., 2012; Mkandawire *et al*., 2013). According to Taylor & Emmambux (2010), soluble fibers (10.0 – 25.0 %) and insoluble fibers (75.0 – 90.0%) are the major sources of non – starch polysaccharides (6.0 – 15.0g/100g). Determination of carbohydrates, ash content and moisture content using an oven has been done using the OMA (David *et al*., 2016; Jaworski *et al*., 2015; Koyuncu *et al*., 2014; Popping & Diaz-Amigo, 2014).

2.11.2 Evaluation of proteins, crude fibre and fats

Sorghum proteins contain high levels of non-polar amino acids like proline, alanine and leucine (Stonestreet *et al*., 2010). The availability of proteins, minerals and starch

in sorghum is reduced by the presence of tannins (McIntosh & Vancov, 2010). Analysis of proteins has been done using kjaldhal method as described in the official methods of analysis (Ape *et al*., 2016). Crude fibre consists of lignin and cellulose with some minerals (Dhingra *et al*., 2012). In sorghum, crude fibre has been determined by first treating the sample using an acid followed by an alkali (Vasquez *et al*., 2016). Loss in weight is then determined to give the crude fibre content in the samples (Verma & Patel, 2013). Fats in sorghum have been evaluated using a solvent extraction method according to OMA (Okunlola *et al*., 2019).

2.11.3 Tannin evaluation

Tannin in sorghum varieties is condensed and constituted by polymers or oligomers of Catechin (Cardoso *et al*., 2017). Its content varies between 0.2 – 48.0 mg/g among sorghum varieties with black testa sorghum having high amounts (Wu *et al*., 2012). Modified vanillin HCl methanol method has been used to quantitatively estimate the tannin content in sorghum, expressed as Catechin equivalent (Dykes, 2019). Tannin levels of 0.4 – 3.5 mg/100mg and 0.02 – 0.19 mg/100mg Catechin equivalent have been reported in type III (high tannin sorghum varieties) and type II (low tannin sorghum varieties) sorghums respectively (Dykes & Rooney, 2011). Omondi *et al*. (2012) reported tannin levels of 0.81% C.E, 0.03% C.E, 2.22% C.E and 1.2% C.E in Gadam, Kari/Mtama-1, Seredo and Serena respectively.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Site description

The study was carried out at the University of Embu research farm between November 2019 and July 2021. The experimental site lies at 0°35'25'' S and 37° 25'31'' E with an altitude of 1463 m above sea level. The site lies between the upper midland 2 (UM2) and UM3 Agro-ecological zones in the South-eastern slopes of the Mount Kenya region. The area has a bimodal rainfall pattern and receives rainfall amount of 1230 mm annually. Long rains occur between March and June while short rains occur between October and December (Jaetzold *et al*., 2007). The mean maximum temperature of the area is 25°C while the mean minimum temperature is 14.1°C; this gives the mean annual temperature of 19.6°C. Soils found in the area are obtained from basic volcanic rocks and are *Humic Nitisols* (Jaetzold *et al*., 2007). They are well-aerated, deep and have a clay texture that is friable with average to high inherent fertility (FAO, 2011).

3.2 Assessing the grain yield attributing traits of hybrid sorghum lines

3.2.1 Sorghum seed materials

Elite sorghum varieties Gadam, Kari/Mtama-1, Serena, and Seredo were obtained from the Kenya Agricultural and Livestock Research Organization (KALRO) seed unit at Katumani. A germination test was done in the laboratory to determine the seeds' germination rate and their viability using blotter method (Shakshi *et al*. 2014).

3.2.2 Synchronization of heading time

Sorghum varieties namely Gadam, Kari/Mtama-1, Serena and Seredo heads at 45, 58, 71, and 70 days respectively (Thomas *et al*., 2003; Mwadalu & Mwangi, 2013). Due to this difference in the heading time of sorghum, the sowing of the varieties was staggered over three weeks in a randomized complete block design (RCBD) in the field. This was to ensure that when Gadam sorghum heads there was an accompanying pollen donor flowering and vice versa in the reciprocals.

3.2.3 Development of F_1 hybrid seeds

At the stage of the emergence of the flower panicle from the flag leaf, Gadam sorghum was used in the reciprocal crossing with each of the three selected varieties (Serena, Seredo, and Kari/Mtama-1) to get F_1 seeds. The main crosses were Gadam x Serena,

Gadam x Seredo and Gadam x Kari/Mtama-1 while reciprocal crosses were Serena x Gadam, Seredo x Gadam and Kari/Mtama-1 x Gadam. Reciprocal cross was done to test the presence of cytoplasmic effects. Manual emasculation and artificial pollination were carried out as described by Rooney (2004). First, ten plants at the stage of panicle emergence from flag leaf of each variety were tagged. Five plants were sampled to be the source of pollen (male donor) while the other five plants were used as pollen recipients or female parents in each of the varieties in the cross. First, the tip of the glume was cut using a pair of scissors to open it to facilitate the removal of anthers and later cross-pollination (Kim *et al*., 2016; Seo *et al*., 2019). This was followed by carefully removing the anthers from the chosen flowers using a pair of fine-tip forceps. The panicle of the selected male parent in each variety was then covered using a khaki paper bag a day before anthers dehisce. In the following morning at 8.00 am, the bag was tapped to allow maximum pollen collection. The pollen collected was then dusted onto the emasculated head and covered with a well-labeled butter paper bag (indicating the date of pollination and parents involved) to prevent any unwanted pollination. Pollen dusting was repeated on each flower for two to three days to increase the level of pollination. Forceps were always sterilized by dipping them in 70% ethanol and wiping them with Whatman tissue before emasculation of a new floret to avoid cross-contamination.

3.2.4 Experimental layout, design and crop management

Sorghum hybrid lines developed in section 3.2.3 were used as plant materials while parents were the controls. They were sown in Randomized Complete Block Design (RCBD). The experiment had three replicates. Each treatment had an experimental plot, measuring 0.75m by 2.0 m with 0.5 m alleys between the plots and between the blocks. Sowing ground was pulverized using a hoe to achieve a moderately smooth seedbed with a fine tilth. Drilling of seeds was done in rows at a spacing of 75 cm between rows and later thinned to 20 cm between plants (Adams *et al*., 2015; Ottman, 2016). After planting, all the standard crop husbandry practices were observed. This included application of triple super phosphate (TSP) fertilizer at a rate of 100 kg per hectare at sowing (Hanway & Olson, 2012) and topdressing at 40 days after sowing with nitrogenous fertilizer, calcium ammonium nitrate (CAN) at a rate of 20 kg per hectares (Thivierge *et al*., 2015). Other field practices such as weeding, watering, disease, and pest management were performed as per Bonin *et al*. (2016) and Peerzada

et al. (2017). Five plants in each test entry (hybrids and parents) were selected and tagged before flowering for the evaluation of yield attributing traits.

3.2.5 Data collection

Data on yield traits that include rate of germination, plant height (cm), days to heading, days to flowering, the number of tillers per plant, number of reproductive tillers per plant, days to maturity, length of the panicle, the weight of full panicle, weight of 1000 seeds, and panicle compactness was collected.

3.2.6 Traits evaluation

Yield traits were evaluated using sorghum descriptors (IBPGR & ICRISAT, 1993). Germination rate was calculated in percentage (%) as:

$$\%\ seeds\ germination = (number\ of\ seeds\ that\ germinated\ per\ each\ petri\ dish \div total\ number\ of\ seeds\ that\ were\ placed\ in\ each\ petri\ dish) \times 100 \ldots\ldots\ldots (1)$$

Plant height was measured in centimeters using a tape measure as the length between the surface of the soil to the tip of the panicle. Days to heading were determined as the difference in days from emergence to panicle initiation. Days to flowering were taken as the total number of days from sowing to when each tagged plant reached the half bloom phase. Panicle length was measured in centimeters as the length from the panicle's base to the top while the weight of a thousand seeds was determined by weighing a thousand seeds in each treatment at 12 % moisture content. The weight of full panicle was determined by weighing the weight of panicles with the grains after harvesting at physiological maturity. The number of tillers per plant was counted as the total number of stalks that originated from the main stem. The number of reproductive tillers was determined by counting the individual tillers that produced reproductive heads in each treatment. Days to maturity were taken as the total number of days from sowing to when the seeds of each tagged plant reached the physiological maturity stage. This was indicated by turning black of layer on the base of the kernel. Panicle compactness was determined at the physiological stage on 15 plants sampled from each treatment in each replicate using a scale of 1 to 3 where 1 = loose panicle, 2 = semi-loose panicle, and 3 = compact panicle (Sulistyawati *et al*., 2019).

3.2.7 Estimates of heterosis

The better parent (BP) heterosis and mid parent (MP) heterosis were estimated in percentage in an excel for quantitative traits between genotypes as per the method described by Falconer & Mackay (1996):

$$Mid\ parent\ heterosis\ (\%) = [(F1 - MP)/MP] \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

$$Better\ parent\ heterosis\ (\%) = [(F1 - BP)/BP] \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

where, F_1 = mean value of F_1 hybrid, MP = average mean value of the two parents in the cross, BP = mean value of the better parent in the cross, % = percentage, and x = cross multiplication sign.

3.2.8 Data analysis

Data collected was subjected to one way ANOVA using R statistical software. Data on panicle compactness was also subjected to one way ANOVA after transformation using $\log_{10}$ (x + 1). Mean separation was done using Tukey's Studentized Range (HSD) at 95% level of confidence. The degree of relationship between the quantitative characters was assessed using the Pearson correlation coefficient in the R package vegan. A t-test analysis was carried out using XLSTA version 2020 to test whether heterosis was significantly different than zero. The difference between the means was done at 95% confidence interval.

3.3 Determining the compatibility between Gadam and hard coat tannin sorghum

3.3.1 Experimental layout, design and crop management

Plant material was the six sorghum hybrid lines developed in 3.2.3 above, where F_1s were used as treatments with parents (Gadam, Kari/Mtama-1, Seredo and Serena) used as controls. Experimental design, layout and crop management were done as described in 3.2.4. Five plants in each treatment were later selected and tagged before flowering for the evaluation of compatibility.

3.3.2 Data collection

Data was collected from 5 tagged plants in each test entry (hybrids and parents) per each replicate. Parameters included grain filling percentage (GFP) and 100 seed weight.

3.3.3. Compatibility determination

To determine compatibility, the panicles of the five tagged plants in each treatment (parents and hybrids) were cut at maturity then the total number of glumes were counted against the filled glumes. Then, the compatibility (in form of grain filling percentage) was calculated using the formula described by Chen *et al*., 2019:

$$\% \, GF = (total \, number \, of \, filled \, glumes \div total \, number \, of \, glumes)100 \ldots (4)$$

The weight of a hundred seeds was determined by weighing a hundred seeds harvested at physiological maturity and dried to 12 % moisture content for each treatment.

3.3.4 Data analysis

Data collected were subjected to one way ANOVA using R statistical software. Mean separation was done using Tukey's Studentized Range (HSD) at 95% level of confidence.

3.4 Determining changes in tannin gene expression of hybrid sorghum lines

3.4.1 Collection of samples

Grain samples of experimental plant materials (parents together with their hybrids) planted in section 3.3 were used. The Gadam x Kari/Mtama-1 hybrid line and its reciprocal were included as the control treatments due to their low tannin content. Five plants in each variety were selected randomly and tagged for determining the level of tannin gene expression both at the tannin and RNA levels. Grain samples were taken at the flowering stage, milk stage, soft dough stage, hard dough stage, and physiological maturity stage. They were collected from each parental material and six F_1 hybrid lines under study as follows; Grains from the top spikelets were cut using a pair of sterilized scissors, thereafter, they were separated from the chaff and then put in polythene bags well labeled. Samples for tannin gene analysis were stored in liquid nitrogen at -196^{o}C in the laboratory until use whereas tannin content analysis, seeds at each stage were ground using a high-speed universal disintegrator (FW80-I) in the laboratory to produce fine flour. The flour was then kept at 4^{o}C in polythene bags till use.

3.4.2 Gene analysis

3.4.2.1 Extraction of RNA

Total RNA was extracted from sorghum tissues using the ISOLATE II RNA Plant Kit (Bioline) according to the manufacturer's instructions. Gel electrophoresis was done

to check the quality of total RNA using 1% (w/v) agarose gel as follows; First, 1% agarose gel was prepared by adding 100ml of 1X Sodium Borate buffer to 1g of Agarose in a conical flask. The mixture was then heated in a microwave for 3 minutes to dissolve the agarose. After this, the molten gel was allowed to cool and later ethidium bromide (visualization dye) was added to a final concentration of 500 µL. The gel solution was mixed thoroughly by swirling gently. A comb was inserted on the slots in the provision on the gel-casting plate. It was positioned at 0.5-1.0 mm above the base of the gel-casting plate to form wells when the agarose gel is cast on the casting plate without perforating it through. Warm agarose gel solution was then poured into the gel casting plate. The gel was left for 20-45 minutes at room temperature to completely polymerize. Then, a small amount of electrophoresis buffer was poured on the gel-casting plate to facilitate easy removal of the gel without sticking. The gel slap was laid into the electrophoresis tank and the electrophoresis buffer was added to a depth approximately 1.0 mm above the gel. The RNA samples were mixed with a loading dye followed by loading the sample mixture into the wells of the submerged gel using a disposable micropipette. Three (3) µL of 50 base pair ladder [Gene Ruler (Thermo Scientific®, USA)] was loaded into the electrophoresis gel alongside other samples. The gel electrophoresis tank was then covered with its lid. The gel tank was then closed and electrical leads were attached such that the RNA migrates towards the positive anode. The gel was allowed to run for forty minutes at a voltage of 110 V. Thereafter, gel visualization was done using a UV trans illuminator (Bioline USA Inc. USA). Once the quality was confirmed to be good, RNA was converted to cDNA.

3.4.2.2 Synthesis of complementary DNA and Polymerase Chain Reaction

The first complementary DNA (cDNA) strand was synthesized using the following reagents; 5 µL of PCR premix PureTaq Ready-To-Go PCR bead dissolved in 25 µL molecular water, 1 µL of Oligo $(dT)_{18}$ primer, 1 µL of RiboSafe RNase Inhibitor, 1 µL of Tetro reverse transcriptase (200 u/µL) and 1.5 µL of template RNA to a total reaction volume of 9.5 µL prepared in a sterile RNase free tube on ice. The above mixture was mixed gently by pipetting. Thereafter, the mixture was incubated at 45^{o}C for 30 minutes. The reaction was then terminated by incubating at 85^{o}C for 5 minutes chilled on ice. The cDNA was then stored at -20^{o}C till use.

The following two primers, Sbct5 (5′-CCCGATTTCTCCACCCCATGGACCTACC-3′) and Sbct6 (5′-CACCATGGTACCAACCTTGTCAGACCCT-3′) forward and reverse respectively were designed as per Wu. *et al*. (2012). They were used to amplify the *Tan1* gene from cDNA synthesized from sorghum grain samples. The PCR was done as per the procedure described by Mbuvi (2017) with some moderations. The PCR was done using 14.5 µL reaction mixture comprising of 1.5 µL of template cDNA, 1.5 µL of each forward and reverse primers, and 10 µL of MyTaq premix comprising of 0.2 µL MyTaq DNA polymerase, 2 µL of 5x MyTaq reaction Buffer consisting of 15mM $MgCl_2$, 5mM dNTPs, enhancers and stabilizers and top up with 7.8 µL of water. Polymerase reaction was then done in an eppendorf thermal cycler under the following conditions: initial denaturation at 95^{o}C for 1.0 minute, 30 cycles consisting of denaturation at 95^{o}C for 15 seconds, annealing at 50^{o}C for 15 seconds and extension of 72^{o}C for 10 seconds and an elongation time of 4 minutes at 72^{o}C.

Gel electrophoresis was done to visualize PCR products using 1% (w/v) agarose gel. Three microliters of PCR product were mixed with 1 µl of loading dye (Bioline USA Inc. USA). The products were electrophoresed alongside 3 µl of 1 kb ladder (Bioline USA Inc. USA). The gel was then allowed to run for forty minutes at a voltage of 110 V and then visualized using an ultra-violet trans illuminator (Bioline USA Inc. USA) and photographed.

3.4.3 Tannin evaluation

The experiment to evaluate tannin content at flowering, milk, soft dough, hard dough and physiological maturity stages was done in the laboratory in three replicates. Tannin content at each stage was determined using the modified vanillin-HCl assay method of Price *et al*. (1978) using a digital spectrophotometer (ME 801) as described by Dykes (2019). Briefly, before sample analysis, a standard curve was run using the Catechin solution (1000 ppm). To prepare the standard calibration curve, 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of the Catechin solution was added into test tubes and then diluted to 1.0 mL using methanol. All the tubes were then placed simultaneously in the thermosetted water bath set at 30^{o} C and five (5.0) mL of the vanillin reagent was added to each tube at an interval of 1.0 minute. After 20 minutes of incubation period, the absorbance of the coloured intensity for each tube was measured at 500 nm using a digital spectrophotometer. Always, the methanol blank was used to adjust the machine

to zero absorbance. The slope of the line was determined using Catechin concentration (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) as the x-axis and the absorbance values as the y-axis in Microsoft excel (Figure 4.1, Figure 4.2, Figure 4.3, Figure 4.4 and Figure 4.5). The coefficient of determination of the regression model (r^2) of the curve was determined. For sample analysis, three replicates of 0.3 g each of freshly ground sorghum seed samples were weighed and transferred into centrifuge tubes. Then, 8 mL of 1% HCl in methanol was added to each tube. The contents were mixed on a vortex mixer for 10 seconds and then each tube was placed in a water bath for 20 minutes. After the first 10 minutes of incubation, each tube was vortexed again for 10 seconds and placed back into the water bath for the remaining incubation period. Each tube was removed exactly after 20 minutes of incubation and mixed on a vortex mixer for 10 seconds immediately after removal from the water bath. The samples were then centrifuged at 4000 x *g* for 10 minutes. The supernatant was then decanted carefully avoiding the ground sample to be transferred into the supernatant. Two 1 mL aliquots were taken from the supernatant and each placed into a clean separate test tube. In total, there were 60 tubes. Among them, 30 were labeled 'blank' tubes while the other 30 were labeled 'sample' tubes. All the tubes were simultaneously placed into the thermosetted water bath set at 30^{o}C and five (5.0) mL of the vanillin reagent was added to each 'sample' tube while five (5.0) mL of 4% HCl in methanol was added to each blank tube at an interval of 1.0 minute as indicated by Dykes (2019). Each set (a sample and a blank tube) was allowed to stay in the water bath for 20 minutes. The absorbance of the colored intensity for each 'sample' and 'blank' tube was then read exactly after 20 minutes at 500 nm using a digital spectrophotometer. The methanol blank was used to adjust the machine to zero absorbance. To determine the final tannin content, the value of the 'blank' was subtracted from the value of the 'sample'. Tannin concentration (mg/g) was calculated using the quadratic equation obtained from the standard calibration curve;

$Y = 7 * 10^{-5} x + 0.0096$

Where; Y= absorbance, X = concentration.

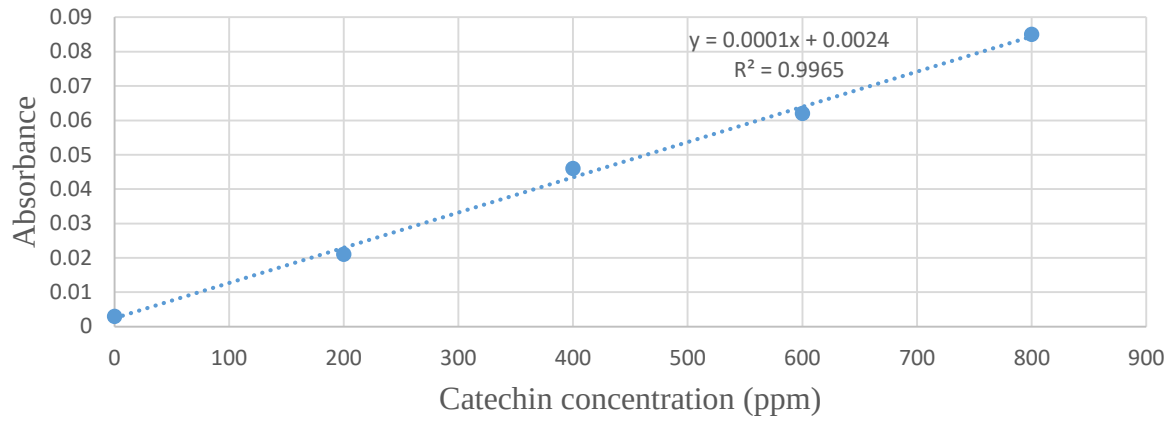

Figure 4.1: Standard calibration curve- flowering stage

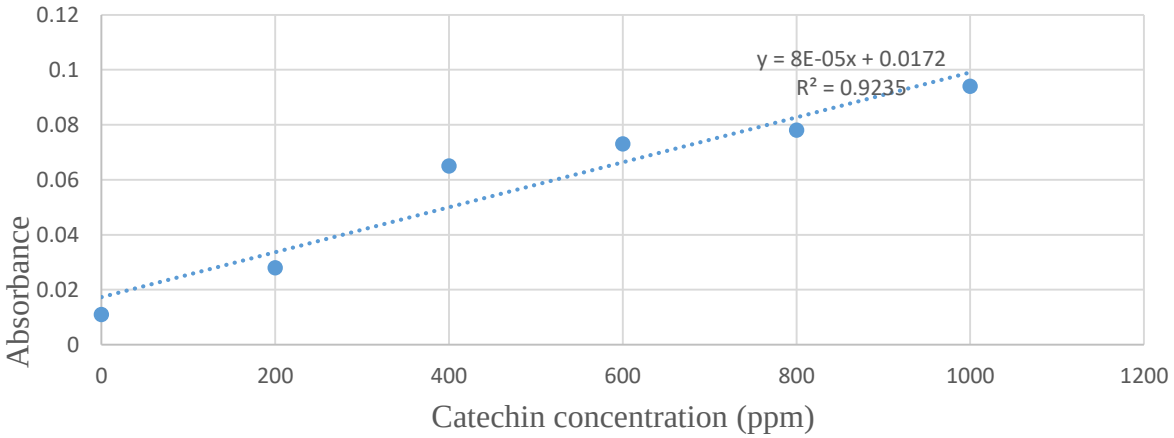

Figure 4.2: Standard calibration curve- milk stage

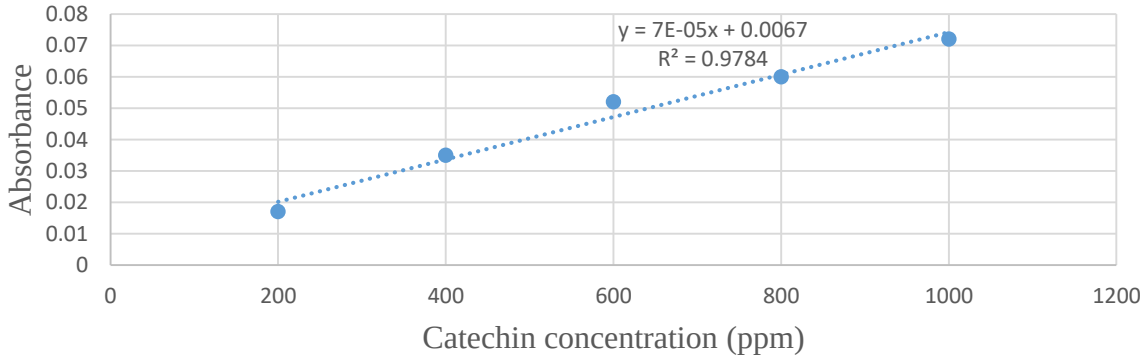

Figure 4.3: Standard calibration curve - soft dough stage

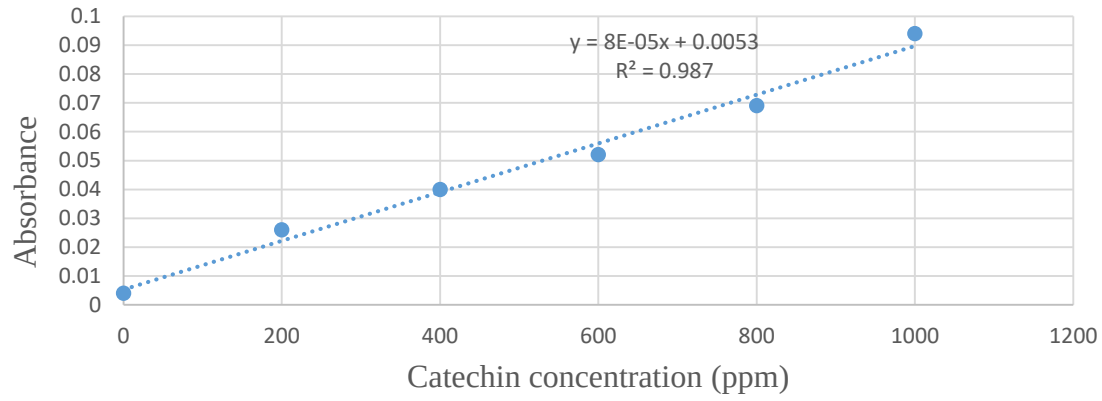

Figure 4.4: Standard calibration curve - hard dough stage

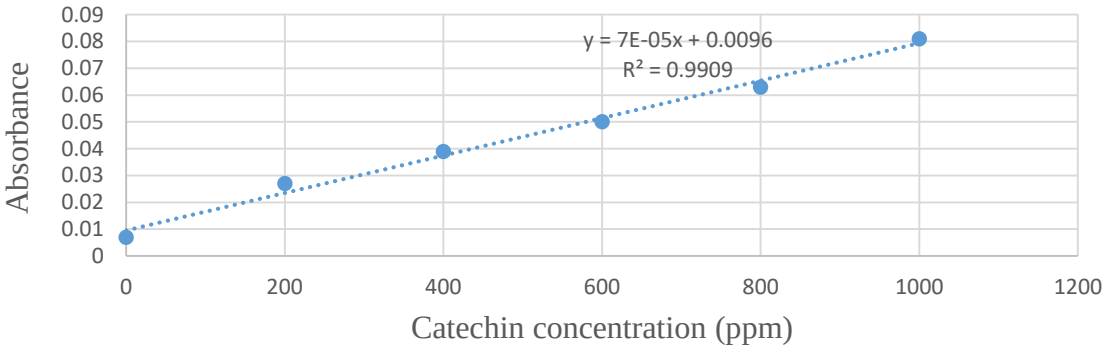

Figure 4.5: Standard calibration curve - physiological maturity stage

3.4.4 Data analysis

Data on tannin were subjected to ANOVA using R statistical software while mean separation was done using Tukey's Studentized Range (HSD) at 95% level of confidence.

3.5 Determining the nutritional levels and tannin content of hybrid sorghum lines

Experimental material was the whole mature grains of each hybrid sorghum line and their parents planted as per section 3.3.1. Cleaning of sorghum grains was done manually to remove the impurities. Seeds were hand–threshed, cleaned and ground using a high-speed universal disintegrator (FW80-I) to produce fine flour. The flour was then kept at 4^{o} C in polythene bags till use. Crude protein, total carbohydrates, fat, crude fibre, tannin, moisture and ash contents were determined. Crude protein was

determined using the modified Kjeldahl method of Cope (1889) as adopted by Sarkar & Haldar (2005). Fat content was determined as per the method of AOAC 945:16 (W Horwitz, 2000), as adopted by Tasie & Gebreyes (2020) with slight modification that included evaporating the major portion of the solvent inside the fume hood. The crude fibre was determined as per the method of AOAC 962:09 (Horwitz & Latimer, 2005) as supported by the Food Safety and Standards Authority of India (FSSAI, 2016). Ash content was determined as per the method of AOAC 923:03 (Horwitz & Latimer, 2005). Moisture content was determined from seeds harvested at physiological maturity. It was determined based on the method of AOAC 925:10 (W Horwitz, 2000) as adopted by Tasie & Gebreyes (2020) with slight modifications that included drying the samples in the drying oven for 2 hours at 105^{o}C. Total carbohydrates from the sorghum samples were obtained as per Pearson (1976) by subtracting the obtained figure of moisture content, fats, ash content, proteins from 100 % as follows;

Total carbohydrate (%) = [100-(moisture (%) + fats (%) + ash (%) + proteins (%))]

Tannin content was determined using the modified vanillin-HCl assay method (Price *et al*. 1978; Dykes, 2019).

3.5.1 Data analysis

Data collected were subjected to one-way analysis (ANOVA) in R statistical software (R Development Core Team, 2015). Tukey's Studentized Range (HSD) at 95% confidence level was used in mean separation (De Mendibru, 2019). Pearson's correlation was done to compare the degree of association between the traits analyzed. Analysis for nutrients content was expressed in g/100g while for tannin content it was expressed in mg/100g.

CHAPTER FOUR

RESULTS

4.1 Grain yield attributing traits of hybrid sorghum lines

4.1.1 Variations in quantitative and qualitative characters

The results on plant height among F_1 hybrids and their parents are shown in table 4.1. The cross between Gadam and Serena recorded the lowest plant height mean among hybrids in the growth period. However, it was not significantly different from its reciprocal cross (Serena x Gadam) apart from in week 2 and 4, when it recorded 30.713 cm and 58.401 cm respectively. This was significantly different from its reciprocal (Serena x Gadam) which recorded a mean plant height of 45.773 cm and 81.313 cm in week 2 and 4 respectively. In weeks 5-6, Gadam x Serena recorded significantly lower mean plant height than all other crosses apart from its reciprocal cross. Also, it was significantly lower than parental lines in the study apart from Gadam. The cross Kari/Mtama-1 x Gadam recorded the highest plant height with a mean of 159.473 cm at week 6. This was not significantly ($p<0.001$) different from Gadam x Kari/Mtama-1 (142.293 cm) in the same growth period. The cross Kari/Mtama-1 x Gadam had the highest mean plant height among the F_1 hybrids from week 3-6, but with a significant difference observed with the Gadam parent.

The results on the rate of germination, panicle length, days to heading, flowering, maturity, the number of tillers per plant, number of reproductive tillers, weight of full panicle, and weight of 1000 seeds are shown in Table 4.2. There were no significant variations observed in the rate of germination among the crosses and their reciprocals. In terms of panicle length, significant variations ($p<0.01$) were observed among the crosses, reciprocals and their parents. Kari/Mtama-1 x Gadam recorded the highest panicle length of 23.727 cm which was significantly different from its Gadam parent (20.200 cm) but not Kari/Mtama-1 parent. Among the crosses, the panicle length of Kari/Mtama-1 x Gadam was only significantly higher than Serena x Gadam that had 20.667 cm. It was also significantly higher than Serena that had 19.733 cm. All the crosses had significant variations ($p<0.001$) with their reciprocals in days to flowering and days to heading. The cross Gadam x Kari/Mtama-1 (with 43.800 days) recorded significantly low days to heading than all other F_1 hybrids. Gadam x Kari/Mtama-1 with 52.200 days was the earliest to flower and was significantly lower than its parents Gadam and Kari/Mtama-1 and also its reciprocal that recorded 65.267, 70.200 and

67.933 days respectively. In terms of days to maturity, the cross Gadam x Serena recorded significantly lower days to maturity (87.000 days) than its reciprocal that had 103.267 days. Gadam x Kari/Mtama-1 (93.600 days) had no significant difference with its reciprocal cross Kari/Mtama-1 x Gadam (97.933 days). Crosses Gadam x Serena and Gadam x Seredo recorded significantly lower days to maturity than their reciprocal crosses. All the crosses did not differ significantly ($p>0.05$) in the number of tillers per plant, number of reproductive tillers, weight of full panicle, and weight of 1000 seeds.

Panicle compactness among the crosses and their reciprocals is shown in Table 4.3. A significant difference ($p<0.001$) was observed among the crosses and their reciprocals apart from Gadam x Serena and its reciprocal that both recorded a mean of 2.773 for compact panicle. Among the parents, the highest mean recorded for semi-loose and loose panicles was 2.773 for Seredo and Kari/Mtama-1 respectively. Also, the parents Gadam and Serena recorded the highest mean of 2.773 for compact panicles. The cross Gadam x Seredo exhibited a mean panicle compactness values of 1.843 for compact and 2.366 for semi-loose panicle. Its reciprocal cross exhibited a mean panicle compactness values of 2.366 for compact and 1.843 for semi-loose panicle. The cross Gadam x Kari/Mtama-1 had a mean panicle compactness values of 1.782 for compact and 2.395 for loose while its reciprocal cross had 2.483 for compact and 1.596 for loose panicle.

Table 4.1: One way analysis of variance showing means of plant height variable for the six weeks

Treatments	PH (WK1) (Mean ± S.E)	PH(WK2) (Mean ± S.E)	PH (WK3) (Mean ± S.E)	PH(WK4) (Mean ± S.E)	PH (WK5) (Mean ± S.E)	PH (WK6) (Mean ± S.E)
Gadam	$19.347^{c} \pm 3.838$	$29.327^{d} \pm 3.867$	$46.360^{d} \pm 5.500$	$63.880^{de} \pm 2.933$	$85.633^{ef} \pm 7.620$	$103.287^{f} \pm 4.181$
Serena	$32.420^{a} \pm 2.471$	$47.893^{ab} \pm 6.053$	$79.445^{ab} \pm 1.727$	$106.260^{ab} \pm 3.973$	$125.933^{bcd} \pm 2.646$	$144.747^{bcd} \pm 2.177$
Seredo	$24.801^{abc} \pm 4.583$	$33.720^{bcd} \pm 6.464$	$81.493^{ab} \pm 3.950$	$110.753^{ab} \pm 3.733$	$135.260^{abc} \pm 2.601$	$151.007^{abc} \pm 0.903$
Kari/Mtama-1	$31.187^{a} \pm 1.522$	$48.927^{a} \pm 3.806$	$94.053^{a} \pm 3.177$	$126.187^{a} \pm 3.031$	$155.693^{a} \pm 5.028$	$170.347^{a} \pm 2.864$
Gadam x Serena	$23.860^{abc} \pm 1.607$	$30.713^{cd} \pm 3.227$	$43.533^{d} \pm 4.536$	$58.401^{e} \pm 3.247$	$79.100^{f} \pm 6.454$	$99.480^{f} \pm 2.212$
Gadam x Seredo	$20.133^{c} \pm 3.854$	$35.160^{abcd} \pm 4.391$	$63.893^{bcd} \pm 6.872$	$97.067^{bc} \pm 5.878$	$110.253^{cde} \pm 5.206$	$127.127^{de} \pm 6.484$
Gadam x Kari/Mtama-1	$27.827^{abc} \pm 3.671$	$44.533^{abc} \pm 5.718$	$76.067^{abc} \pm 3.850$	$106.987^{ab} \pm 3.585$	$124.340^{bcd} \pm 2.627$	$142.293^{bcde} \pm 2.775$
Serena x Gadam	$29.287^{ab} \pm 1.911$	$45.773^{ab} \pm 2.639$	$56.563^{cd} \pm 5.985$	$81.313^{cd} \pm 5.304$	$102.440^{def} \pm 9.015$	$120.473^{ef} \pm 7.101$
Seredo x Gadam	$19.080^{c} \pm 1.301$	$29.680^{d} \pm 1.131$	$71.420^{abc} \pm 4.066$	$99.780^{bc} \pm 5.540$	$117.873^{bcd} \pm 3.993$	$136.013^{cde} \pm 6.608$
Kari/Mtama-1 x Gadam	$22.227^{bc} \pm 3.114$	$38.920^{abcd} \pm 8.399$	$84.293^{ab} \pm 3.852$	$114.467^{ab} \pm 4.541$	$143.127^{ab} \pm 5.587$	$159.473^{ab} \pm 5.591$
P – Value	0.031^{*}	0.048^{*}	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$

Key: Means with the same letter within the column are not significantly different. * Significant at 5%; *** significant at 0.1%. S.E: standard error; PH: plant height; WK: week

Table 4.2: One way analysis of variance showing means of quantitative growth and yield variables

Treatment	RG (%) (Mean ± S.E)	DTH (Mean ± S.E)	DTF (Mean ± S.E)	DTM (Mean ± S.E)	NTPP (Mean ± S.E)	NRT (Mean ± S.E)	PL (Mean ± S.E)	WFP (Mean ± S.E)	W1000S (Mean ± S.E)
Gadam	$70.000^{ab} \pm 2.887$	$55.733^{de} \pm 0.371$	$65.267^{ef} \pm 0.267$	$97.667^{de} \pm 0.267$	$1.533^{ab} \pm 0.266$	$1.333^{a} \pm 0.176$	$20.200^{b} \pm 0.854$	$76.063^{a} \pm 2.892$	$34.573^{a} \pm 4.099$
Serena	$60.000^{ab} \pm 2.887$	$65.067^{ab} \pm 0.521$	$74.533^{a} \pm 0.371$	$109.867^{b} \pm 1.593$	$1.400^{ab} \pm 0.462$	$1.067^{a} \pm 0.371$	$19.733^{b} \pm 0.285$	$80.283^{a} \pm 9.361$	$32.863^{a} \pm 3.260$
Seredo	$58.333^{b} \pm 3.333$	$68.600^{a} \pm 1.206$	$75.867^{a} \pm 0.581$	$116.753^{a} \pm 1.888$	$1.267^{ab} \pm 0.067$	$1.000^{a} \pm 0.115$	$20.980^{ab} \pm 0.591$	$87.065^{a} \pm 7.427$	$30.317^{a} \pm 0.725$
Kari/Mtama-1	$71.667^{a} \pm 1.667$	$61.600^{bc} \pm 0.115$	$70.200^{cd} \pm 0.917$	$99.400^{cde} \pm 1.311$	$0.600^{b} \pm 0.000$	$0.467^{a} \pm 0.067$	$21.273^{ab} \pm 0.254$	$70.090^{a} \pm 7.375$	$37.910^{a} \pm 2.285$
Gadam x Kari/Mtama-1	$68.333^{ab} \pm 1.667$	$43.800^{f} \pm 1.331$	$52.200^{g} \pm 0.529$	$93.600^{e} \pm 1.026$	$1.667^{ab} \pm 0.240$	$1.267^{a} \pm 0.176$	$22.553^{ab} \pm 0.625$	$82.519^{a} \pm 1.204$	$40.013^{a} \pm 1.679$
Gadam x Serena	$65.000^{ab} \pm 2.887$	$53.800^{e} \pm 0.400$	$63.200^{f} \pm 0.416$	$87.000^{f} \pm 0.987$	$2.467^{a} \pm 0.696$	$1.800^{a} \pm 0.346$	$21.260^{ab} \pm 0.171$	$84.157^{a} \pm 1.585$	$38.867^{a} \pm 6.130$
Gadam x Seredo	$61.667^{ab} \pm 1.667$	$56.333^{de} \pm 0.133$	$66.967^{e} \pm 0.418$	$96.200^{e} \pm 1.114$	$2.067^{ab} \pm 0.133$	$1.667^{a} \pm 0.406$	$21.433^{ab} \pm 0.817$	$91.667^{a} \pm 3.028$	$38.013^{a} \pm 3.574$
Kari/ Mtama-1 x Gadam	$70.000^{ab} \pm 2.887$	$58.133^{cd} \pm 0.371$	$67.933^{de} \pm 0.067$	$97.933^{de} \pm 0.481$	$1.200^{ab} \pm 0.115$	$0.933^{a} \pm 0.06$	$23.727^{a} \pm 0.747$	$78.503^{a} \pm 8.735$	$40.313^{a} \pm 1.291$
Serena x Gadam	$61.667^{ab} \pm 1.667$	$63.733^{b} \pm 0.333$	$71.400^{bc} \pm 0.231$	$103.267^{cd} \pm 1.157$	$1.933^{ab} \pm 0.581$	$1.667^{a} \pm 0.133$	$20.667^{b} \pm 0.636$	$89.602^{a} \pm 3.444$	$35.680^{a} \pm 1.288$
Seredo x Gadam	$60.000^{ab} \pm 2.887$	$64.000^{b} \pm 1.249$	$73.400^{ab} \pm 1.102$	$104.667^{bc} \pm 1.622$	$2.067^{ab} \pm 0.406$	$1.600^{a} \pm 0.577$	$21.713^{ab} \pm 0.654$	$94.521^{a} \pm 5.304$	$33.910^{a} \pm 2.612$
P-Value	0.006^{**}	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	0.077^{NS}	0.087^{NS}	0.009^{**}	0.157^{NS}	0.384^{NS}

Means with the same letter within the column are not significantly different. **, *** significant at 1 % and 0.1% respectively; NS: not significant. S.E: standard error; RG: rate of germination; DTH: days to heading; DTF: days to flowering; DTM: days to maturity; NTPP: number of tillers per plant; NRT: number of reproductive tillers; PL: panicle length; WFP: weight of full panicle; W1000S: weight of a thousand seeds; %: percentage

Table 4.3: One way analysis of variance showing means of qualitative variable

Treatments	Panicle compactness		
	Compact	Semi-loose	Loose
	(Means ± S.E)	(Mean ± S.E)	(Mean ± S.E)
Gadam	$2.773^{a} \pm 0.000$	$0.000^{d} \pm 0.000$	$0.000^{d} \pm 0.000$
Serena	$2.773^{a} \pm 0.000$	$0.000^{d} \pm 0.000$	$0.000^{d} \pm 0.000$
Seredo	$0.000^{d} \pm 0.000$	$2.773^{a} \pm 0.000$	$0.000^{d} \pm 0.000$
Kari/Mtama-1	$0.000^{d} \pm 0.000$	$0.000^{d} \pm 0.000$	$2.773^{a} \pm 0.000$
Gadam x Serena	$2.773^{a} \pm 0.000$	$0.000^{d} \pm 0.000$	$0.000^{d} \pm 0.000$
Gadam x Seredo	$1.843^{c} \pm 0.333$	$2.366^{b} \pm 0.333$	$0.000^{d} \pm 0.000$
Gadam x Kari/Mtama-1	$1.782^{c} \pm 0.577$	$0.000^{d} \pm 0.000$	$2.395^{b} \pm 0.577$
Serena x Gadam	$2.773^{a} \pm 0.000$	$0.000^{d} \pm 0.000$	$0.000^{d} \pm 0.000$
Seredo x Gadam	$2.366^{b} \pm 0.333$	$1.843^{c} \pm 0.333$	$0.000^{d} \pm 0.000$
Kari/Mtama-1 x Gadam	$2.483^{b} \pm 0.577$	$0.000^{d} \pm 0.000$	$1.596^{c} \pm 0.577$
P – Value	<0.001***	<0.001***	<0.001***

Key: Means with the same letter within the column are not significantly different. *** Significant at 0.1%; S.E: standard error.

4.1.2 Pearson correlations between quantitative traits

Both positive and negative significant ($p<0.05$) correlations were observed between the quantitative variables that included the rate of germination, weight of full panicle, the weight of a thousand seeds, days to heading, days to flowering, days to maturity, number of tillers per plant, number of reproductive tillers, plant height and panicle length (Table 4.4). The rate of germination correlated negatively with the weight of the full panicle ($r = -0.790$, $p<0.007$). However, it correlated positively with a thousand seed weight ($r = 0.675$, $p<0.032$). Days to heading correlated positively to days to maturity ($r = 0.814$, $p<0.004$) and days to flowering ($r = 0.990$, $p<0.001$) though, it correlated negatively to a thousand seed weight ($r = -0.757$, $p<0.011$). Days to flowering correlated positively with days to maturity ($r = 0.770$, $p<0.009$). The two were found to be negatively correlated with a thousand seed weight at $r = -0.724$, $p<0.018$ and $r = -0.858$, $p<0.001$ respectively. The number of tillers per plant correlated positively with the number of reproductive tillers ($r = 0.980$, $p<0.001$). Weight of full panicle was found to correlate positively with number of tillers per plant ($r = 0.736$, $p<0.015$) and number of reproductive tillers ($r = 0.751$, $p<0.012$). Plant height correlated negatively with both number of reproductive tillers ($r = -0.832$, $p<0.003$) and number of tillers per plant ($r = -0.793$, $p<0.006$). There was a significant and positive correlation between the panicle length and a thousand seed weight ($r = 0.678$, $p<0.031$).

Table 4.4: Pearson correlation between quantitative variable

Variables	Rate of germination	Days to heading	Days to flowering	Days to maturity	Number of tillers/plant	Number of reproductive tillers	Panicle length	Weight of full panicle	Weight of a thousand seeds
Days to heading	-0.564								
Days to flowering	-0.556	**0.990**							
Days to maturity	-0.605	**0.814**	**0.770**						
Number of tillers per plant	-0.436	-0.269	-0.238	-0.398					
Number of reproductive tillers	-0.438	-0.225	-0.197	-0.349	**0.980**				
Panicle length	0.399	-0.426	-0.416	-0.365	-0.066	-0.128			
Weight of full panicle	**-0.790**	0.170	0.176	0.179	**0.736**	**0.751**	0.011		
Weight of a thousand seeds	**0.675**	**-0.757**	**-0.724**	**-0.858**	0.096	0.045	**0.678**	-0.255	
Plant height	0.135	0.282	0.258	0.437	**-0.793**	**-0.832**	0.386	-0.406	0.014

Values in bold are different from 0 with a significance level of p = 0.05.

4.1.3 Estimates of heterosis

Heterosis was tested for each quantitative trait among the crosses (Table 4.5). Significant variations ($p<0.019$) were observed between better parent (BPH) and mid-parent heterosis (MPH) for plant height. The MPH ranged from -19.89% for Gadam x Serena to 16.56% for Kari/Mtama-1 x Gadam. For the same trait, the BPH ranged from -31.27% for the cross Gadam x Serena to -6.38% for Kari/Mtama-1 x Gadam. Days to heading, days to flowering and days to maturity in the crosses between Gadam and other parents had lower days compared to their reciprocals (Table 4.5). This is illustrated in MPH for days to heading whereby, Gadam x Serena, Gadam x Seredo and Gadam x Kari/Mtama-1 recorded -10.93, -9.38 and -25.34 respectively that was lower than their reciprocal crosses. The days to heading varied from -25.34% for Gadam x Kari/Mtama-1 to 5.52% for Serena x Gadam. For the same trait, BPH ranged from -28.90% to -2.05% for the F_1 hybrids respectively. The MPH for days to flowering varied from -22.93% in Gadam x Kari/Mtama-1 to 4.01% in Seredo x Gadam. The BPH varied from -25.64% for Gadam x Kari/Mtama-1 to -3.23% for Kari/Mtama-1 x Gadam for the same trait. The crosses Gadam x Serena, Gadam x Seredo and Gadam x Kari/Mtama-1 recorded negative MPH values of -9.59, -5.10, and -22.93% respectively for days to flowering compared to positive values in their reciprocal crosses. All F_1 hybrids exhibited a negative MPH and BPH for days to maturity. For the same trait, the MPH ranged from -16.16% for Gadam x Serena to -0.48% for Serena x Gadam while BPH ranged from -20.81% for Gadam x Serena to -1.48% for Kari/Mtama-1 x Gadam. All F_1 hybrids exhibited positive values both for MPH and BPH for panicle length. The MPH ranged from 3.51% for Serena x Gadam to 14.42% for Kari/Mtama-1 x Gadam while BPH ranged from 2.16 % for Gadam x Seredo to 11.54% for Kari/Mtama-1 x Gadam. The use of Gadam as the female parent other than Gadam x Serena resulted in a smaller panicle length than in the reciprocal crosses.

Positive MPH was also observed for all the F_1s in the number of reproductive tillers, the number of tillers per plant, panicle length, the weight of full panicle, and a thousand seed weight (Table 4.5). However, the heterosis was not significant except for the weight of full panicle. The lowest MPH values of 3.67% for the number of reproductive tillers and 12.52% for the number of tillers per plant were recorded in Kari/Mtama-1 x Gadam. The cross Gadam x Serena recorded the highest MPH of

50.00 % for the number of reproductive tillers and MPH of 68.22% for the number of tillers per plant. The BPH ranged from -21.72% for Kari/Mtama-1 x Gadam to 60.93% for Gadam x Serena and -30.01% for Kari/Mtama-1 x Gadam to 35.03% for Gadam x Serena for the number of tillers per plant and number of reproductive tillers respectively. Significant variations ($p<0.031$) were recorded between MPH and BPH for the weight of full panicle. MPH ranged from 7.43% for the cross Kari/Mtama-1 x Gadam to 15.89% for Seredo x Gadam while BPH ranged from 3.21% for the cross Kari/Mtama-1 x Gadam to 11.61% for Serena x Gadam. For the weight of a thousand seeds, the MPH ranged from 4.52% for Seredo x Gadam to 17.16% for Gadam x Seredo while BPH ranged from -1.92% for Seredo x Gadam to 12.42% for Gadam x Serena. However, for the same trait, the use of Gadam as a female parent resulted in superior MPH and BPH compared to its reciprocal crosses apart from Gadam x Kari/Mtama-1.

Table 4.5: Mid parent (MPH) and better parent heterosis (BPH) in percentage

Crosses	PH		RG		DTH		DTF		DTM		PL		NTPP		NRT		WFP		W1000S	
	Percent of heterosis over																			
	MPH	BPH	MPH	BPH	MPH	BPH	MPH	BPH	MPH	BPH	MPH	BPH	MPH	BPH	MPH	BPH	MPH	BPH	MPH	BPH
Gadam x Serena	-19.89	-31.27	0	-4.65	-10.93	-17.32	-9.59	-15.21	-16.16	-20.81	6.48	5.25	68.22	60.93	50.00	35.03	7.66	4.83	15.27	12.42
Gadam x Seredo	-0.02	-15.81	-3.90	-11.90	-9.38	-17.88	-5.10	-11.73	-10.27	-17.60	4.09	2.16	47.64	34.83	42.91	25.06	12.39	5.29	17.16	9.95
Gadam x Kari/Mtama-1	4.00	-16.47	-3.53	-4.65	-25.34	-28.90	-22.93	-25.64	-5.01	-5.84	8.76	6.02	56.31	8.74	40.78	-4.95	12.92	8.49	10.41	5.55
Kari/Mtama-1 x Gadam	16.56	-6.38	-1.18	-2.33	-0.91	-5.63	0.29	-3.23	-0.61	-1.48	14.42	11.54	12.52	-21.72	3.67	-30.01	7.43	3.21	11.23	6.34
Serena x Gadam	-2.86	-16.77	-5.13	-11.90	5.52	-2.05	2.15	-4.20	-0.48	-6.01	3.51	2.31	31.81	26.09	38.92	25.06	14.62	11.61	5.82	3.20
Seredo x Gadam	6.97	-9.93	-6.49	-14.29	2.95	-6.71	4.01	-3.25	-2.37	-10.35	5.45	3.49	47.64	34.83	37.16	20.03	15.89	8.56	4.52	-1.92
Mean	0.79	-16.10	-3.37	-8.29	-6.35	-13.08	-5.20	-10.54	-5.82	-10.35	7.12	5.13	44.02	23.95	35.57	11.70	11.82	7.00	10.74	5.92
Standard deviation	12.16	8.53	2.42	4.98	11.38	10.09	10.03	8.92	6.24	7.48	4.04	3.50	19.52	28.02	16.25	24.44	3.54	3.10	5.00	5.05
P-Value	**0.019**		0.055		0.304		0.352		0.281		0.383		0.180		0.074		**0.031**		0.128	

Key: PH: plant height; RG: rate of germination; DTH: days to heading; DTF: days to flowering; DTM: days to maturity; PL: panicle length; NTPP: number of tillers per plant; NRT: number of reproductive tillers; WFP: weight of full panicle; W1000S: weight of a thousand seeds. A t-test was done at 95% confidence interval to test whether heterosis was significantly different than zero. Values in bold are significant at level alpha = 0.05

4.2 Compatibility between Gadam and hard coat tannin sorghum

Grain filling percentage and a hundred seed weight among the crosses, reciprocals and their parents are shown in Table 4.6. Serena x Gadam with 98.399 recorded significantly higher grain filling percentage other than Gadam x Kari/Mtama-1 (98.177). The cross (Serena x Gadam) had no significant difference with parental lines, apart from Kari/Mtama-1. This parental line (Kari/Mtama-1) had the lowest grain filling percentage that was significantly lower than all others, apart from Serena. Crosses and their reciprocals had a significant difference in grain filling percentage (Figure 4.1). The cross Serena x Gadam had the best grain filling percentage that was significantly higher than its reciprocal cross. All crosses scored higher 100 grain seed weight than their parents. However, the difference was not significant at $p>0.05$. The average weight of a hundred seeds for the crosses was 3.323 g higher than that of the parents however, their average grain filling percentage was lower than that of the parents.

Table 4.6: One way analysis of variance showing means of a hundred seed weigh and grain filling percentage.

Treatment	W100S (Mean ± S.E)	GFP (%) (Mean ± S.E)
Gadam	$2.967^{a} \pm 0.080$	$98.519^{a} \pm 0.098$
Serena	$2.943^{a} \pm 0.162$	$98.275^{ab} \pm 0.101$
Seredo	$2.520^{a} \pm 0.170$	$98.485^{a} \pm 0.107$
Kari/Mtama-1	$3.313^{a} \pm 0.270$	$97.914^{bc} \pm 0.163$
Average for parents	2.936	98.298
Gadam x Kari/Mtama-1	$3.410^{a} \pm 0.127$	$98.177^{ab} \pm 0.095$
Gadam x Serena	$3.330^{a} \pm 0.209$	$97.195^{de} \pm 0.107$
Gadam x Seredo	$3.360^{a} \pm 0.344$	$96.789^{e} \pm 0.100$
Kari/ Mtama-1 x Gadam	$3.550^{a} \pm 0.148$	$96.175^{f} \pm 0.114$
Serena x Gadam	$3.190^{a} \pm 0.131$	$98.399^{a} \pm 0.077$
Seredo x Gadam	$3.100^{a} \pm 0.387$	$97.569^{cd} \pm 0.064$
Average for F_1s	3.323	97.384
P-Value	0.135^{NS}	$<0.001^{***}$

Key: W100S = One hundred seed weight and GFP = grain filling percentage. Means with the same letter within the column are not significantly different. *** significant at 0.1%; NS: not significant. S.E: standard error; % percentage.

4.3 Changes in tannin gene expression of hybrid sorghum lines

4.3.1 Tannin gene analysis

Tannin gene (*Tan1*) expression was analyzed from sorghum grain samples taken at flowering stage, milk stage, soft dough stage, hard dough stage and physiological maturity. *Tan1* was expressed in all the stages, but the expression levels varied among the stages. The expression levels were low at the flowering stage (Plate 4.1) compared to other stages, and this increased up to the soft dough stage where it reached the peak and thereafter it started decreasing. The amplification with primer for tannin gene had a band for all plant lines (Plate 4.1), although faint for Gadam x Seredo and Seredo x Gadam. The amplification was faint for parental Gadam and the cross Gadam x Kari/Mtama-1 at the milk stage (Plate 4.2). Tannin gene expression was highest at soft dough stage (Plate 4.3) and the decline was observed at hard dough stage and physiological maturity stage (Plate 4.4 and Plate 4.5). At the physiological maturity stage (Plate 4.5), the primer for tannin gene was not detected in the parental Kari/Mtama-1 and the crosses Gadam x Kari/Mtama-1, Kari/Mtama-1 x Gadam and Gadam x Serena.

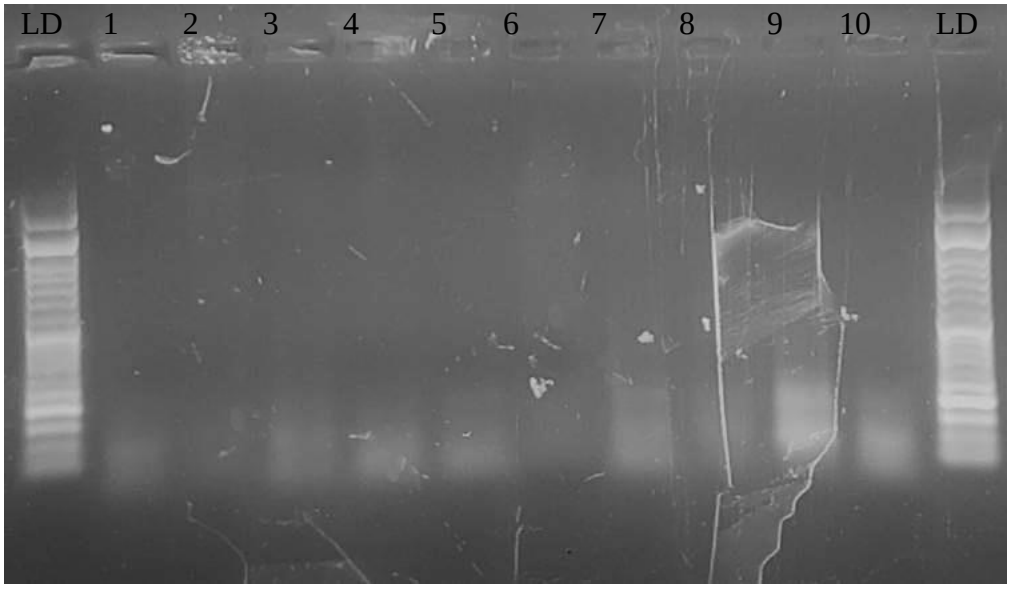

Plate 4.1: RT-PCR products for *Tan1* gene of sorghum hybrid lines and their parents at flowering stage. Numbers 1-10 refer to banding after amplification with primers specific for tannin gene in sorghum samples as follows; 1. Gadam x Serena; 2. Gadam x Seredo; 3. Gadam; 4. Serena; 5. Seredo; 6. Seredo x Gadam; 7. Kari/Mtama-1 x Gadam; 8. Kari/Mtama-1; 9. Gadam x Kari/Mtama-1; 10. Serena x Gadam. LD in extreme right and left banding refer to DNA ladder.

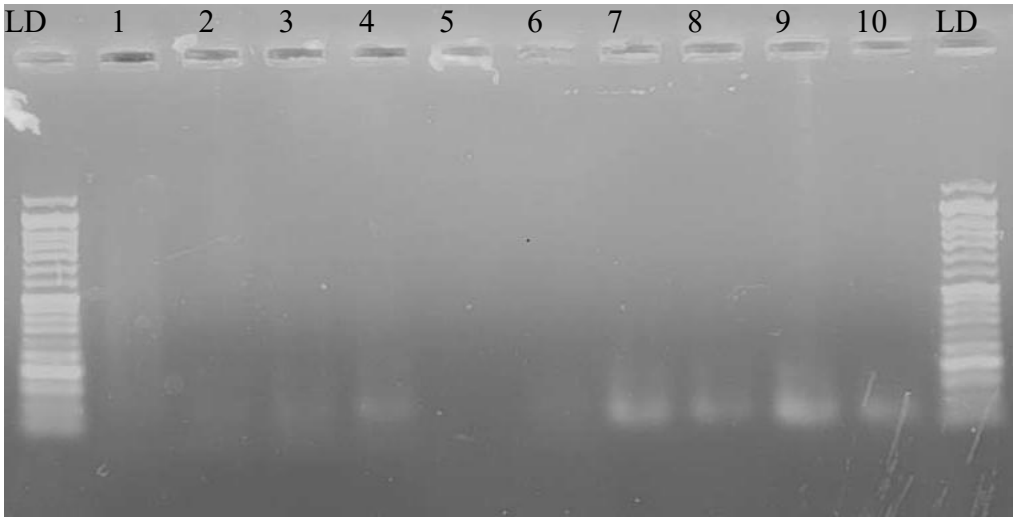

Plate 4.2: RT-PCR products for *Tan1* gene of sorghum hybrid lines and their parents at milk stage**.** Numbers 1-10 refer to banding after amplification with primers specific for tannin gene in sorghum samples as follows; 1. Seredo; 2. Gadam; 3. Kari/Mtama-1; 4. Gadam x Serena; 5. Gadam x Kari/Mtama-1; 6. Kari/Mtama-1 x Gadam; 7. Gadam x Seredo; 8. Serena; 9. Seredo x Gadam; 10. Serena x Gadam. LD in extreme right and left banding refer to DNA ladder.

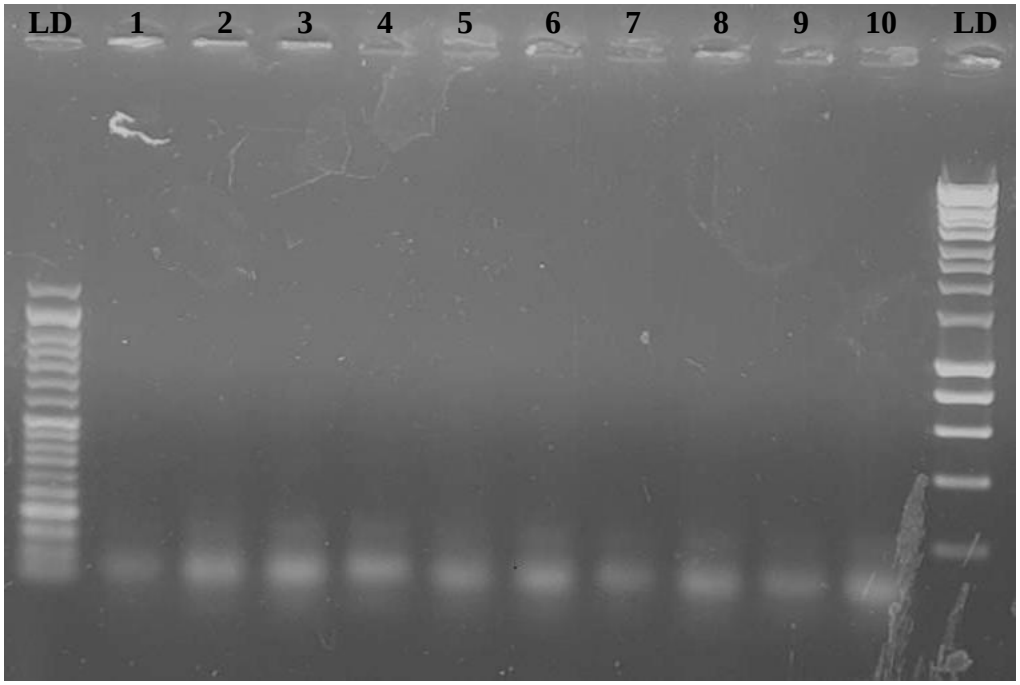

Plate 4.3: RT-PCR products for *Tan1* gene of sorghum hybrid lines and their parents at soft dough stage. Numbers 1-10 refer to banding after amplification with primers specific for tannin gene in sorghum samples as follows; 1. Serena; 2. Seredo; 3. Kari/Mtama-1; 4. Seredo x Gadam; 5. Kari/Mtama-1 x Gadam; 6. Serena x Gadam; 7. Gadam x Kari/Mtama-1; 8. Gadam x Serena; 9. Gadam x Seredo; 10. Gadam. LD in extreme right and left banding refer to DNA ladder.

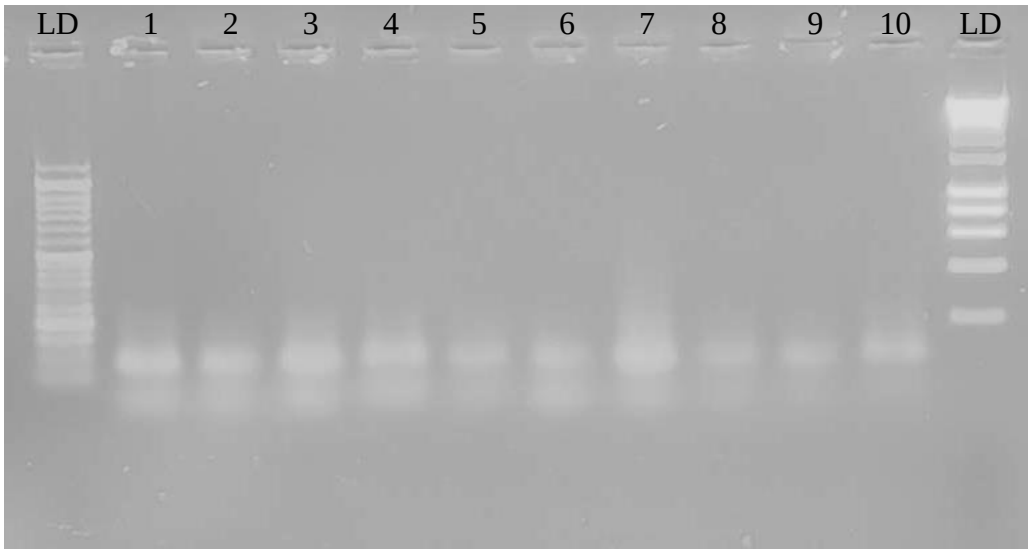

Plate 4.4: RT-PCR products for *Tan1* gene of sorghum hybrid lines and their parents at hard dough stage**.** Numbers 1-10 refer to banding after amplification with primers specific for tannin gene in sorghum samples as follows; 1. Serena; 2. Seredo; 3. Gadam x Kari/Mtama-1; 4. Kari/Mtama-1 x Gadam; 5. Gadam; 6. Serena x Gadam; 7. Gadam x Seredo; 8. Kari/Mtama-1; 9. Gadam x Serena; 10. Seredo x Gadam. Gadam. LD in extreme right and left banding refer to DNA ladder.

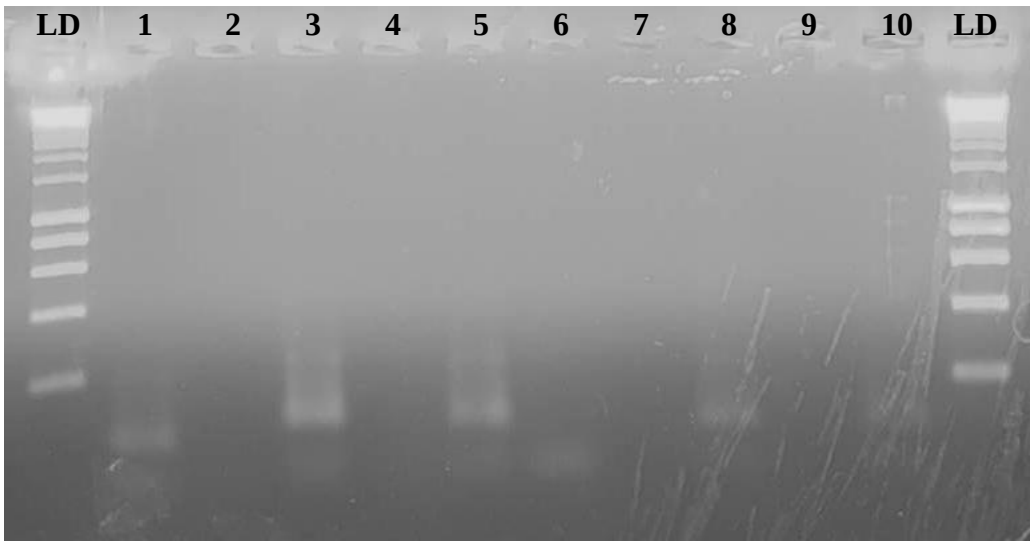

Plate 4.5: RT-PCR products for *Tan1* gene of sorghum hybrid lines and their parents at physiological maturity stage**.** Numbers 1-10 refer to banding after amplification with primers specific for tannin gene in sorghum samples as follows; 1. Serena; 2. Gadam x Serena; 3. Gadam; 4. Kari/Mtama-1 x Gadam; 5. Seredo; 6. Seredo x Gadam; 7. Kari/Mtama-1; 8. Serena x Gadam; 9. Gadam x Kari/Mtama-1; 10. Gadam x Seredo. LD in extreme right and left banding refer to DNA ladder.

4.3.2 Tannin content analysis

Significant variations ($p<0.001$) in the level of tannin were observed at milk stage, soft dough stage, hard dough stage and physiological maturity stage among the crosses and their reciprocals (Table 4.7). The highest tannin levels were recorded at the soft dough

stage with the cross Seredo x Gadam recording the highest amount (1.223) though, it did not differ significantly from all other F_1 hybrids except the cross Gadam x Kari/Mtama-1, Kari/Mtama-1 x Gadam and Gadam x Serena. At this stage also, the cross Gadam x Seredo and its reciprocal differed significantly from the parental Seredo that recorded tannin levels of 1.785. It is noteworthy that the tannin levels started decreasing at the hard dough stage to the physiological stage among the F_1 hybrids and their parents (Figure 4.6). At physiological maturity stage, the crosses Gadam x Kari/Mtama-1, Gadam x Serena and Kari/Mtama-1 x Gadam recorded the lowest tannin levels of 0.177, 0.281 and 0.106 respectively. This differed significantly from all other F_1 hybrids and the parents except Kari/Mtama-1 that had 0.034 (Table 4.7).

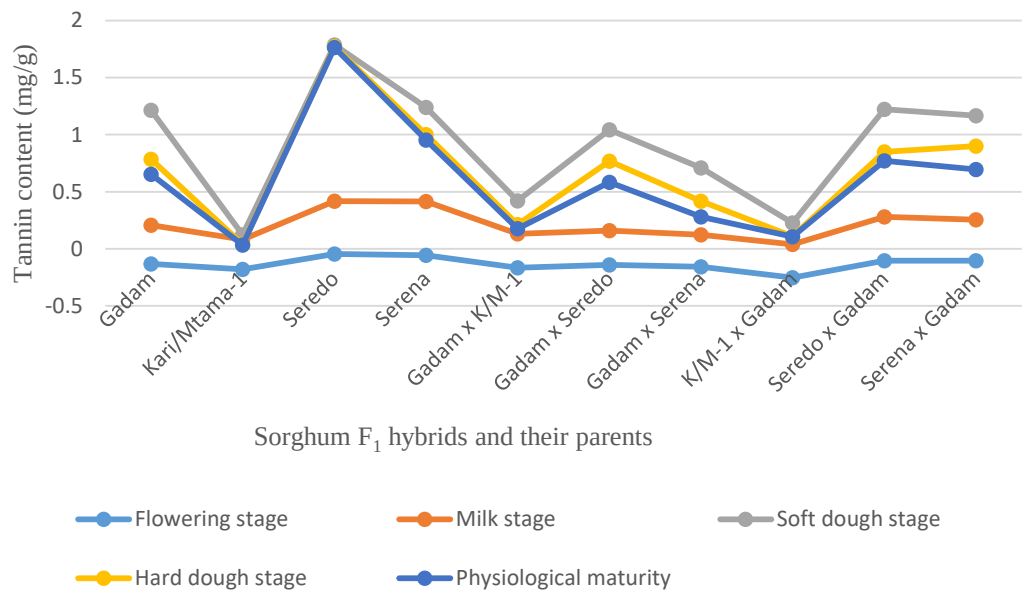

Figure 4.6: Tannin content across the five stages

Table 4.7: One way analysis of variance showing means of tannin content across the five stages

Treatments	FS (Mean ± S.E)	MS (Mean ± S.E)	SDS (Mean ± S.E)	HDS (Mean ± S.E)	PS (Mean ± S.E)
Gadam	$-0.131^{a} \pm 0.003$	$0.206^{abc} \pm 0.040$	$1.214^{b} \pm 0.034$	$0.784^{bc} \pm 0.128$	$0.653^{c} \pm 0.064$
Kari/Mtama-1	$-0.181^{a} \pm 0.029$	$0.081^{c} \pm 0.017$	$0.123^{c} \pm 0.027$	$0.038^{d} \pm 0.011$	$0.034^{d} \pm 0.000$
Seredo	$-0.044^{a} \pm 0.060$	$0.418^{a} \pm 0.111$	$1.785^{a} \pm 0.097$	$1.771^{a} \pm 0.047$	$1.763^{a} \pm 0.130$
Serena	$-0.057^{a} \pm 0.081$	$0.414^{ab} \pm 0.112$	$1.238^{b} \pm 0.066$	$1.000^{b} \pm 0.122$	$0.953^{b} \pm 0.031$
Gadam x Kari/Mtama-1	$-0.167^{a} \pm 0.024$	$0.131^{abc} \pm 0.004$	$0.419^{c} \pm 0.062$	$0.213^{d} \pm 0.047$	$0.177^{d} \pm 0.000$
Gadam x Seredo	$-0.141^{a} \pm 0.048$	$0.160^{abc} \pm 0.033$	$1.042^{b} \pm 0.124$	$0.768^{bc} \pm 0.114$	$0.582^{c} \pm 0.029$
Gadam x Serena	$-0.157^{a} \pm 0.009$	$0.123^{bc} \pm 0.007$	$0.709^{c} \pm 0.100$	$0.417^{cd} \pm 0.111$	$0.281^{d} \pm 0.041$
Kari/Mtama-1 x Gadam	$-0.254^{a} \pm 0.040$	$0.039^{c} \pm 0.015$	$0.228^{c} \pm 0.053$	$0.109^{d} \pm 0.025$	$0.106^{d} \pm 0.036$
Seredo x Gadam	$-0.104^{a} \pm 0.042$	$0.281^{abc} \pm 0.041$	$1.223^{b} \pm 0.223$	$0.850^{bc} \pm 0.175$	$0.771^{bc} \pm 0.034$
Serena x Gadam	$-0.104^{a} \pm 0.023$	$0.256^{abc} \pm 0.069$	$1.166^{b} \pm 0.025$	$0.901^{bc} \pm 0.145$	$0.696^{bc} \pm 0.081$
P-Value	0.078^{NS}	0.0013^{**}	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$

Key: Means with the same letter within the column are not significantly different. ** Significant at 1%; *** significant at 0.1%; NS: not significant. S.E= standard error%: percentage. FS: flowering stage; MS: milk stage; SDS: soft dough stage; HDS: hard dough stage; PS: physiological stage.

4.4 Nutritional levels and tannin content of sorghum F_1 hybrids and their parents

The crude protein, fat content, crude fibre, ash content, total carbohydrates, moisture and tannin content of F_1 hybrids is shown in Table 4.8. Crude protein ranged from 5.323% to 10.390%, which was significantly different between the crosses, reciprocals and the parents at $p<0.001$. The lowest value (5.323 %) was recorded from the parent Seredo. The reciprocal cross Kari/Mtama-1 x Gadam gave the highest value of 10.390%, which was significantly different from Gadam x Kari/Mtama-1 that recorded a value of 9.770%. Parents, crosses and reciprocals also differed significantly ($p<0.001$) for fat, crude fibre, ash and carbohydrates with values ranging from 1.691% to 2.299%, 2.230% to 3.520%, 1.215% to 1.360%, and 76.790% to 85.677% respectively. The cross Kari/Mtama-1 x Gadam recorded the highest fat content value of 2.299%, although not significantly different from Gadam x Kari/Mtama-1 that had a value of 2.159%. The cross Gadam x Serena recorded the highest value of 3.433% for crude fibre and this differed significantly from Serena x Gadam (2.953%). The highest ash content value of 1.619% was recorded in the cross Gadam x Kari/Mtama-1, which was not significantly different from Kari/Mtama-1 x Gadam that recorded a value of 1.333%. The cross Seredo x Gadam recorded the highest value of 84.503% for total carbohydrates content and this was significantly different from all F_1 hybrids except from the cross Serena x Gadam. The F_1s scored carbohydrate content less than that of Gadam except for Serena x Gadam and Seredo x Gadam. All crosses showed a significant difference with their reciprocals. Moisture content in the F_1 hybrids and the parents differed significantly at $P<0.01$. Kari/Mtama-1 x Gadam recorded the highest value of 8.600% which was significantly different ($P<0.01$) from all other F_1 hybrids apart from Gadam x Kari/Mtama-1 that recorded a moisture content value of 8.100. Tannin content varied significantly ($p<0.001$) among the F_1 hybrids and their parents. The detectable tannin content ranged from 0.034 mg/g for Kari/Mtama-1 to 1.763 mg/g for Seredo. The F_1 hybrids Seredo x Gadam and Serena x Gadam recorded the highest tannin content value of 0.771 and 0.696 respectively. The crosses had significantly lower or equal tannin content to their parents apart from Gadam x Seredo and its reciprocal.

Pearson correlations of proximate compositions of sorghum was done as shown in Table 4.9. A significant positive correlation was observed between crude protein and

moisture content ($r = 0.661$, $p<0.038$), crude fibre and carbohydrates ($r = 0.746$, $p<0.013$), crude fibre and tannin ($r = 0.664$, $p<0.036$) and between tannin content and carbohydrates ($r= 0.900$, $p<0.001$). Tannin content correlated negatively with crude protein, fat and moisture content. The correlation was only significant for crude protein ($r = -0.886$, $p<0.001$) and moisture content ($r = -0.740$, $p<0.015$). A negative correlation was also observed between crude protein and carbohydrates ($r = -0.953$, $p<0.001$), moisture content and crude fibre ($r = 0.938$, $p<0.001$), and moisture content and carbohydrates ($r = -0.853$, $p<0.002$).

Table 4.8: One way analysis of variance showing nutritional compositions and tannin of hybrids and their parents.

Treatments / Material	Crude protein (%) (Mean ± S.E)	Fat (%) (Mean ± S.E)	Crude fibre (%) (Mean ± S.E)	Ash (%) (Mean ± S.E)	Moisture (%) (Mean ± S.E)	Carbohydrate (%) (Mean ± S.E)	Tannin (mg/g) (Mean ± S.E)
Gadam	$7.660^{e} \pm 0.127$	$2.050^{ab} \pm 0.066$	$3.180^{ab} \pm 0.092$	$1.318^{b} \pm 0.040$	$7.200^{c} \pm 0.100$	$81.772^{c} \pm 0.171$	$0.653^{c} \pm 0.064$
Kari/Mtama-1	$10.133^{ab} \pm 0.073$	$2.197^{ab} \pm 0.046$	$2.230^{e} \pm 0.058$	$1.215^{b} \pm 0.028$	$9.667^{a} \pm 0.333$	$76.788^{f} \pm 0.236$	$0.034^{d} \pm 0.000$
Serena	$5.900^{g} \pm 0.000$	$1.931^{bc} \pm 0.058$	$3.150^{ab} \pm 0.100$	$1.329^{ab} \pm 0.081$	$5.993^{de} \pm 0.007$	$84.847^{a} \pm 0.047$	$0.953^{b} \pm 0.031$
Seredo	$5.323^{h} \pm 0.073$	$2.114^{ab} \pm 0.070$	$3.520^{a} \pm 0.111$	$1.453^{ab} \pm 0.032$	$5.433^{e} \pm 0.088$	$85.677^{a} \pm 0.165$	$1.763^{a} \pm 0.130$
Gadam x Kari/Mtama-1	$9.770^{bc} \pm 0.070$	$2.159^{ab} \pm 0.082$	$2.663^{cd} \pm 0.100$	$1.619^{a} \pm 0.056$	$8.100^{b} \pm 0.100$	$78.352^{e} \pm 0.162$	$0.177^{d} \pm 0.000$
Gadam x Serena	$9.557^{cd} \pm 0.073$	$2.034^{ab} \pm 0.046$	$3.433^{a} \pm 0.083$	$1.227^{b} \pm 0.014$	$7.103^{c} \pm 0.103$	$80.079^{de} \pm 0.188$	$0.281^{d} \pm 0.041$
Gadam x Seredo	$9.227^{d} \pm 0.037$	$2.116^{ab} \pm 0.034$	$3.197^{ab} \pm 0.075$	$1.352^{ab} \pm 0.084$	$6.427^{d} \pm 0.073$	$80.878^{d} \pm 0.152$	$0.582^{c} \pm 0.029$
Kari/Mtama-1 x Gadam	$10.390^{a} \pm 0.110$	$2.299^{a} \pm 0.104$	$2.493^{de} \pm 0.063$	$1.333^{ab} \pm 0.071$	$8.600^{b} \pm 0.1h00$	$77.378^{f} \pm 0.244$	$0.106^{d} \pm 0.036$
Serena x Gadam	$6.443^{f} \pm 0.064$	$2.167^{ab} \pm 0.028$	$2.953^{bc} \pm 0.055$	$1.349^{ab} \pm 0.058$	$6.413^{d} \pm 0.049$	$83.628^{b} \pm 0.087$	$0.696^{bc} \pm 0.081$
Seredo x Gadam	$6.343^{f} \pm 0.124$	$1.691^{c} \pm 0.087$	$2.280^{de} \pm 0.087$	$1.360^{ab} \pm 0.070$	$6.103^{d} \pm 0.058$	$84.503^{b} \pm 0.188$	$0.771^{bc} \pm 0.034$
P – Value	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	0.005^{**}	$<0.001^{***}$	$<0.001^{***}$

Key: Means with the same letter within the column are not significantly different. ** Significant at 1%; *** significant at 0.1%. S.E= standard error; mg/m: milligram per gram; %: percentage.

Table 4.9: Pearson correlation of proximate compositions of sorghum

Variables	CP	F	CF	A	M	CHO
F	0.553					
CF	-0.547	0.121				
A	-0.140	0.065	0.146			
M	**0.661**	0.133	**-0.938**	-0.339		
CHO	**-0.953**	-0.470	**0.746**	0.196	**-0.853**	
T	**-0.886**	-0.344	**0.664**	0.232	**-0.740**	**0.900**

Key: Values in bold are different from 0 with a significance of p = 0.05. CP: crude protein; F: fat; CF: crude fibre; A: ash; M: moisture; CHO: carbohydrates; T: tannin

CHAPTER FIVE

DISCUSSION

5.1 Grain yield attribute traits of hybrid sorghum lines

There was a significant ($p<0.05$) difference displayed among the F_1s in height at week 1 to 6 (Table 4.1). This indicates different growth rates during the vegetative stages of plant growth. The sorghum plant is characterized by three growth stages (GS) namely, GS I or vegetative, GS II or reproductive structure formation, and GS III or grain filling stage (Tom Gerik *et al*., 2003). Among the parents, Serena and Kari/Mtama-1 had significantly faster vegetative growth than Gadam sorghum. In week 4 – 6, the cross Gadam x Serena had a significantly lower vegetative growth rate than all other crosses apart from its reciprocal. The vegetative growth stage in sorghum plants is under genetic control and it is sensitive to daylight length (Takamizo *et al*., 2012; ICRISAT,1984). The F_1 hybrids Kari/Mtama-1 x Gadam, Gadam x Kari/Mtama-1, Seredo x Gadam and Gadam x Seredo had larger panicle length compared to their parental genotypes. This can be attributed to heterosis for this trait. Significant and positive heterosis has been reported in panicle length (Jadhav & Deshmukh, 2017) which is in line with the observation in this study.

The F_1 hybrids showed significant days to flowering, days to heading, and days to maturity at $p<0.001$ (Table 4.2). The floral initiation and maturity in sorghum are under facultative gene control at the loci Ma_1 to Ma_4, Ma_5, and Ma_6 (Quinby, 1966; Quinby, 1967; Rooney & Aydin, 1999). This explains the difference in flowering and heading time among hybrid lines under study. The cross Gadam x Kari/Mtama-1 recorded significantly lower values for days to heading and days to flowering compared to the parental genotype Gadam and Kari/Mtama-1. This shows that crossing influenced earliness in heading and flowering. This is supported by Mohammed *et al*. (2015) who observed earliness for anthesis in sorghum hybrids compared to their parents. Early flowering was recorded in the cross Gadam x Kari/Mtama-1 compared to Gadam x Serena. However, the days to maturity for the two crosses were significantly different. The observed difference can be attributed to differences in sensitivity in the flowering phase. Flowering time is both under environmental and genetic control (Murphy *et al*., 2011). The crosses Gadam x Seredo, Gadam x Kari/Mtama-1 and their reciprocals exhibited high variability in panicle compactness compared to their parents. This

suggests that both non-additive and additive gene action control this trait. Similar results have been reported by Karanja *et al*. (2014); Mohammed *et al*. (2015, 2018) in sorghum.

The results in crosses where Gadam was the male parent gave a more domineering influence, especially in plant height, days to heading, days to flowering, length of the panicle, and days to maturity. This is evidence that besides the direct genetic effects, the maternal effects were also involved in the inheritance of these characters. Cytoplasmic effects in crossing have been reported in many crops including sorghum, rice and maize. In sorghum, it influences morphological and agronomic traits such as days to flowering and plant height (Mohammed *et al*., 2015). In rice breeding, cytoplasmic effects have been reported for various traits including plant regeneration rates (Chu & Croughan, 1989), milling quality traits (Shi & Zhu, 1995), crossability (IRRI., 1994), number of panicles (Wu, 1968), heterosis (Young & Virmani, 1990), filled grain ratio (Tao *et al*., 2011) and grain weight (Chandraratna & Sakai, 1960; Soomrith *et al*., 1979). In maize breeding, it has been reported for tassel length, leaf area, plant height and ear height (Calugar & Rotar, 2016). This supports differences in agronomic traits between crosses and their reciprocals in this study.

The degree of correlation among the quantitative characters studied is key in the selection of useful characters towards improving the productivity of sorghum. For example, a positive and significant correlation was observed between the days to heading and days to maturity and days to flowering indicating that earliness in anthesis and maturity in sorghum can be attained through selection for fewer days to heading. A similar positive correlation has been reported by Rutayisire *et al*. (2020) in sorghum. A positive and significant correlation observed between the number of tillers per plant with the number of reproductive tillers and weight of full panicle indicates that the number of reproductive tillers and weight of full panicle in sorghum can be increased through selection for more number of tillers per plant. Also, a positive correlation between the weight of a thousand seeds and the panicle length indicates that seed weight in sorghum can be attained through selection for sorghum lines with higher

panicle length. These traits are considered as the main characters towards improving rice and sorghum grain yield (Ali *et al*., 2011; Reddy *et al*., 2013; Ratna *et al*., 2015).

The negative correlation between plant height with the number of tillers per plant and reproductive tillers indicates that increased branching in sorghum is associated with dwarfism. Richards (1988) reported a negative correlation between plant height and tiller number. However, the association between tillering with other leaf and plant size traits has not been fully elucidated. Amare *et al*. (2015) while working on variability for yield, yield-related attributes and relationship among sorghum traits, reported that plant height correlated negatively with days to heading and days to flowering although the correlations were not significant. Increased number of tillers especially in rice has been reported to lead to decreased height, although, the association is not strong, as the total number of tillers and final plant height is coordinated by both hormones, genetic and environmental factors (Liao *et al*., 2019).

Different variations for better parent heterosis and mid parent heterosis were recorded for the traits under study among the F_1 hybrids. In the current study, crosses Gadam x Serena, Gadam x Seredo and reciprocal cross Serena x Gadam exhibited negative MPH and BPH for plant height which resulted in shorter hybrids. The shortness in plant height among F_1 hybrids suggests that there could be a dominance or additive gene for dwarfness. Quinby & Karper (1954) while working on sorghum reported that, tallness is a partially dominant gene and that four recessive, non-linked brachytic dwarfing genes control plant height. This is aligned with the works of Shukla *et al*. (2017) who reported sorghum hybrids with shorter height than their parental genotypes. Negative BPH in days to heading, maturity and flowering in sorghum is an indication of early anthesis and maturity in sorghum hybrids than their parental genotypes. Negative heterosis for days to flowering and maturity has been reported in sorghum (Mindaye *et al*., 2016; Chikuta *et al*., 2017; Crozier *et al*., 2020). Positive MPH recorded for the weight of full panicle, the number of reproductive tillers, the weight of a thousand seeds, and the number of tillers per plant across all F_1 hybrids is an indication of possible yield improvement of Gadam sorghum through hybridization.

Sorghum yield improvement using hybrid has been reported in China and India (Ashok *et al*., 2019).

In this research, the difference between the performance of crosses and their reciprocals was observed. For example, the use of Gadam as a female parent gave a better 1000 grain weight in all the crosses apart from Gadam x Kari/Mtama-1. Grain filling was better where Gadam was the male parent apart from Kari/Mtama-1 x Gadam where its reciprocal performed better indicating paternal gene influence. Therefore, in sorghum hybrid seed production, the choice of maternal or paternal parent is significant.

5.2 Compatibility between Gadam and hard coat tannin sorghum

Compatibility is the highest degree of fitness between the male and female gametes that results in fertilization in the flowering plants (Puurtinen *et al*., 2005). Among the F_1 hybrids, Serena x Gadam recorded the highest values for grain filling percentage. This is an exhibition of the superiority of the cross compared to other crosses. The crosses Gadam x Seredo, Seredo x Gadam, Gadam x Serena and Kari/Mtama-1 x Gadam recorded significantly ($p<0.001$) lower grain filling percentages compared to their parents indicating incompatibility in the crosses for this trait. Wide compatibility is a desired trait in crossing (Ji *et al*., 2005; Priyadarshi *et al*., 2018). Low grain filling has been observed in crops such as rice due to F_1 sterility that is caused by parental incompatibility (Mizuta *et al*., 2010) and this limits the breeding of new lines. Sub-functionalization of duplicated genes or loss of reciprocal genes between isolated populations has been reported to be the major cause of parental incompatibility or genetic incompatibility among crop species (Bikard *et al*., 2009; Mizuta *et al*., 2010; Yamagata *et al*., 2010) have identified three examples of hybrid incompatibility in Arabidopsis and rice crops occasioned by reciprocal gene loss. Incompatibility in rice has been reduced by the inclusion of the S^{5n} gene for wide compatibility (Yang *et al*., 2009). The inclusion of a similar gene in sorghum could lead to a more positive grain filling percentage in F_1 hybrids. Also, post-zygotic barriers such as abnormal growth of endosperm or embryo abortion have been reported to lead to inter-specific incompatibility (Tonosaki *et al*., 2016). Endosperm abnormality has been thought to cause embryo abortion. Embryo abortion depends on the direction of hybridization, cross combination of species and parental ploidy levels (Kinoshita, 2007). Embryo rescue techniques such as ovule, ovary and embryo have been developed to overcome

the post-zygotic barriers (Van *et al*., 1991). Hybrid sterility has been reported to be caused by reduced chromosome pairing that occurs during meiosis (Tonosaki *et al*., 2016). This barrier therefore, can be restored through somatic or zygotic chromosome doubling through cell cycle disruption by the use of antimitotic agents including oryzalin, colchicine and trifluralin (Dhooghe *et al*., 2011). This emphasizes the need to have Gadam sorghum with a wide compatibility gene for use in crossing with a wide range of germplasm for increased yield.

5.3 Tannin gene expression in hybrid sorghum lines

Tannin in F_1 hybrids was observed to decline with the maturity of the grains (Plate 4.1 to 4.5). Studies on the mechanism of tannin gene expression are necessary for determining the stage of the hybrid lines seed maturity when tannin starts to decline. Changes in tannin levels influence sorghum nutritional levels and birds damage (Gilani *et al*., 2012; Xie *et al*., 2019). Studies of tannin sorghum using association analysis, meta quantitative trait locus (QTL) analysis, and a functional complementation test have revealed that the biosynthesis of tannin in sorghum is controlled by nucleotide polymorphisms in the *Tan1* gene that codes a WD40 protein (Wu *et al*., 2012). In the current study, RNA was used to track the expression levels of *Tan1* gene to identify the stage at which it is at the maximum. Expression levels of Tan_1 gene were low at the flowering stage and this increased up to the soft dough stage where it reached the peak and decreased in later stages. The implication is that the seeds are more vulnerable to birds' damage at and after the soft dough stage (Mofokeng & Shargie, 2016; Ruelle & Bruggers, 1982). The findings of this study are in agreement with those of Wu *et al*. (2012) who after analyzing tannin gene expression in sorghum tissues that included immature panicle before heading, mature leaf from a flowering plant, seed coat 15 days after pollination and seed coat 30 days after pollination, reported that the expression levels of *Tan1* gene increased during panicle and seed coat development. In this current study, analysis of RNA levels tallied with tannin content levels. When tannin content was measured quantitatively using the modified vanillin-HCl test, the maximum tannin levels were observed at the soft dough stage and declined in later stages indicating synchrony between RNA levels and tannin levels. This is in harmony with the observation by William *et al*. (1981) who reported that tannin content in grain sorghum is high at the soft dough stage and that it decreases as the seed matures. Bird damage is the major problem facing sorghum farming in Kenya (Kagwiria *et al*.,

2019). Sorghum is likely to be more exposed to birds' damage at the point when tannin starts declining therefore indicating that manual bird control methods like scaring need to be introduced.

5.4 Nutritional levels and tannin content of hybrid sorghum lines

Sorghum is a major source of proteins, carbohydrates, fats and crude fibre, necessary for human development and health (Duodu *et al*., 2003; Jakobek, 2015). Determining the nutritional and anti-nutritional properties of sorghum hybrid lines would aid in the selection of lines with moderate tannin levels with high food value to be used in sorghum hybrid seed production programs. In the current study, there were significant variations ($p<0.001$) both in protein content, crude fibre, fat, ash, carbohydrates and tannin content among the F_1 hybrids. The significant variations exhibited by F_1 hybrids in ash content suggest different amounts of mineral content in F_1 hybrids. Ash content indicates the total amount of mineral content found in a sample (Jimoh & Abdullahi, 2017). Among the F_1 hybrids, the cross Gadam x Kari/Mtama-1 recorded the highest ash content value of 1.619% while the lowest ash content value of 1.227% was recorded in the cross Gadam x Serena. The differences in ash content among the F_1 hybrid sorghum could be attributed to the genotype as well as the amount and nature of ions available in the soil where the plant is growing (Akinsola, 1993). Different values for ash content among sorghum varieties has been reported by various researchers for instance: 1.90% to 1.97% (Gassem & Osman, 2003), 1.43% to 1.61% (Chung *et al*., 2011), 1.01% to 1.56% (Abu *et al*., 2001), 0.80% to 2.50% (Moharram & Youssef, 1995) and 0.99% to 1.71% (Pontieri *et al*., 2012). In the current study, the fat content ranged from 1.691% in the reciprocal cross Seredo x Gadam to 2.299% in the cross Kari/ Mtama- x Gadam. Results in this study agree with the works of Okoh *et al*. (1982) who reported that fat content ranged from 1.38 to 3.70. However, the findings of this study differ from the findings of Buffo *et al*. (1998), while working on the proximate analysis of sorghum varieties, documented fat content range of 3.44 to 4.90%.

Crude fibre is a major portion of carbohydrates that cannot easily be digested. Among the F_1 hybrids, the highest crude fibre value of 3.433% was recorded in the cross Gadam x Serena while the lowest value of 2.493% was recorded in the reciprocal cross Kari/Mtama-1 x Gadam. Crude fibre content varying from 1.0% to 3.4% (Jambunathan

et al., 1981), and 2.166% to 8.587% (Tasie & Gebreyes, 2020) have been reported. The differences in crude fibre in this study and other studies could be attributed to the environment where the crop was grown as well as the type of genotype or the method used in the analysis. Crude fibre is capable of holding oil and water (Elleuch *et al*., 2011) thus varieties with high crude fibre content can be useful in yield enhancement and also in making products that need hydration. However, these varieties may not have high food value since high crude fibre binds minerals together reducing their efficiency for absorption and sometimes leading to minerals deficiency as well as imbalances (Oliveira *et al*., 2009).

In the current study, protein content among the F_1 hybrids ranged from 6.343% in Seredo x Gadam to 10.390% in Kari/Mtama-1 x Gadam. The variability in protein content among the F_1 hybrids could be attributed to the genetic make-up of the hybrids as well as their macromolecules composition especially the tannins. The findings of this study on protein content are in agreement with those of Badigannavar *et al*. (2016); Jambunathan *et al*. (1981) and Pontieri *et al*. (2012) who reported protein content varying from 5.25 to 14.53%, 4.4 to 21.1% and 7.44 to 9.66% respectively. Other researchers have documented sorghum protein content range of 10.3% to 14.9% (Johnson *et al*., 2010), 9.06% to 18.58% (Hamad, 2006), 11.23% to 13.42% (Chung *et al*., 2011), and 9.06% to 18.58% (Okrah, 2008). Differences in the amount of protein content among the studies are due to the differences in the genotype and environment (Deosthale *et al*., 1972).

In cereals, moisture content of less than 15% ensures long-term storage of the grains without loss of quality or viability that might occur as a result of molding caused by high moisture content (Onimawo *et al*., 2003). In this study, cross Kari/Matam-1 x Gadam recorded the highest moisture content of 8.600% while the cross Seredo x Gadam recorded the lowest moisture content of 6.103%. This suggests that moisture content in sorghum can be attributed to genotype. Carbohydrates content for hybrids had a significant difference with their reciprocals. Seredo x Gadam recorded carbohydrates content significantly different from all F_1 hybrids except from the cross Serena x Gadam. The choice of male or female parent has been reported to influence

the levels of carbohydrate for the materials under study. Reciprocal combining ability for days to flowering, 100 seed weight and plant height has been reported in sorghum (Mohammed *et al*., 2015). This is an indication that carbohydrate content in sorghum can also be under a similar maternal influence.

The crosses Gadam x Kari/Mtama-1, Gadam x Serena, and Kari/Mtama-1 x Gadam recorded significantly lower tannin content compared to the standard line, Gadam (Table 4.8). This is an indication that tannin can be down regulated through hybridization. Tannin is one of the major antinutritional factors available in sorghum (Hariprasanna *et al*., 2015). It has been reported to bind proteins together and inhibit many enzymes in *in vitro* assays reducing their efficiency of utilization and digestion (Emmambux & Taylor, 2003; Frazier *et al*., 2010). Besides, it makes the sorghum grains remain bitter thus reducing the sensory of many food products (Coelho *et al*., 2007; Tasie & Gebreyes, 2020). This differed from the findings of Omondi *et al*. (2012) who reported tannin levels of 0.81% C.E, 0.03% C.E, 2.22% C.E and 1.2% C.E in Gadam, Kari/Mtama-1, Seredo and Serena respectively. The variations observed between the results could be due to differences in the method used in the analysis. However, tannin content range of 0.106 mg/g to 0.771 mg/g observed among the F_1 hybrids is within the findings by Moharram & Youssef (1995), who reported sorghum tannin content range of 0.02 g/100g to 2.69 g/100g.

Reduced tannin in hybrid grains is desirable since it affects protein availability. A significant negative correlation was observed between tannin and proteins (Table 4.8). Sorghum proteins are less digestible compared to those of other cereal crops like maize (Xiong *et al*., 2019). This poor digestibility is due to phenolic compounds mainly tannins that are found in most sorghum varieties (Duodu *et al*., 2003). Tannins have been thought to interact with proteins through hydrophobic interactions and hydrogen bonding and it has been reported to bind and precipitate most proteins, at least 12 times their own weight of proteins (Butler *et al*., 2011; Jakobek, 2015).

5.5 Conclusion

In conclusion, a better performance was observed in some traits where hybrid outperformed parents. One of them was cross Gadam x Serena that had short stature

and also matured early compared to their parents. Both the mid parent and better parent heterosis were observed, however, they did not differ significantly in most quantitative traits assessed except for the plant height and weight of full panicle. All the F_1 hybrids have desirable negative BPH heterosis for plant height. Traits such as days to heading, flowering and maturity, and a thousand grain weight were significant between hybrid and their reciprocals. This is an indication of a maternal influence on these traits. Days to heading, flowering, and maturity correlate positively.

In the current study, wide compatibility among the sorghum varieties assessed was not noticeable. Tannin gene is at the pick of expression at the soft dough stage of grain formation. This is indicated by the maximum presence of RNA. Tannin content was highest at the soft dough stage. The decline of the level of RNA associated with tannin gene and also tannin content, observed at the soft dough stage, is desired because it is an indication of improved nutrient availability as sorghum matured.

This study also confirmed that the choice of maternal and paternal parent influence crude proteins, crude fibre and carbohydrates. Tannin is highly influenced by hybridization. This was demonstrated by significantly lower values recorded in all the F_1 hybrids compared to their high tannin sorghum varieties, Serena and Seredo. Among the F_1 hybrids, Gadam x Serena has low tannin content. Carbohydrates, crude fibre and tannins correlate positively in grain sorghum.

5.6 Recommendations based on this study

Based on the conclusions of this study, the following recommendations have been made:

1. Heterosis can be utilized to improve the growth and yield of sorghum.
2. There is a need to include a wide compatibility gene in sorghum breeding for increased interspecific crossing and hence yield.
3. Tannin levels started decreasing at the hard dough stage. This is an indication that manual birds' control methods need to be enhanced to reduce their infestation on grains.
4. The F_1 hybrids showed a relatively higher levels of nutrients than parent plants hence a good choice for breeding programs.

5.7 Recommendations for further studies

1. Hybrid lines need to be tested in different locations and for a longer time to test for stability in heterosis.
2. Tannin levels in the F_1 hybrids need to be evaluated over a longer time to determine the effect of hybridization on tannin content

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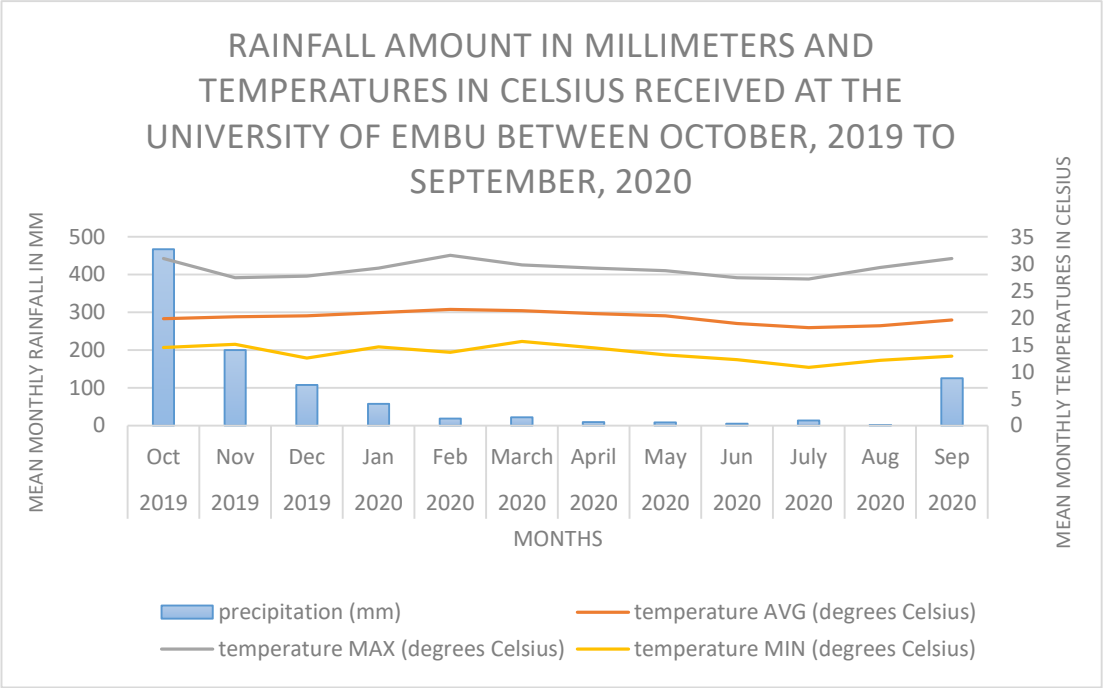
APPENDICES

Appendix 1: Rainfall amount (mm) and temperatures (˚C) received at the University of Embu during the growth period



Appendix 2: Blotter method for testing the seeds' germination rate and its viability

1. Three pieces of blotting paper were placed in petri-dish 9 cm diameter and fold.
2. Distilled water was sprinkled on blotter paper to moisten them.
3. Then, the seeds were placed in each petri-dish and incubated at 25^{o} C.
4. The experiment was replicated thrice.
5. Germination rate was calculated in percentage as;

$$\%\ seeds\ germination = (number\ of\ seeds\ that\ germinated\ per\ each\ petri\ dish \div total\ number\ of\ seeds\ that\ were\ placed\ in\ each\ petri\ dish) \times 100 \ldots\ldots (5)$$

Appendix 3: Manual for RNA extraction from tissues using the Isolate II RNA Plant Kit (Bioline)

1. First, 100 mg of the sample was ground under liquid nitrogen to a fine powder using a mortar and pestle.
2. 350 µL Lysis Buffer RLY and 3.5 µL β-ME were added to a maximum of 100 mg ground tissue and vortexed vigorously.
3. Isolate II filters (violet) were then placed in a 2mL collection tube (supplied) and lysate was loaded and centrifuged (1 min at 11,000 x g). This was done to reduce the viscosity and to clear the lysate. The filtrate was then transferred to a new 1.5 mL microcentrifuge tube (not supplied).
4. Isolate II filter was discarded and 350 µL ethanol (70%) was added to the homogenized lysate. The mixture was then mixed by pipetting up and down (5 times). This step was done to adjust the binding conditions.
5. To bind RNA, for each preparation, one Isolate II RNA plant column (blue) was placed in a 2 mL collection tube and the lysate was added. Care was taken to ensure that all the lysate was loaded on the column. This was followed by centrifuging the lysate at 11,000 x g for 30s. The column was then placed in a new 2 mL collection tube.
6. 350 µL Membrane Desalting Buffer (MEM) was added and centrifuged at 11,000 x g for 1 min to dry the membrane.
7. DNase I reaction mixture was prepared in a sterile 1.5 ml microcentrifuge tube (not supplied): For each isolation, 10 µL reconstituted DNase I was added to 90 µL Reaction Buffer for DNase I (RDN). The mixture was then mixed by gently flicking the tube. 95 µL DNase I reaction mixture was applied directly onto the center of the silica membrane of the column. This was followed by incubation the mixture at room temperature for 15 min.
8. Silica membrane was washed three times and then dried. In the first washing, 200 µL Wash Buffer RW1 was added to the Isolate II RNA plant column and then centrifuged for 30s at 11,000 x g. The column was then placed in a new collection tube (2 mL). In the second washing, 600 mL Wash Buffer RW2 was added to the Isolate II plant column and centrifuged for 30s at 11,000 x g. The

flow-through was discarded and then the column was placed back into the collection tube. In the third washing, 250 µL Wash Buffer RW2 was added to the Isolate II RNA plant column and then centrifuged at 11,000 x g for 2 min. The membrane was then dried completely followed by placing the column into a nuclease-free 1.5 mL collection tube (supplied).

9. The RNA was then eluted in a 60 µL RNase-free water (supplied) and centrifuged at 11,000 x g for 1 min.

Appendix 4: Manual for tannin content, protein, fats, crude fibre, ash and moisture content determination in sorghum samples

4.1 Crude protein analysis

Protein content was determined using the modified Kjeldahl method of Cope (1889) as described by Sarkar & Haldar (2005). Briefly, 10.0g of ground sample was taken into a Kjeldahl flask (800 mL). 35 mL of the Sulphuric acid-salicylic acid mixture was then added. The contents were shaken and left to stand for 30 minutes for nitrate to react with the salicylic acid. Then 2g of zinc dust was added into the flask and the contents were then heated gently on low flame for 5 minutes taking care to avoid frothing. The mixture was then cooled and 10g of digestion mixture was added and the process of digestion was continued for three hours gradually raising the temperature until the solution became clear and acquired a grayish blue colour. After three hours of digestion, the solution was cooled and 300 mL of distilled water was added slowly, with intermittent shaking. The solution was further cooled to the heat of dilution. The flask was then fit to the distillation apparatus. 100 mL of concentrated sodium hydroxide (usually 40%) and several pieces of granulated zinc were added to the flask. One teaspoon of glass beads was also added. The flask was then connected to the distillation head and 150 mL was distilled into 25 mL of standard Sulphuric acid solution 0.1 (N) containing methyl red indicator. The distilled sample was titrated using standard alkali 0.1 (N) sodium hydroxide until the first appearance of the yellow colour. The blank was performed in the exactly same manner without the sample. The deduced nitrogen value was then multiplied by a conversion factor for protein from % nitrogen which is 6.25 to get protein content.

% Crude protein = [(S-T) x N x 1.4/w] 6.25

Where,

S = blank titration, mL standard sodium hydroxide required for 25mL sulphuric acid used for receiving the distillation of the blank

T = titration of the sample, mL standard sodium hydroxide required for 25mL sulphuric acid used for receiving the distillation of the sample

N = Normality of standard alkali

w = sample weight in grams

4.2 Fats analysis

Fat content was determined as per the method of AOAC 945:16 (W Horwitz, 2000), soxhlet extraction method as described by Tasie & Gebreyes (2020) with slight modification. First, 2g of the sample was transferred into the cellulose extraction thimble. The top of the thimble was then plugged with fat-free absorbent cotton wool. The assembling of soxhlet extraction apparatus was done and the flat bottomed flask (quick fit) was filled with petroleum ether (extraction solvent). The fat was then extracted for four hours. After the extraction process, the flask containing the extracted fat was removed and then taken into the fume hood to evaporate the major portion of the extraction solvent. The flasks were dried inside the oven (WGLL-45BE) at 105^{o} C to a constant weight to remove any traces of residual solvent. The flasks were then removed, cooled inside the desiccator and weighed. The fat content was calculated in percentage as;

% fat = [(W2-W1)/W] 100

Where,

W2 = Weight of the receiver flask and fat deposit

W1= Weight of the empty receiver flask only

W = Weight of the sample taken for the test

4.3 Crude fibre analysis

The crude fibre was determined according to AOAC 962:09 (William *et al*., 1970) as per FSSAI (2016). Briefly, 3g of ground sample (with fat content < 10) was weighed into a thimble and put in a soxhlet extractor and extracted with 250 ml of petroleum ether for two hours. The extracted sample was then air-dried and transferred to a dry flat bottomed flask (quick fit). 200 ml of dilute Sulphuric acid (1.25%) was poured into the beaker and brought to boil. The whole of the boiling acid was then transferred to the flask containing the defatted material and the flask was connected to a water cooled reflux condenser. Glass beads were added in the flask to avoid bumping. The contents of the flask were heated and allowed to boil for 30 minutes. The flask was rotated frequently taking care to keep the material from remaining on the sides of the flask and out of contact with the acid. After 30 minutes of boiling, the flask was removed and filtered using a gooch crucible filter (G-2). Some quantity of sodium hydroxide (1.25%) was brought to boil and then 200 ml of the boiling solution was

used to wash the residue on the crucible filter into the flask. The flask was then connected immediately to the reflux condenser to boil the content for 30 minutes. Glass beads were added to avoid bumping. After 30 minutes of boiling, the flask was removed and the content was filtered through the gooch crucible filter (G-2). The residues were first washed thoroughly with boiling water and then with 15 ml of ethanol. The gooch crucible and the contents were dried in an oven (WGLL-45BE) at 105^{o} C until a constant weight was achieved. It was then cooled and weighed. The process of drying was repeated at an interval of 30 minutes, cooling and weighing until the difference between two consecutive weighings was less than 1g. The contents of the gooch crucible (G-2) were then incinerated in a muffle furnace (SX2-2-17TP) until all carbonaceous matter was burnt. The gooch crucible (G-2) containing the ash was then cooled at room temperature and weighed. The crude fibre was calculated in percentage as;

% Crude fibre = [(W1-W2)/W] 100

Where,

W1 = Weight in grams of gooch crucible and contents before ashing

W2 = Weight in grams of gooch crucible containing asbestos and ash

W = Weight in grams of the dried material taken for the test

4.4 Ash content analysis

Ash content was determined according to AOAC 923:03 (William *et al*., 1970). Briefly, clean and dry crucibles were weighed and the weights recorded as W1. 4 g of sample was then accurately weighed into the crucibles after tearing the crucible weight. The materials in the crucible were first ignited using a hot plate maintained at 450^{o} C till charred. The materials containing crucibles were then transferred to a muffle furnace (BioBase MC5=12) maintained at 550^{o} C to continue with the process of ignition for 3 hours to attain the light-grey ash. The crucibles were then removed from the muffle furnace, cooled inside the desiccator and weighed. Ash content was then computed in percentage as;

% Ash = [(W2-W1)/W] 100

Where,

W2= Weight in grams of the crucible with the ash after ignition in the muffle furnace

W = Weight in grams of the sample taken for the test

W1 = Weight in grams of the empty clean and dry crucible

4.5 Moisture content analysis

Moisture content was determined according to AOAC 925:10 (W Horwitz, 2000) as described by Tasie & Gebreyes (2020) with some modifications. Empty and dry aluminum glass Petri-dishes were accurately weighed followed by a sample addition of 2.0g and reweighed. The sample in the Petri-dishes was then placed in an oven (WGLL-45BE) for 2 hours at 105^{o} C. The time was reckoned from the moment the oven attained 105^{o} C after placing the Petri-dishes. The Petri-dishes were then removed from the oven after 2 hours, cooled in a desiccator and weighed. The Petri-dishes were placed back in the oven at an interval of 30 minutes till a constant weight was achieved. Moisture content was then computed in percentage as;

% moisture = [(WI-W2/ W] 100

Where,

W1= Weight in grams of the petri-dish with sample before drying

W2 = Weight in grams of the petri-dish with the sample after drying

W = Weight in grams of the sample taken for the test

4.6 Tannin content analysis

Tannin content was determined using the modified vanillin-HCl assay method of Price *et al*. (1978) using a digital spectrophotometer (ME 801) as described by Dykes (2019). Briefly, before sample analysis, a standard curve was run using the Catechin solution (1000 ppm). To prepare the standard curve, 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of the Catechin solution was added into test tubes and then diluted to 1.0 mL using methanol. All the tubes were then placed simultaneously in the thermosetted water bath set at 30^{o} C and five (5.0) mL of the vanillin reagent was added to each tube at an interval of 1.0 minute. After 20 minutes of incubation period, the absorbance of the coloured intensity for each tube was measured at 500 nm using a digital spectrophotometer. Always, the methanol blank was used to adjust the machine to zero absorbance. The slope of the line was determined using Catechin concentration (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) as the x-axis and the absorbance values as the y-axis in Microsoft excel. The coefficient of determination of the regression model (r^2) of the

curve was determined. For sample analysis, three replicates of 0.3 g each of freshly ground sorghum samples were weighed and transferred into centrifuge tubes. Then, 8 mL of 1% HCl in methanol was added to each tube. The contents were mixed on a vortex mixer for 10 seconds and then each tube was placed in a water bath for 20 minutes. After the first 10 minutes of incubation, each tube was vortexed again for 10 seconds and placed back into the water bath for the remaining incubation period. Each tube was removed exactly after 20 minutes of incubation and mixed on a vortex mixer for 10 seconds immediately after removal from the water bath. The samples were then centrifuged at 4000 x *g* for 10 minutes. The supernatant was then decanted carefully avoiding the ground sample to be transferred into the supernatant. Two 1 mL aliquots were taken from the supernatant and each placed into a clean separate test tube. In total, there were 60 tubes. Among them, 30 were labeled 'blank' tubes while the other 30 were labeled 'sample' tubes. All the tubes were simultaneously placed into the thermosetted water bath set at 30^{o}C and five (5.0) mL of the vanillin reagent was added to each ‘sample’ tube while five (5.0) mL of 4% HCl in methanol was added to each blank tube at an interval of 1.0 minute as indicated by Dykes (2019). Each set (a sample and a blank tube) was allowed to stay in the water bath for 20 minutes. The absorbance of the coloured intensity for each 'sample' and 'blank' tube was then read exactly after 20 minutes at 500 nm using a digital spectrophotometer. The methanol blank was used to adjust the machine to zero absorbance. To determine the final tannin content, the value of the 'blank' was subtracted from the value of the 'sample'. Tannin concentration (mg/g) was calculated using the quadratic equation obtained from the standard calibration curve;

$Y = 7 * 10^{-5} x + 0.0096$

Where; Y= absorbance, X = concentration.