EVALUATION OF SORGHUM (*Sorghum bicolor* L. Moench) GENOTYPES FOR ALUMINIUM TOXICITY TOLERANCE USING MORPHOLOGICAL AND MOLECULAR MARKERS

BY

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DEPARTMENT OF PLANT SCIENCE AND CROP PROTECTION
FACULTY OF AGRICULTURE
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This thesis is my original work and has not been submitted for award of a degree in any other University.

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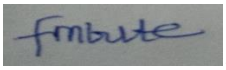
Signature: …  …… **Date: 2nd August 2023**

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DEDICATION

The work is dedicated to my spouse, Jeniffer Kipkech and children, Bradley Kipkoech, Zaddock Berur, Britney Jerop and Laura Jepkemoi whose support, encouragements and understanding steered the successful completion of the studies.

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LIST OF ABBREVIATIONS AND ACRONYMS

AACT	Aluminium Activated Citrate Transporter
AFLP	Amplified Fragment Length Polymorphism
ALMT	Aluminium Activated Malate Transporter
ANOVA	Analysis of Variance
CRD	Completely Randomized Design
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization of the United Nations
FSRL	Final Seminal Root Length
GOK	Government of Kenya
ICRISAT	International Crops Research Institute for Semi-Arid Tropics
IFPRI	International Food Policy Research Institute
ISRL	Initial Seminal Root Length
KAVES	Kenya Agricultural Value Chain Enterprises
KCSAP	Kenya Climate Smart Agriculture Project
LSD	Least Significant Difference
MAB	Marker Assisted Breeding
MAS	Marker Assisted Selection
MATE	Multidrug and Toxic Compound Extrusion
MOA	Ministry of Agriculture
NLWRA	National Land and Water Research Audit
NSRL	Net Seminal Root Length
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RSRL	Relative Seminal Root Length
RTI	Root Tolerance Index
SDS	Sodium Dodecyl Sulfate
SSR	Simple Sequence Repeats
USAID	United States Agency for International Development
^{0}C	Degrees Celsius
µM	Micromolar
µmol	Micromole
ml	Milliliter
mM	Millimolar
M	Molar

GENERAL ABSTRACT

Aluminium (Al) toxicity is listed among the leading edaphic factors limiting production of sorghum in acidic soils (pH<5.0). It affects the apical root cell multiplication and elongation, hence, inhibiting the absorption of water and mineral elements which eventually leads to poor plant growth, yield and kernel quality. Liming, a most adopted remedy against Al toxicity has proved costly and unsustainable, however, identification and utilization of tolerant genotypes could sustainably aid in management of the constraint. Magnavaca solution screening was used in phenotypic evaluations of 14 selected lines for Al toxicity tolerance. Among the genotypes were the sensitive and the tolerant checks. Seeds for each genotype were pre-germinated in an incubator and initial seminal root lengths (ISRL) taken 4 days upon germination. Selected seedlings were then laid out in a completely randomized design (CRD) with 2 treatment levels of Al; 0 and 148 µM bearing pH levels of 4.3. Final seminal root lengths (FSRL) of the seedlings were taken 5 days after exposure to Aluminium and together with initial root lengths used to compute the net seminal root length (NSRL), relative seminal root length (RSRL), root tolerance index (RTI) and % response to Al that were applied in establishing the tolerance status for the genotypes in reference to the provided standard scales. Results from ANOVA showed that genotypes varied significantly in response to the Aluminium treatment. Genotypes exhibited significant ($P<.001$) variability in growth of roots under Aluminium stress. Genotypes *Gadam* and *Wagita* were found to be tolerant, *Macia* and *Kiboko local 2* moderately tolerant while a remainder of 8 genotypes expressed sensitivity. To validate sorghum genotypes with Al tolerance genes, specific SSR markers linked to Al tolerance in sorghum were used. DNA was isolated from each of the genotypes following the CTAB protocol, quantified using a nanodrop spectrophotometer and subjected to polymerase chain reactions. DNA amplicons were detected through agarose gel electrophoresis where band patterns of the genotypes were analyzed in respect to the checks. Genotypes with band pattern identical to the tolerant check were categorized as in possession of Al tolerance genes and vice versa. Identification of tolerant genotypes was largely achieved through marker *Xtxp34*. Unlike markers *Sb5_236, Sb6_342* and *Sb6_34*, the marker was polymorphic and specific to the targeted gene locus linked to Al tolerance. Band patterning due to the marker clearly discriminated between tolerant and sensitive genotypes and

strongly associated with genotypes' tolerance status established via nutrient solution screening. Based on the marker (*Xtxp34*), genotypes *Gadam*, *Wagita* and *Macia* had identical band pattern to the tolerant check *IS 41764* and were considered Al tolerant. The rest of the genotypes shared the same band pattern with the sensitive check *Seredo* hence categorized as Al sensitive. Contrary to nutrient solution screening, the applied markers did not present genotype *Kiboko local 2* as tolerant indicating that there could be other gene variants for Al tolerance in this genotype that are yet to be ascertained. The observed existence of variability and potential for Al tolerance in sorghum germplasm provides basis for selection of parental lines for breeding against the stress.

CHAPTER ONE
INTRODUCTION

1.1 Background information

Sorghum (*Sorghum bicolor* L. Moench); also named the great millet is a grass species cultivated largely for its grain for human and animal consumption. It is a C_4 crop performing well under temperature conditions of 25-31°C, rainfall of 500-800 mm throughout the growing season and favoured by most of the soil types with pH levels ranging between 5.5-8.5 (Balole and Legwalia, 2006; Xiao *et al*., 2021). It is native to Africa, ranked at the fifth position among the most crucial cereal crops of the globe after maize, wheat, barley and rice (Shahbandeh, 2020). It comes second after maize as the key origin of day-to-day energy requirements for over 300 million people occupying the African sub-Saharan region (Bhosale *et al*., 2011; Macauley and Ramadjita, 2015).

Africa's sorghum production is approximated at about 20 million metric tonnes per annum. Nigeria takes the lead in the continent with production averaging at 6.57 million metric tonnes per annum (Shahbandeh, 2021). In Kenya, sorghum is considered an indigenous subsistence crop mainly cultivated in areas experiencing low rainfall by about 250,000 farmers with farm size range of 0.5-0.6 hectare and mean production of below 1.5 tonnes per hectare (KAVES, 2013; GOK, 2009). Its consumption in the country stands at approximately 83,000 tonnes per annum with about 53% of the total grain supply being utilized as food inform of flour while 24% is value added into other commodities (MOA, 2011; Mailu and Mulinge, 2016). Its grain is processed into a range of food products like fermented porridge, semi-leavened bread and dumplings. Sorghum is also utilized as feed, biofuel and in beer producing industries as a substitute to barley (Rao *et al*., 2019).

Sorghum is versatile and hardy. It thrives relatively well under low moisture and soil fertility conditions and stands out for its lower costs of production. It is recognized among the food security crops and serves to improve rural livelihoods in arid and semiarid areas (World Bank, 2005; Mwadalu and Mwangi, 2013). Despite the positive attributes, optimal sorghum production is still impeded by a number of factors; inadequacy of superior

cultivars, insect pests, diseases, parasitic weeds, prolonged droughts, soil salinity and acidity. There has been an increase in acreage under sorghum production in Kenya from about 123,000 to 173,174 hectares, however, yields have remained as low as 0.54 t ha^{-1} contrasted to a global mean of about 2.5 t ha^{-1} (GOK, 2010; Chepng'etich *et al*., 2014).

Aluminium toxicity is the leading impediment to productivity of sorghum in soils of lower pH (Yang *et al*., 2013). Aluminium (Al^{3+}) interferes with normal root growth. Poorly grown roots are unable to efficiently absorb water and dissolved minerals from the soil rendering the plants moisture stressed and deficient of required nutrients especially Phosphorus (P) (Le *et al*., 2008; Penn *et al*., 2019). This culminates into poor growth and overall development of the crop which subsequently causes low yield and reduced value of the kernels (Vitorello *et al*., 2005; Du *et al*., 2021). Application of lime to mitigate the problem has proved costly and unsustainable, nevertheless, utilization of tolerant genotypes could sustainably aid in the management of the constraint. Tolerant genotypes maintain relatively high growth of roots in presence of Aluminium toxicity as they are able to exclude Aluminium ions from their root systems. They release large amounts of Al-binding materials namely malic and citric acids that chelates the toxic Al^{3+} forming Alumino-carboxylate compounds that plants are unable to take up from the rhizosphere (Goncalves *et al*., 2005; Wei *et al*., 2021).

In Kenya, Aluminium toxicity accounts for up to 35% drop in sorghum grain yield (Too, 2014). Observations made have also shown that the levels of Aluminium ions (Al^{3+}) present in acidic soils of Kenya could also be detrimental to a number of maize and wheat varieties released for commercialization (Kisinyo *et al*., 2014). Therefore, knowledge on mechanisms for Al tolerance coupled with authentication of tolerant genotypes for use in acidic soils is highly desirable for sustainable crop production and protection of soils from excessive use of lime.

1.2 Statement of the problem

Soil acidity is a condition in which soil pH is lower than the neutral level. Acidic soils (pH<5.0) are characterized by deficiency of basic cations, especially Ca^{2+} and Mg^{2+}. Approximately a half of potentially productive soils globally are acidic. These soils majorly cover tropical and subtropical areas of most third world countries where food sufficiency has not been realized (Bian *et al*., 2013). In Kenya, such soils have claimed up to 8.0 million hectares equivalent to 13% of potential agricultural land (Obura *et al*., 2010). They are widely distributed and cover most parts of Northern Rift Valley, Western and Nyanza regions (Kanyanjua *et al*., 2002) which are the major sorghum growing areas.

Aluminium toxicity limits crop production in acidic soils worldwide. The toxic Al^{3+} inhibit root cell division, elongation and root membrane permeability. Upon penetration into the root and within the plasma membrane, the trivalent cation (Al^{3+}) is attracted to the opposite charges of phospholipid bilayers causing stiffness, altered membrane functioning and intensified oxidative tensions. Basically, such biological root cell adjustments result into poor growth and development of roots hence low intake of water and nutrients that eventually affect sorghum yield and quality of the kernels (Cicero *et al*., 2018). Approximately 40% of grain yield loss in sorghum is linked to Al toxicity (Too, 2014).

Application of lime to mitigate the problem has been reported to be costly and unsustainable (Akinrinde, 2006; Matonyei *et al*., 2020), nevertheless, utilization of Al tolerant genotypes could sustainably aid in management of the constraint. Development of Al tolerant sorghum lines has been an area of focus to breeders, however, limited knowledge on tolerance status among the known genotypes has been an impediment (Kumar *et al*., 2011). Therefore, evaluation of available germplasm is necessary in order to identify potential sources of genes for development and deployment of superior cultivars against Al toxicity thus sustainably improving sorghum production in acidic soils.

1.3 Justification of the study

Aluminium toxicity limits sorghum production in acidic soils, thus, appropriate application of lime for optimum production of the crop is paramount, however, lime application as the sole way of managing acidic soils in most agricultural systems is costly and unsustainable or it may take long before desirable results are achieved (Matonyei *et al*., 2020). Excessive application of lime also induces shortage of particular soil elements and may negatively impact a given species of crops in the rotation (Ernani *et al*., 2004).

Development and utilization of high yielding Aluminium tolerant genotypes emerges as the most favorable socioeconomic and environmentally friendly strategy in curbing Al toxicity in acidic soils, hence, screening and identification of tolerant genotypes is necessary especially in developing world characterized by food inadequacy partly caused by acidic soils (Christou and Twyman, 2004).

Methodical identification of tolerant genotypes coupled with breeding of the germplasm to endure toxic levels of Aluminium is essential for improved and sustained production in order to adequately cater for rising demand from consumers for both quality and variety of sorghum products. The interventions are not only relevant towards sustainable improvement of production and contribution to a food secured nation but also building the resilience of the crop on verge of climate change.

1.4 Research objectives

1.4.1 Broad objective

To contribute to enhanced production of sorghum through identification of genotypes tolerant to Aluminium toxicity.

1.4.2 Specific objectives

The specific objectives of the study were:

1. To identify sorghum genotypes tolerant to Aluminium toxicity using morphological markers.
2. To validate sorghum genotypes with Aluminium tolerance genes using specific simple sequence repeats (SSR) markers.

1.5 Hypotheses

1. Genotypes expressing Al toxicity tolerance do not exist.
2. Genotypes with Al toxicity tolerance genes cannot be validated with SSR markers.

CHAPTER TWO
LITERATURE REVIEW

2.1 Botany of sorghum (*Sorghum bicolor* L.)

Sorghum is a cereal crop traced to the grass (Gramineae) family. It is an annual/perennial grass species distributed in most areas of the globe owing to its capacity to cope in a vast range of environments. It is a C_4 plant capable of carrying out the process of photosynthesis at higher rates and tolerates drought (Reddy *et al*., 2009; Verma *et al*., 2018) a trait that points out to its adaptations to conditions dominant in its center of origin (North eastern Africa), specifically regions of Sudan and modern Ethiopia (Dillon *et al.*, 2007; Ananda *et al*., 2020). Sorghum is majorly self-pollinating species with two sets of chromosomes (2n=2x=20). Cross-pollination occurs by nature from 6-14% in respect to a genotype, type of the panicle and strength and direction of the blowing winds (House, 1980; Schmidt *et al*., 2013). *Sorghum bicolor* is categorized into 5 basic races basing on the differences in morphology of the flowers. The five basic races including bicolor are cultivated and are identified through matured spikelet and sometimes by head type (Li *et al*., 2020).

2.2 Challenges to production of Sorghum

The production of sorghum is faced by a number of constraints which differ in degree and combination from one area to another. These setbacks are majorly as a result of living and non-living factors at play throughout the semi-arid tropics (House, 1984; Gasura *et al*., 2015).

2.2.1 Biotic challenges

Infections caused by a number of pathogens, field pests, parasitic weeds and inferior cultivars have been cited to be the major biotic factors lowering sorghum production within the expansive range of environments where the crop is grown. The most common and notorious diseases attacking sorghum are caused by fungus and includes anthracnose (*Colletotrichum sublineolum*), leaf blight (*Helminthosporium turcicum*), downy mildew

(*Peronosclerospora sorghi*), gray leaf spot (*Pyricularia grisea*), rough leaf spot (*Cercospora sorghi*), smuts and Fusarium head mold (*Fusarium spp*.) (Njoroge *et al*., 2018). Generally, pests attacking sorghum crop in the field are categorized into soil and seedling pests (*Schyzonycha spp*.), leaf and stalk- boring pests (*Spodoptera exempta*) and panicle and seed pests. Parasitic weeds and more specifically striga species (*Striga hermonthica*) is known to considerably lower yield and grain quality among the susceptible sorghum genotypes. Under high disease severity rating (severity>5) based on a scale of 1-9 where 1 represents absence of disease, anthracnose causes yield loss of up to 15%, leaf blight 1.5%, rust causes a loss of 15.2% and leaf spots 27% (Ngugi *et al*., 2002). In areas infested by parasitic striga weed, over 90% loss of sorghum grain yield has been recorded (Temesgen, 2021).

2.2.2 Abiotic challenges

Drought and edaphic factors namely soil texture, unavailability of nutrients, salinity and acidity hampers the production of sorghum in sub-Saharan Africa (Doggett,1988; Tari *et al*., 2013). Acidic soils are associated with root growth inhibitors and low fertility. Growth of sorghum in such conditions is restricted by Aluminium toxicity and phosphorus insufficiency that are responsible for over 35% grain yield loss (Too,2014). The inadequacy and poor access to superior sorghum cultivars to withstand pressure from these stresses have too contributed to the overall loss in sorghum grain yield per unit area (USAID, 2006; Njagi *et al*., 2019).

2.3 Soil acidity

Soil acidity occurs when cations of basic elements which bind to the soil colloids are interchanged with hydrogen ions (H^+) which upon increase in their concentration leads to low pH of the soil. The condition is commonly associated with tropics on account of what the base rock is composed of, high levels of weathering and leaching of basic cations. Soils developing from non-calcareous parent material are inherently acidic due to percolation of

rain water down the soil horizons together with basic cations leaving the top soil layer acidic (Caulda *et al.*, 1988; Akinrinde, 2006; Alnaimy *et al*., 2023).

The process of soil acidification occurs naturally but accelerated by agricultural activities (Kennedy, 1986; Tkaczyk *et al*., 2020). Inefficient use of nitrogen, export of alkalinity in produce and accumulation of organic matter are known to be the main contributors to the condition (Williams, 1980; Agegnehu *et al*., 2021). Heavy and continuous application of nitrogenous fertilizers (Ammonium Sulphate) makes the soil acidic due to its conversion into nitrate (Kochian, 2002). Leaching of too much nitrates unutilized by plants down beyond the upper soil layers leads to replacement of basic cations within the soil horizons leaving the acidic hydrogen ions in the surface layer (Helyar and Porter, 1989; Cameron *et al*., 2013). In hot environments characterized by dry summer months, nitrates build up as a result of nitrogenous mineralization in decaying plant materials.

Removal of produce from the land takes with it a lot of alkaline material. Failure to supplement this lost alkalinity brings about soil acidification. For instance, an 8^{th} of a hectare harvest of lucerne in a year demands an application of not less than a half a tonne per hectare of lime to restore former status. This translates to additional 20-30% of production costs (Slattery *et al.,* 1991).

Soil pH should ideally be maintained at above 5.5 and 5.0 for top and subsoil, respectively. At these levels, growth of plants, soil microbial activities and availability of essential nutrients e.g., Phosphorus and Molybdenum are favoured. In other terms, a negative deviation from the level of 5.5 is accompanied by a number of edaphic constraints to crop production. Differences exists on response to varied levels of soil pH by species and genotypes within a species. Secondly, Aluminium and Manganese solubilized in acidic soils are toxic to plants. Also, below the level of 5.5, Phosphorus and Molybdenum are fixed hence unavailable to plants (Penn *et al*., 2019). These factors cumulatively contribute to poor growth of plants, reduced yield and quality of crop product.

Closer to 50% of arable lands in the world constitute acidic soils (pH<5.5) that are widely spread (Wei *et al*., 2021). Australia accounts for up to 90 million hectares of the land that is affected. West Australia and New South Wales is accountable for 12-24 million hectares under extremely acidic soils with pH value of ≤4.8 (NLWRA, 2001), hence, Aluminium toxicity remains a challenge to production of crops in these areas. For Kenya's case, such soils have claimed up to 8.0 million hectares of potential agricultural land with Aluminium saturation ranging in between 8 and 61% where saturation of 20% greatly affects most plant species (Obura *et al*., 2010; Kisinyo *et al*., 2014). They are widely distributed and cover most parts of Northern Rift Valley, Western and Nyanza regions (Kanyanjua *et al*., 2002, Osundwa, 2013) among other major areas of sorghum production. A study in Kenya has revealed that Al toxicity associated with acidic soils is responsible for 30-40% loss in sorghum grain yield (Too, 2014).

2.4 Aluminium (Al) toxicity

Aluminium (Al) occupies third position in abundance after Oxygen (O) and Silicon (Si) among the elements found in Earth (Kochian, 1995; Bolt *et al*., 2020). It is plenty of all metallic elements and makes up to 7% of the planet's mass. It exists in large amounts as Alumino-silicate deposits and little amount in solution form. Aluminium is released from the minerals of the soil under low pH conditions occurring in three forms but Al $(H_2O)6^{3+}$ is always responsible for toxicity (Kinraide, 1995; Mossor-Pietraszewska, 2001; Chowra *et al*., 2017). Aluminium is highly soluble with decrease in soil pH (<5) and it is under these conditions that the trivalent cations (Al^{3+}) are exchangeable and available but toxic and non-essential to plants. In acidified soils (low pH), the amounts of phytotoxic cations of Aluminium are increased whereas under elevated pH levels (>5.5), Al $(OH)_2^+$ and Al $(OH)^{2+}$ that are harmless dominate (Delhaize and Ryan, 1995).

The toxic cation is present in both solution and the sites for cation exchange where its tendency to exchange with other soluble cations is high. $A1^{3+}$ interferes with a number of physiological and cellular activities through its multiple interactions with the components of cell wall, cytomembrane and cytoplasm. The threshold at which its availability to plants

starts to pose serious implications is above 0.4 ppm, with a few adapted crop varieties withstanding levels greater than 1000 ppm (Weller, 2000).

2.4.1 Aluminium toxicity effects in plant growth and development

Aluminium toxicity limits production of crops in acidic soils of the globe (Shao, 2010; Rasheed *et al*., 2020). Restricted root growth is the most prevalent manifestation and extensively adopted determinant of Al attack in plants. The toxic Al^{3+} mainly targets the tip of the plant root (distal area of transition zone) where it interferes with the normalcy of the processes of cell division and extension. This leads to less developed root system which in turn affects intake of water and nutrients, ultimately, capability of plants to tolerate moisture stress and produce optimally are affected (Miguel *et al*., 2010; Mishra *et al*., 2020). A significant drop in grain yield was reported by Duncan *et al.* (1980) from experimental trials set to determine effects of Aluminium on sorghum yield at soil pH <5. Reports by Gallardo *et al*. (1999) also indicated that there was 50% decline in grain yield for sensitive barley (*Hordeum vulgare*) genotypes grown in natural acidic soil of pH<5.0 as compared to 30% loss recorded from tolerant genotypes under the same conditions.

Toxicity of cells by Aluminium in plants has been studied and well described (Delheize and Ryan, 1995; Jaskowiak *et al*., 2019). Interactions between Al^{3+} with the components making up the cell wall leads to undesirable changes on its properties and functioning. Cellular selectivity, ability to extend and enzymatic activities are altered leading to poor growth of roots thus inefficiency in uptake of water and mineral elements. Aluminium toxicity majorly attacks the cytoplasmic homeostasis causing disruptions which may be responsible for restriction of cell multiplication and eventually root elongation as a result of interference with dependent physiological and biochemical processes. Dependent phospholipase C whose role is to act on lipid substrate phosphate dylinositol-4, 5-biphosphate was found to be inhibited by Aluminium toxicity in wheat root tips. Root exposure to Aluminium leads to cellular oxidative stress. Components of the cell including lipids, enzymes and nucleolar materials are oxidized in existence of reactive oxygen

species resulting into unprogrammed death of cells (Delheize & Ryan, 1995; Achary *et al*., 2008; Bera *et al*., 2019).

Root lateralization/ branching is also limited by Aluminium toxicity (Foy, 1992; Rahman and Upadhyaya, 2021). Studies by Parker (1995) clearly described ways in which plants respond to Al toxicity; a slowed plant growth and later severe Aluminium impacts on growth of roots. Based on experiments performed, acid soil stress in sorghum genotypes is largely linked to Al toxicity (Tan *et al.*, 1992; Bhalerao and Prabhu, 2013). Under acidic soil conditions, root extension and development are greatly disabled which is closely related to decline in produce among Al sensitive genotypes. Following the less growth of roots are development of tumors and discoloration. In severe conditions, roots appear brown or black, brittle and very stubby (Mossor-Pietraszewska *et al*., 1997; Gupta *et al*., 2013). Latest reports indicate that Al toxicity negatively affects soil rhizobia thus restricting nodule formation and fixation of nitrogen in legume species.

Carver and Ownby (1995) noted that Aluminium induces drought susceptibility and inefficient utilization of available soil nutrients by plants. The uptake of mineral salts particularly Calcium (Ca) and Magnesium (Mg) is curtailed in presence of solubilized Aluminium in acidic soil leading to deficiencies of these elements. In addition to this, Phosphorus (P) is fixed, hence, phosphorus deficiency sets in as a condition associated with Al toxicity (Moustaka *et al*., 2016). Toxicity of Aluminium also causes a decline in the concentration of chlorophyll and the rates of photosynthesis and transpiration (Ohki, 1986).

Plants growing in acidic soils present visible nutrient deficiency symptoms. Sorghum plants thriving under such conditions show Magnesium (Mg) deficiency disorders including overall stunting, stems turning purple (anthocyanin), chlorotic leaf veins and necrotic leaf apices (Foy *et al.*, 1992). In addition, Al toxicity manifests as limited Calcium (Ca) ion mobility within the plant, triggering a condition known as leaf curling (Foy *et al*., 1992; Rout *et al*., 2001). According to observations made by Grundon *et al.* (1987) on the symptoms and internal concentrations, Magnesium rather than Calcium or Phosphorus

deficiency associated with effects of Al toxicity at pH range of 3.9-4.8 was evident in sorghum plants.

2.5 Management of Aluminium toxicity in acidic soils

Aluminium toxicity impedes growth of plants in soils of low pH (<5). Application of Calcium (lime) has been extensively employed to alleviate the menace and boost crop yield; however, it has proved uneconomical and not sustainable in most agricultural systems. An average of 4 - 8 tonnes per hectare of lime is required to lower the soil acidity and provide optimum pH for growth of plants (Kisinyo *et al*., 2010). Another challenge experienced with application of lime is that the amendment of subsoil acidity demands large quantities of lime which should be mixed thoroughly into the deeper soil horizons thus furthering the operational costs. Moreover, excessive application of Calcium induces shortage of particular soil elements and may negatively impact a given species of crops in the rotation since different species react differently to various levels of soil pH (Ernani *et al*., 2004; Whitten *et al.*, 1997). Run-off pollution caused due to lime application is also undesirable (Jawad *et al*., 2014).

Farmers especially from tropical regions who are incapacitated to obtain the required commercialized lime or base fertilizers have resolved in application of organic matter to ameliorate soil acidity, however, regular application of compost and manure in recommended quantities to correct acidic soils is disadvantaged by other demands on sources of the organic matter which includes but not limited to animal feeds, fuel and construction (IFPRI, 2010).

Promising results have been observed through application of mineral nutrients including Magnesium (Mg) and Sulfur (S). Magnesium has the ability to prevent the movement of Aluminium via the cytoplasmic membrane in root apices. Sulfur on the other hand is able to minimize toxicities of Manganese (Mn) and Aluminium (Al). Silicon and some biological fertilizers and industrial byproducts have also shown significant ability to mitigate the toxicity of Aluminium in soils of low pH (Li *et al*., 2010; Ahmad *et al*., 2022),

however, they have too proved to be costly and may not provide sustainable agricultural systems. Knowledge on continuous soil nutrient interactions is required which poses additional costs from seeking frequent soil tests services.

A review on these available methods of managing Aluminium toxicity in acidic soils indicates that all the options apart from breeding against the constraint comes with serious shortcomings. Screening, identification and development of high yielding tolerant plant genotypes presents themselves as the feasible approaches in management of the stress.

2.6 Breeding against Aluminium toxicity in cereals

Efforts to breed cereals for Aluminium toxicity tolerance has registered a success. The process entails sourcing of germplasm, screening to identify desirable parents, selection of ideal breeding programme and rescreening to confirm successful transfer of genes.

2.6.1 Sourcing of germplasm

Collection and characterization of desirable parental lines are crucial initial steps in any successful breeding programme. Majority of crop varieties tolerant to Aluminium toxicity were gathered from stronger acidic soils of the globe (Caniato *et al*., 2011; Zishiri *et al*., 2022). Reports for instance show that in among the hundreds of bread wheat races obtained from different nations, approximately 30 genotypes gathered from strongly acidic soils of Nepal exhibited high tolerance to Aluminium toxicity. Such phenomenon was greatly linked to selection by nature and humans involved in early agriculture (Stodart *et al*., 2007) leading to a logical conclusion that screening of plant genotypes collected from areas dominated by acidic soils remains the most appropriate approach in initial breeding strategies against Al toxicity.

In modern crop world, breeding programmes have applied mutagens in attempts to achieve the desired gene variations against Aluminium toxicity. Focusing on barley (*Hordeum*

vulgare), treatments by mutagens N-methyl-N-nitrose urea (MNH) and sodium azide produced over ten variants with high tolerance to Aluminium toxicity (Nawrot *et al*., 2001; Kubo *et al*., 2022). Somaclonal variations through cell and tissue cultures and genetic engineering technology have also proved their significant relevance in generation of Al tolerant genotypes (Dhamendra *et al*., 2011). In sorghum, maize and rice, the technologies through in vitro cultures have yielded lines that are tolerant to the Al^{3+} toxicity (Foy *et al*., 1993; Sibov *et al*., 1999; Rai, 2022).

2.6.2 Germplasm screening for Aluminium toxicity tolerance

Successful gathering of germplasm is followed by their screening for Aluminium tolerance to distinguish between tolerant and sensitive lines. Most of the screening activities have utilized either of the two major methods; field evaluations and controlled conditions specifically nutrient solution cultures and tissue or cell cultures (Deborah and Tesfaye, 2003), however, nutrient solution culture is commonly utilized in screening plant genotypes for Aluminium tolerance (Raman and Gustafton, 2011; Abate *et al*., 2013). The operating principle when using this screening method is the comparison of seedling root growth in a set of two nutrient solutions; one with Aluminium treatment and the other without (Too *et al*., 2020). This method presents advantages in that root systems of seedlings are easily accessed, allows control over pH, availability of nutrients and light source and is not destructive when measuring root growth (Carver and Ownby, 1995). Majority of nutrient solution screening in cereals are done using Magnavaca and Yoshida solution cultures where Magnavaca's is used in screening sorghum, wheat and maize while Yoshida's in screening of rice (Magnavaca *et al*., 1987; Yoshida *et al*., 1976).

Aluminium tolerance screening involving nutrient solutions are conducted following two bioassays; staining of root tip and growth of roots. Under root tip staining, a chemical known as haematoxylin is largely used (Raman and Gustafson, 2011). Procedure for haematoxylin root staining reveals Al susceptible genotypes through formation of complexes as a result of buildup of Al^{3+} in root apices of such genotypes. Genotypes that

are highly susceptible bear large amounts of Al^{3+} in their root tips thus exhibit intense purple coloration of the stain (Deborah and Tesfaye, 2003; Abate *et al*., 2022).

Measurements of root extension have also been extensively used in differentiating Al tolerant from sensitive genotypes with use of nutrient solution screening. The method puts in to consideration parameters such as root extension/ growth, root tolerance index or relative root growth (Carver and Ownby, 1995; Khu *et al*., 2012). Measurements of growth of roots are based on ability of roots to elongate or extend growth in presence of Al toxicity. Genotypes having high root growth under the conditions are regarded as tolerant. Root tolerance index is a ratio of growth of roots in presence of Aluminium to growth of roots in absence of Aluminium (Hede *et al*., 2001; Kichigina *et al*., 2017). In respect to the parameter, genotypes with high root tolerance index (>0.90) are categorized as tolerant to Al toxicity. Comparisons between the two nutrient solution assays shows that root growth parameters are more dependable and frequently used in determining Al^{3+} toxicity resistance in plants (Hede *et al*., 2002; Butare *et al*., 2012).

In vitro technique is also available for screening plants for Aluminium tolerance. The method relies on cell culture in identification of Aluminium tolerant genotypes. Screening is conducted through evaluation of developing callus derived from distinct genotypes under acidified media bearing various levels of Aluminium besides the controls (acidic media without Aluminium) (Deborah and Tesfaye, 2003; Too *et al*., 2020). Although the technique is effective, it is associated with high expenses involved when screening some species. In addition to this, screening at pH ranges of 4 is accompanied by challenges including failure of agar to solidify when autoclaved (Conner and Meredith, 1985).

Greenhouse screening using controlled acidic soils has also been applied in identification of Aluminium tolerant genotypes, however, they are normally performed after nutrient solution screening. Recommendations guides that acidic soils utilized for screening should be obtained from target area of production (Carver and Ownby, 1995). The outline of the protocol is that plants are grown in soil treated with lime alongside soil that has not received lime then scores for parameters namely root tolerance index, root growth, shoot dry matter

or root dry matter are recorded and computed to determine tolerance (Liu, 2005). Acid soil screening has an advantage over solution screening since it factors in other edaphic factors accompanying Aluminium toxicity in acidic soils.

Development of Aluminium toxicity tolerant crop cultivars should put in to consideration the targeted soils in areas of production. Screening of germplasm should be conducted in the affected fields in order to generate varieties that are well adapted and highly productive. Like greenhouse screening, field evaluations for Aluminium toxicity tolerance are performed in a set of two conditions; all test genotypes are grown in a plot treated with lime and another that has not been limed (Too *et al*., 2020). Genotypes are then evaluated on target traits e.g., grain yield reported as a yield ratio of plot without lime to yield from plot treated with lime (Carver and Ownby, 1995). Field evaluations are prone to challenges emanating from other stresses including crop diseases and spatial variabilities existing in acidic soils that have effect on the final output.

Genetic markers also take a greater part in crop improvement programmes against Aluminium toxicity. They are instrumental in monitoring of expression of alleles of interest and in studies involving genetic diversity (Raman and Gustafson, 2011; Too *et al*., 2018). Comparative mapping studies have depicted existence of similarities in genomes of sorghum, rice, barley, wheat, maize and oats. This has provided an opportunity to assess their tolerance to Aluminium toxicity using a group of common molecular markers in linkage to tolerance to the stress (Raman and Gustafton, 2011). Markers linked to major genes *SbMATE, HvMATE, TaALMT* and *ZmMATE* have been generated. These genes are responsible for Aluminium resistance in sorghum, barley, wheat and maize. Markers that have been developed and more often used in cereals include Simple Sequence Repeats (SSR) and Amplified Fragment Length Polymorphism (AFLP) (Mosajc *et al*., 2001; Nguyen *et al*., 2002; Wayima *et al*., 2019). Genetic markers have been helpful in improving efficiency of conventional plant breeding.

2.6.3 Breeding *Sorghum bicolor* L. against Aluminium toxicity

In breeding work carried out by Too *et al.* (2020), *Seredo* a highly farmer preferred Aluminium sensitive commercial variety and *ICSR 110*, an Al tolerant genotype obtained from ICRISAT-Kenya were systematically crossed to generate subsequent first and second filial populations. Evaluations involving the F_1, F_2 segregating individuals together with the parental lines for Aluminium tolerance were then carried out under two conditions; nutrient solution culture with and without Aluminium and field conditions in plots with and without lime. Percentage relative root growth (% RRG) and percentage responses to Aluminium (% Response) (Caniato *et al*., 2007) were assessed through nutrient solution screening at 148µM Al and pH of 4.3 while grain yield per plant was assessed in acidic soil at the same pH level. Results obtained showed that F_1 hybrids and some F_2 individuals acquired tolerance to Al toxicity with some outperforming the tolerant parent *ICSR 110* in both setups. This therefore demonstrated potential transfer of Aluminium tolerance into sensitive but high yielding sorghum genotype providing an avenue for more breeding trials to sustainably combat Al toxicity stress in acidic soils dominant within the tropics.

Conventional breeding has been highly instrumental in improvements made on sorghum (Grootboom *et al.*, 2010). Breeding approaches practiced under the conventional breeding of sorghum are the classical evaluations and upgrading of available genetic resources, pedigree selection, recurrent selection, backcrossing and creation of hybrids through cytoplasmic and genetic male sterility techniques (Reddy, 2019). Plant biotechnology methods namely molecular genetics, genomics and plant transformations are currently being sought in attempts to improve sorghum more efficiently (Deshpande *et al*., 2016).

Transgenes have successfully been introduced to *Sorghum bicolor* with utilization of *Agrobacterium tumefaciens* mediated transformations and biolistic methods of gene transfer (Elkonin *et al*., 2016. The current and future intentions of plant breeders are to equip the crop with necessary traits of agronomic values ranging from insect and disease resistance, striga resistance, drought resistance and Aluminium toxicity tolerance (Windpassinger *et al*., 2015). By the fact that transfer of Aluminium tolerance between the

sorghum genotypes has been successful, extensive collection and identification of more tolerant genotypes is paramount in order to comprehend the extent of availability of gene donors to enable generation of superior cultivars.

2.7 Genetics of Aluminium toxicity tolerance in cereal species

Research on genetic tolerance to Al in some cereals of economic importance has been prioritized and findings documented. Aluminium tolerance traits in wheat, rye, sorghum, barley and oat have been found to be under control of various genes from Multi-drug and Toxin Compound Extrusion (*MATE*) and Aluminium Activated Malate Transporter (*ALMT*) lineage of genes (Magalhaes *et al*., 2004; Navakode *et al*., 2009; Duan *et al*., 2022). Narrowing to sorghum (*Sorghum bicolor*), a locus named *Alt SB* underlying the *SbMATE* (gene responsible for Aluminium tolerance) has been comparatively mapped on chromosome 3. However, it is not known whether sorghum populations solely rely on this locus or variant Aluminium tolerance genes as noted among members of grass family possessing conserved genomic areas hosting Aluminium tolerance genes. The *Alt SB* locus contains multiple alleles that confers a remarkable range of variations in sorghum towards Aluminiun toxicity resistance (Caniato *et al*., 2007; Hufnagel *et al*., 2018) and normally co-segregates with Al ion dependent release of citric acid from the roots. In barley, *HvAACT1* a gene from MATE genealogy encodes for citric acid release in counteraction of Aluminium attack (Furukawa *et al*., 2007; Zhou *et al*., 2013).

Plant genes from the two families; MATE and ALMT encode synthesis of membrane transporter proteins also known as cellular membrane anionic channels that aid in secretion of organic acids by root cells on encountering solubilized Aluminium in acidic soils (Sasaki *et al.,* 2004; Du *et al*., 2021). Documented research findings indicate that physiological mechanisms are accountable for turning on of citric, malic and oxalic acids (Ma *et al*., 2014). When roots of tolerant sorghum genotypes encounter Al ions, they are induced to produce significant volumes of organic acids through the protein /anionic channels encoded by Al tolerance genes. The organic acids play a role of chelating Al^{3+} thus greatly reducing its chances of being attracted to oppositely charged components of cellular membranes and

the cell wall. Bound Al^{3+} results in to formation of compounds of Al and organic acids otherwise Alumino-carboxylate complexes that cannot move into the plant through the roots (Kochian *et al*., 2005; Chauhan *et al*., 2021).

Molecular markers serve a crucial role in pointing out presence or absence of genes of interest allowing for upgrading of genotypes. Different molecular markers exist and are categorized as either dominant or codominant. They have been designed and utilized in the crop world including cereals (Idrees and Irshad, 2014). Polymerase chain reaction (PCR) based markers for instance SSRs are advantageous as they are codominant, require minimal quantity of DNA, rapid and easy to use, hence, ideal for genotyping processes (Dokupilova *et al*., 2013). More often, AFLP and SSR markers are mostly applied in cereals owing to their power to widely cover the genome and provide large polymorphic information content at an affordable cost. SSR markers in linkage with genes responsible for tolerance to Aluminium toxicity in sorghum have been recognized by way of QTL analysis, genetic linkage and association mapping which have promised speedy generation of superior cultivars that yield more as well as tolerant to toxic Al levels (Magalhaes *et al*., 2007). The summary of the available information on genes in control of anion transporter proteins for Aluminium toxicity resistance in cereals is shown in Table 2.1.

Table 2.1: Genes encoding anion transporter proteins for Aluminium resistance in cereals

Gene	Plant Species	Reference
TaALMT1	Wheat (*Triticum aestivum* L.)	Sasaki *et al*., 2004
ScALMT1	Rye (*Secale cereale* L.)	Fontecha *et al.,* 2007
HvMATE	Barley (*Hordeum vulgare* L.)	Wang *et al.,* 2007
SbMATE	Sorghum (*Sorghum bicolor* L.)	Magalhaes *et al.,* 2007

Genes from MATE and ALMT families responsible for Aluminium toxicity tolerance.

2.8 Mechanisms of Al^{3+} tolerance in crop plants

Plants tolerate Aluminium through two major mechanisms; exclusion from its root tissues (external tolerance) and sequestration or conversion of Al^{3+} that has permeated the plasmalemma into non-toxic form, a mechanism also termed as internal resistance (Hartwig *et al.*, 2007; Wei *et al*., 2021).

The available external means of Aluminium tolerance in plants are; low cation exchange capacity otherwise curtailed substitution of existing cations by Al^{3+} on cell wall, selectiveness of membranes on what to move in and out of the cell, plant induced pH barrier formation at root area and exudation of chelates, ligands, phosphates and phenolic compounds (Kochian, 1995; Taylor, 1991; Yan *et al*., 2022). Al-chelating ligands, for instance organic acids secreted in good amounts by resistant genotypes creates a compound known as Alumino-carboxylate that is impossible for plants to absorb through the roots. Cereal species employ the efflux of the organic acids as a strategy to cope with Aluminium stress (Ryan *et al.,* 2009; Chauhan *et al*., 2021). Sorghum genotypes that are tolerant to the stress have been found to produce high volumes of citric acids.

The internal resistance mechanism operates within the cytoplasm of the cell through detoxification or paralysis of the mobility of Aluminium that has gained entry into the plant cells (Taylor, 1995; Wei *et al*., 2021). Evolution and boosting of Al tolerance anti-enzymes, vacuole sectioning and binding of Aluminium cytoplasmic matrix are the possible internal mechanisms. Tolerant sorghum genotypes utilize silicon accumulated in their systems to detoxify Al^{3+} that has gained entry into the cell. Silicon binds Al^{3+} leading to formation of Alumino-silicate complexes that are non-toxic to the cell (Hodson and Sangster, 1993).

2.9 Screening methods for Aluminium toxicity tolerance under soil acidic conditions

Selection and breeding for Al toxicity tolerance is integral towards increasing sorghum production in acid soils. This therefore demands for a rapid and reliable method for distinguishing between susceptible and resistant genotypes.

Variety of methods are available for assessing plant's tolerance to Aluminium toxicity. Greenhouse and laboratory-based procedures are mostly utilized since they are less destructive and suitable for screening plants from seedling to flowering stage. Two main methods of screening exist; soil and nutrient solution culture. Field evaluations, though necessary, are uneconomical and greatly interrupted by wide spatial variations in acidic soils which makes it hard to select the soil suitable for screening (Sikirou *et al*., 2015). Furthermore, restriction of root extension being the primary target of Al toxicity, roots are not easily accessed when using soil culture. Therefore, most of the screening work done for tolerance to Aluminium has been carried out using nutrient solution, however, the results from the two methods of screening are always consistent. (Moore *et al*., 1976; Baligar *et al.,* 1985; Richard *et al*., 2015).

For more rapid evaluations, bioassays such as tissue culture, marker assisted screening and haematoxylin staining are utilized, however, the outcomes from these techniques with exception of that from nutrient solution culture, largely, are not in close relationship with performance from the field. Thus, solution culture screening is known to be affordable, fast, reliable and more often used (Magnavaca *et al*., 1987). It enables easy access of roots, precise information on availed nutrients and pH control (Carver and Ownby, 1995). It has been utilized in evaluation of various crops for Al tolerance. They include, cowpea, barley, alfalfa, soybean, sorghum, tomato, rape and maize. Measurements of root growth is the most ideal way for assessing cereal tolerance to Aluminium (Hede *et al*., 2002).

CHAPTER THREE

IDENTIFICATION OF SORGHUM (*Sorghum bicolor* L. Moench) GENOTYPES TOLERANT TO ALUMINIUM TOXICITY USING MORPHOLOGICAL MARKERS

3.1 Abstract

Aluminium (Al) toxicity is cited among the major edaphic factors limiting sorghum production in soils with pH<5.0. It interferes with apical root cell multiplication and elongation, hence, inhibiting the absorption of water and nutrients which eventually leads to poor plant growth, yield and kernel quality. Liming, a most adopted remedy against Al toxicity has proved costly and unsustainable, however, identification and utilization of tolerant genotypes could feasibly aid in management of the menace. Magnavaca solution screening was used in phenotypic evaluation of 14 selected sorghum genotypes for tolerance to Al toxicity. Among the test genotypes were two standard checks, Al-sensitive *Seredo* and Al-tolerant *IS 41764*. The genotypes were laid out in a completely randomized design (CRD) with two levels of Al treatments; 0 and 148 µM bearing pH levels of 4.3. Fifty seeds for each genotype were pre-germinated in an incubator and measurements for initial seminal root lengths (ISRL) taken four days upon germination. Final seminal root lengths (FSRL) were taken 5 days after exposure of seedlings to Al and together with initial root lengths used to compute the net seminal root length (NSRL), relative seminal root length (RSRL), root tolerance index (RTI) and % responses to Al that were applied in establishing the tolerance status of the genotypes in reference to the provided standard scales. Results from ANOVA showed a significant ($P<.001$) decline in grand mean for net root lengths of seedlings at 148µM Al in reference to the control (0 µM Al) indicating persistence of Al toxicity as a serious constraint to sorghum growth in acidic soils. The genotypes exhibited significant ($P<.001$) variability in root growth under Aluminium stress. Genotypes *Gadam* and *Wagita* were found to be tolerant, *Macia* and *Kiboko local 2* moderately tolerant while a remainder of 8 genotypes expressed sensitivity. These results revealed high potential for Al tolerance among the available sorghum germplasm providing basis for selection of tolerant parental lines for breeding against the stress.

3.2 Introduction

Sorghum (*Sorghum bicolor* L. Moench) is a grass species cultivated majorly for its grain and fodder. It is recognized among the food security crops and serves to improve rural livelihoods and vulnerable communities living under harsh climatic conditions of the tropics (World Bank, 2005; Hadebe *et al*., 2017). The major sorghum growing areas in Kenya are dominated by acidic soils characterized by elevated levels of available Al ions (Al^{3+}) (Kisinyo *et al*., 2010). When soils are acidic (pH <5.5), Aluminium minerals are solubilized and become available but harmful to plants (Kochian *et al*., 2005). These soils are deficient of basic cations especially Ca^{2+} and Mg^{2+} lost through leaching of minerals and accelerated by poor capacity of soils to exchange cations (Kidd & Proctor, 2001).

Aluminium toxicity limits sorghum productivity in acidic soils with pH<5.0 (Magalhaes *et al.*, 2004; Rasheed *et al*., 2020). Root cell division and elongation are highly targeted by Aluminium (Al^{3+}) toxicity causing poor root development systems which leads to limited access to water and nutrients and eventually reduced sorghum yield and kernel quality. Amendment of soil acidity to manage Al toxicity is possible through addition of lime, however, this approach is generally uneconomical and not feasible to most farmers (Akinrinde, 2006; Matonyei *et al*., 2020).

Sorghum genotypes vary in tolerance to the toxic Al^{3+} (Ringo *et al*., 2011; Too, 2011) offering opportunity for identification and utilization of tolerant genotypes to improve crop's resilience and productivity in acidic soils. Tolerant sorghum genotypes employ physiological mechanisms to tolerate toxic levels of exchangeable Aluminium cations. The mechanisms are categorized into two; exclusion from its tissues (external tolerance) and sequestration or conversion of Al^{3+} that has permeated the plasmalemma into non-toxic form, a mechanism also termed as internal resistance (Hartwig *et al*., 2007; Wei *et al*., 2021). External tolerance is achieved through release of organic acids that bind Al^{3+} forming Alumino-carboxylate compounds that cannot be taken up by plants. Internal tolerance occurs when Al^{3+} that have entered into the cell are bound by silicon resulting to formation of Alumino-silicate complexes that are non-toxic (Hodson and Sangster, 1993).

The presence of variability in sorghum towards Aluminium tolerance motivates the screening, identification and utilization of tolerant genotypes which will not only enhance and sustain production but also aid in improvement of the crop's resilience against the edaphic stress and protect the soils from negative impacts of excessive liming.

3.3 Materials and methods

3.3.1 Plant materials

A total of 14 sorghum genotypes inclusive of Al tolerant and Al sensitive checks were screened and evaluated for Aluminium toxicity tolerance. The genotypes were selected on the basis of farmer and market preferences and agronomic values they are associated with. The names, sources and descriptions of the genotypes are outlined in Table 3.1.

Table 3.1: Sorghum genotypes selected and screened for Aluminium (Al^{3+}) tolerance

Entry	Genotype	Source	Description
1	KARI Mtama 1	ICRISAT-Kenya	Lowland Commercial Variety. High Yielding (4.0t/ha) & Early Maturing.
2	IS 8193	ICRISAT-Kenya	Sub-humid Commercial Variety. Early Maturing.
3	Seredo	ICRISAT-Kenya	Al Sensitive Check. Moderate Drought Tolerant. Yielding at 2.7t/ha.
4	Serena	ICRISAT-Kenya	Sub-humid Commercial Variety. Drought Tolerant.
5	Gadam	ICRISAT-Kenya	Lowland Commercial Variety. Early Maturing with High Malting Quality. Yielding stands at 3.15t/ha.
6	E 1291	ICRISAT-Kenya	Highland Dual-Purpose Variety. Good Beverage and Silage Quality.
7	E 6518	ICRISAT-Kenya	Highland Commercial Variety. Has High Forage Quality.
8	Macia	ICRISAT-Kenya	Lowland Commercial Variety. High Yielding (4.5t/ha) & Early Maturing.
9	Makueni Local	ICRISAT-Kenya	Lowland Land Race. Drought Tolerant.
10	IS 41764	ICRISAT-Kenya	Aluminium Tolerant Check.
11	Wagita	ICRISAT-Kenya	Sub-humid Local Cultivar. Early Maturing. Yields at 860kg/ha.
12	Kiboko Local 2	ICRISAT-Kenya	Lowland Local Early Maturing Variety.
13	Nakhadabo	ICRISAT-Kenya	Sub-humid Local Cultivar.
14	Tegemeo	ICRISAT-Kenya	Lowland Commercial Variety. Has High Brewing Quality.

ICRISAT= International Crops Research Institute for Semi-Arid Tropics. *Seredo* and *IS 41764* are Aluminium sensitive and Aluminium tolerant checks, respectively.

3.3.2 Experimental site, design and layout

Solution culture screening for phenotypic identification of sorghum genotypes tolerant to Aluminium toxicity was carried out in botany laboratory at University of Eldoret, Kenya. Discrimination between sensitive and tolerant genotypes was based on assessment of parameters related to root growth (Magnavaga *et al*., 1987). Genotypes were laid out in a completely randomized design (CRD) with two levels of Aluminium treatments; 0 and 148µM. The entire experiment was repeated once in order to improve the accuracy of the targeted data. The 0µM Aluminium treatment acted as a standard of comparison to the experimental component in the controlled environment.

3.3.3 Components of screening solution culture and screening chamber

The nutrient solution screening process followed a protocol by Magnavaca *et al*. (1987). In brief, the screening solution was made up of 7 stock solutions prepared prior to the start of the screening process (Magnavaca *et al*., 1987). Solution 7 which is the Aluminium source ($AlK(SO_4)_2.12H_2O$) was prepared and used fresh on day two of screening. Solution 1 was a Calcium source ($Ca(NO_3)_2.4H_2O$ and NH_4NO_3), Solution 2 a potassium source (KCl, K_2SO_4 and KNO_3 dissolved separately), solution 3 Magnesium ($Mg(NO_3)_2.6H_2O$) and solution 4 a Phosphorus source (KH_2PO_4). Solution 5 was an Iron chelate (H_3HEDTA) for 2 liters of stock solution. Iron nitrate as $Fe_3(NO_3)_3.9H_2O$ M.W 404.0 g/mole and Solution 6 a combination of micronutrients in forms of $MnCl_2.4H_2O$, H_3BO_3, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $Na_2MoO_4.2H_2O$ (to make 1 liter). Each tray holding 8 liters of distilled water received 24.64 ml of Calcium source, 18.48 ml of Potassium, 12.32 ml of Magnesium, 2.8 ml of Phosphorus, 12.32 ml of Iron, 6.16 ml micro nutrients and 12.8 ml Aluminium added to each of the half of the trays designated to receive the treatment.

The growth chamber was composed of trays holding nutrient solution, polystyrene rafts (32.5 cm x 32.5 cm) fixed with plastic cups (3.5 cm x 2.5 cm) suspending the seedlings' roots in the solution, source of illumination (550 µmol photons per square meters per second) and air tubes served by an electric air pump to aerate the nutrient solutions continuously for the entire period of screening as shown in Figure 3.1.

Figure 3.1: Screening chamber installed with trays holding screening solution, light source and air tubes supplied by an electric air pump.

3.3.4 Screening procedure and selection criteria for Aluminium tolerance

From each genotype, fifty seeds were set aside and surface sterilized in 1% sodium hypochlorite solution prepared by mixing sodium hypochlorite (NaOCl) and distilled water (dH_2O). Seeds were agitated on a mechanical shaker for about 10 minutes and repeatedly rinsed approximately 8 times with sterile distilled water (dH_2O). They were then sown in moist kitchen paper towels (20 cm x 20 cm) in autoclaved germination tins and placed in an incubator set at persistent temperature of 26 °C and relative humidity of 60% for a span of 96 hours. Upon the conclusion of the set duration, developed seedlings were moved to solution culture without Aluminium and left to stabilize for 24 hours. On the following day, measurements for initial seminal root lengths (ISRL) were taken, seedlings transferred back to the nutrient solution and thereafter Aluminium introduced to 50% of the trays.

Seedling selection was done and only those that had uniform germination transferred to grow in the nutrient solution for 5 days. Genotypes were grown in a completely randomized design (CRD) with 2 Al treatment levels; 0 and 148µM added in form of Al $K(SO_4)_2.12H_2O$). Sorghum screening for Aluminium toxicity tolerance was done at the level of 148µM, a concentration corresponding to 27% available Al^{3+} (Magalhaes *et al*., 2004). The pH for nutrient solution with and without Aluminium was adjusted to 4.3 using an electric pH meter under influence of HCl and NaOH at concentration of 0.1M each. Root length measurements were repeated five days after addition of Aluminium and assigned final seminal root lengths (FSRL).

The ISRL and FSRL were then used to compute the net seminal root length (NSRL), relative seminal root length (RSRL), root tolerance index (RTI) and % response to Al as described in equations 1-4 (Magnavaca *et al*., 1987; Caniato *et al*., 2007).

$$NSRL = FSRL - ISRL \quad \textbf{Equation 1}$$

$$RSRL = \frac{NSRL\ of\ Al\ treated\ plant}{NSRL\ of\ control\ (0\ Al)\ plant}\ x\ 100 \quad \textbf{Equation 2}$$

$$RTI = \frac{FSRL\ of\ Al\ treated\ plant}{FSRL\ of\ control\ (0\ Al)\ \ plant} \quad \textbf{Equation 3}$$

$$\%\ Response = \frac{FSRL\ of\ control\ plants - FSRL\ of\ Al\ treated\ plant}{FSRL\ of\ control\ (0\ Al)\ plant}\ x\ 100 \quad \textbf{Equation 4}$$

Selection criterion for Aluminium tolerance was based on NSRL, RSRL or RTI guided by the standard scales provided by Magnavaca *et al*. (1987) and given in Table 3.2. Results for net seminal root growth are more often considered reliable in discriminating between sensitive and tolerant lines (Magnavaca *et al*., 1987), however, relative variables such as RTI and RSRL are also important as they serve to reveal genetic capability of a genotype to tolerate Al stress (Raman and Gustafson, 2011).

Table 3.2: Standard scales applied in classification of genotypes for Aluminium toxicity tolerance

Parameter	**Scale 1**	**Scale 2**	**Scale 3**
	Tolerant	**Moderately Tolerant**	**Sensitive**
NSRL	≥1.70cm	1.50 – 1.69cm	≤1.49cm
RSRL	≥70%	50 – 69%	≤49%
RTI	>0.90	0.80 – 0.90	<0.80

Source: Magnavaca *et al*., 1987. NSRL= Net seminal root length. RSRL=Relative seminal root length. RTI= Root tolerance index.

3.3.4.1 Data collection

For determination of genotypes' response to Aluminium toxicity in laboratory, data was collected for a number of parameters related to primary root growth. The initial seminal root lengths (ISRL) were taken before the transfer of selected pre-germinated seedlings to the solution culture while final seminal root lengths (FSRL) were taken five days after introduction of Aluminium solution to half of the trays. Root length measurements were

achieved by gently placing the seedling along a ruler on a bench and taking the measurement between the base and the tip of the root.

3.3.4.2 Data analysis

Means for initial root length (ISRL), final root length (FSRL) and net root length (NSRL) were computed using MS excel spreadsheet and subjected to ANOVA using GenStat 14th version following the statistical model given as equation 5.

$Y_{ijk} = \mu + \tau_i + A_j + B_k + \tau A_{ij} + \tau B_{ik} + AB_{jk} + \tau AB_{ijk} + \varepsilon_{ijk}$ ..... **Equation 5**

where Y_{ijk} = observation/ response, μ = overall mean, τ_i = effects due to i^{th} genotype, Aj = effects due to j^{th} Aluminium concentration, B_k = effects due to k^{th} experiment cycle, τA_{ij} = effects due to interaction between i^{th} genotype and j^{th} Aluminium concentration, τB_{ik} = effects due to interaction between i^{th} genotype and k^{th} experiment cycle, AB_{jk} = effects due to interaction between j^{th} Aluminium concentration and k^{th} experiment cycle, τAB_{ijk} = effects due to interaction between i^{th} Genotype, j^{th} Aluminium concentration and k^{th} experiment cycle, ε_{ijk} = random error (deviation from the observed value).

Since data for relative root length (RSRL), root tolerance index (RTI) and % Response to Aluminium were proportions, computed mean performances of the genotypes were transformed before subjecting to ANOVA following the statistical model presented as equation 6.

$Y_{ij} = \mu + \tau_i + B_j + \tau B_{ij} + \varepsilon_{ij}$ ..... **Equation 6**

where Y_{ijk} = observation, μ = overall mean, τ_i = effects due to i^{th} genotype, B_j = effects due to j^{th} experiment cycle, τB_{ij} = effects due to interaction between i^{th} genotype and j^{th} experiment cycle, ε_{ij} = random error.

Means separation was done using least significant difference (LSD) test at 5% level of significance in respect to equation 7.

$$\text{LSD} = t_{\frac{\alpha}{2},\ error\ df} \times \text{SED} \quad \text{..... Equation 7}$$

where t $\frac{\alpha}{2}$, is the *t* value for a significance level of α/2, *error df* is the number of degrees of freedom in the error term of the analysis of variance. SED is the standard error of difference.

3.4 Results

3.4.1 Variability of sorghum genotypes for root growth traits under Aluminium stress

Results from ANOVA revealed that genotypes differed significantly (*P<.001*) in response to Aluminium treatment for important quantitative traits assessed. Mean square ratios for effect of genotype were significant (*P<.001*) for initial seminal root length (ISRL). The effects due to genotype, Aluminium treatment and their interactions were significant (*P<.001*) for final seminal root length (FSRL) and net seminal root length (NSRL) suggesting that the nutrient solution culture influenced genotypic response to Aluminium treatment levels (Table 3.3).

Table 3.3: Analysis of variance for ISRL, FSRL and NSRL among the sorghum genotypes at 0 and 148µM Aluminium

Source of variation	DF	ISRL		FSRL		NSRL	
		MS	F pr.	MS	F pr.	MS	F pr.
Genotype	13	29.909	<.001	55.174	<.001	6.472	<.001
Al Concentration	1	0.108	0.671	99.723	<.001	93.266	<.001
Experiment Cycle	1	0.71	0.276	2.662	0.21	0.622	0.414
Genotype. Al Conc.	13	0.194	0.988	3.829	0.008	3.070	<.001
Genotype. Cycle	13	3.248	<.001	3.942	0.006	8.823	0.568
Al Conc. Cycle	1	0.751	0.263	1.84	0.297	0.240	0.611
Genotype. Al Conc. Cycle	13	0.259	0.956	1.099	0.807	0.627	0.786
Residual	224	0.597		1.681		0.928	
Total	279						

DF= Degree of freedom. MS= Mean squares. ISRL= Initial seminal root length. FSRL= Final seminal root length. NSRL= Net seminal root length. Al Conc.=Al concentration.

Genotypic effect was also highly significant (*P<.001*) for relative seminal root length (RSRL) and root tolerance index (RTI) and significant (*P<0.05*) for percentage response to Aluminium (% reduction in root growth under Al stress) (Table 3.4).

Table 3.4: Analysis of variance for RSRL, RTI and % Response among the sorghum genotypes under Aluminium stress

Source of variation	DF	RSRL		RTI		%RESPONSE	
		MS	F pr.	MS	F pr.	MS	F pr.
Genotype	13	0.3008	<.001	0.0642	<.001	0.1682	0.021
Experiment Cycle	1	0.0349	0.539	0.0299	0.199	0.0706	0.352
Genotype. Cycle	13	0.0595	0.810	0.0206	0.328	0.0510	0.825
Residual	112	0.0920		0.0179		0.0810	
Total	139						

DF= Degree of freedom. MS= Mean squares. RSRL= Relative seminal root length. RTI= Root tolerance index. % Response= Percentage response to Al.

3.4.2 Mean performance of the genotypes

Generally, the effect of Aluminium treatment on final root length (FSRL) and net root length (NSRL) was highly significant (*P<.001*). Significant root growth reductions were recorded at 148µM level of Aluminium. There was considerable decline in overall means for final and net seminal root growth of the genotypes at 148µM Al in respect to the controls (genotypes at 0 µM Al) (Table 3.5). An overall reduction of 1.16 cm was recorded in net root growth due to exposure of the seedlings to Aluminium. This clearly indicated that Aluminium stress restricted growth of roots among the sorghum genotypes in acidic nutrient solution.

Table 3.5: Overall genotypic root growth means across the Aluminium treatments

Al treatment (µM)	ISRL (cm)	FSRL (cm)	NSRL (cm)
0	2.849	5.323	2.474
148	2.779	4.129	1.319
LSD $_{(0.05)}$	**0.182**	**0.808**	**0.227**

ISRL= Initial seminal root length. FSRL= Final seminal root length. NSRL= Net seminal root length.

Genotypic variability in tolerance to Aluminium toxicity was evident among the screened sorghum genotypes since their root growth particularly net root growth (NSRL) varied significantly (*P<.001*) in the nutrient solution with Aluminium. Based on the net seminal root length at 148µM Al and in reference to the provided standard scales, genotypes *Gadam* and *Wagita* were identified to be tolerant with net root lengths of 2.61 cm and 1.71 cm, respectively. Genotype *Gadam* significantly outperformed the tolerant check *IS 41764* whose net root length was 1.74 cm and differed significantly from its tolerant counterparts. Genotypes *Macia* and *Kiboko local 2* were moderately tolerant with net seminal root

lengths of 1.53 cm and 1.62 cm, respectively and did not differ significantly from each other. The rest of the genotypes had their root growth inhibited by the Aluminium treatment where genotypes *E 6518, IS 8193* and *Tegemeo* with net root lengths of 0.43 cm, 0.57 cm and 0.98 cm in the same order were noted to be highly sensitive to the availed concentration of Al^{3+} in comparison with the sensitive check *Seredo* (Table 3.6).

Table 3.6: Effects of Aluminium concentrations and genotypes on NSRL after 5 days of screening in nutrient solution culture (pH 4.3)

Genotype	NSRL (cm)		Tolerance Status
	0 µM Al	148 µM Al	
Gadam	2.98	2.61	T
IS 41764	2.10	1.74	T
Wagita	1.70	1.71	T
Kiboko Local 2	2.71	1.62	MT
Macia	2.78	1.53	MT
KARI Mtama 1	4.30	1.39	S
Serena	2.37	1.38	S
Makueni Local	2.14	1.21	S
E 1291	3.31	1.11	S
Nakhadabo	2.23	1.10	S
Seredo	3.18	1.09	S
Tegemeo	2.20	0.98	S
IS 8193	1.42	0.57	S
E 6518	1.21	0.43	S
General Mean	**2.47**	**1.31**	
LSD (P=0.05)	**0.25**	**0.14**	
CV %	**4.7**	**4.9**	
±SE	**0.96**	**0.56**	

NSRL= Net Seminal Root Length. T= Tolerant; MT= Moderately Tolerant and S= Sensitive. Scales of classification: T; ≥1.70cm. MT; 1.50-1.69cm. S; ≤1.49cm. *IS 41764* and *Seredo* are Al tolerant and Al sensitive checks, respectively.

Genotypes *E 6518* and *IS 8193* also recorded significantly lower net root growth at 0µM Al (Table 3.6) revealing their additional sensitivity to acidity (H^+) of the solution culture itself. The tolerant genotypes had insignificant differences on their net root growth at both levels of Aluminium. Their performance at both 0 and 148µM Al were more less the same bringing out their aspect of tolerance. The genotypes sustained relatively high root growth in presence of Al toxicity.

Relative seminal root length (RSRL), root tolerance index (RTI) and percentage response to Al were also applied as alternative parameters to establish the Aluminium tolerance status for the sorghum genotypes. Similar to net seminal root length, genotypes exhibited significant (*P<.001*) variability on these traits under Al stress. Genotypes *Gadam* and *Wagita* maintained their Aluminium tolerance status by posting highest mean relative root growth scores of 88.1% and 96.83%, respectively (Table 3.7). The figures differed significantly from 82.99% relative root growth score for the Al tolerant check *IS 41764* at *P=0.05*. Moderately tolerant genotypes *Macia* and *Kiboko local 2* scored relative root lengths of 54.95% and 59.43%, respectively, although, there was no significant difference between them. Genotypes *E 6518, E 1291* and *IS 8193* with relative seminal root lengths of 35.73%, 37.33% and 33.88% in the same order had no significant difference from sensitive check *seredo* whose relative seminal root length (RSRL) score stood at 34.28% (Table 3.7).

Genotypes with root tolerance index scores >0.90 were considered tolerant while those with scores <0.80 categorized as sensitive. In this regard, tolerant genotypes *Gadam* and *Wagita* emerged with tolerance index scores of 0.95 and 1.09, respectively. Genotype *Wagita* scored significantly higher root tolerance index than the tolerant check *IS 41764* whose score stood at 0.94. Moderately tolerant genotypes *Macia* and *Kiboko local 2* had their Aluminium tolerance status constant with indices of 0.84 and 0.83, respectively. Genotypes *IS 8193*, *E 1291* and *KARI Mtama 1* were noted to be more sensitive in comparison with the sensitive check *Seredo* whose root tolerance index was 0.68 (Table 3.7). Genotype *Wagita* having significantly higher root tolerance index pointed to its possible ability to tolerate Aluminium concentration higher than 148µM.

Generally, tolerant genotypes responded lowly (<30%) to the Aluminium treatment (148µM) while highly sensitive genotypes had responses >60%. Low response to Aluminium by the tolerant genotypes translated to their low percentage reduction in root growth in presence of Al toxicity. Tolerant genotypes *Gadam*, *Wagita* and *IS 41764* responded at 16.37%, 21.73% and 17.02%, respectively. Moderately tolerant genotypes *Macia* and *Kiboko local 2* responded to the Aluminium treatment at slightly below the

grand mean of 42.46%. They posted responses of 41.02% and 37.58%, respectively. Scores that did not differ significantly from each other. Sensitive genotypes including *IS 8193*, *KARI Mtama 1*, *E 6518* and *E 1291* together with the sensitive check *Seredo* had their percentage responses at over 60%. About 78% of the susceptible genotypes had over 50% response to Aluminium treatment translating to their high % reduction in root growth under the stress (Table 3.7).

There was evidence that Aluminium inhibited root growth/ elongation among the screened sorghum genotypes as there was considerable reduction of net root growth at 148µM Al as compared to the scores of the genotypes at 0 µM Al. There was overall net root growth reduction difference of 1.16cm equivalent to 46.69% reduction in root growth as a result of exposure of genotypes to Al stress.

Table 3.7: Effects of Aluminium concentrations and genotypes on RSRL, RTI and % Response after 5 days of screening in nutrient solution culture (pH 4.3)

Genotype	RSRL (%)	RTI	% Response	Tolerance status
Gadam	88.10	0.95	16.37	T
IS 41764	82.99	0.94	17.02	T
Wagita	96.83	1.09	21.73	T
Kiboko local 2	59.43	0.83	37.58	MT
Macia	54.95	0.84	41.02	MT
KARI Mtama 1	31.99	0.66	67.92	S
Serena	48.08	0.75	46.88	S
Makueni local	47.97	0.76	42.85	S
E 1291	37.33	0.62	62.66	S
Nakhadabo	47.42	0.67	50.58	S
Seredo	34.28	0.67	65.61	S
Tegemeo	44.98	0.69	54.95	S
IS 8193	33.88	0.62	66.07	S
E 6518	35.73	0.75	64.27	S
General Mean	**49.72**	**0.76**	**42.46**	
LSD (P=0.05)	**4.69**	**0.08**	**4.12**	
CV %	**17.6**	**31.3**	**17.2**	
±SE	**2.01**	**0.02**	**1.93**	

RSRL= Relative Seminal Root Length. RTI= Root Tolerance Index. % Response= % Reduction in root growth under Al stress. T= Tolerant; MT= Moderately Tolerant and S= Sensitive. Scales of classification: RSRL: ≥70%; Tolerant. 50-69%; Moderately Tolerant. ≤ 49%; Sensitive. RTI: >0.90; Tolerant. 0.80-0.90; Moderately Tolerant. <0.80; Sensitive. *IS 41764* and *Seredo* are Al tolerant and Al sensitive checks respectively.

3.4.3 Correlation coefficients among the root growth traits of the screened sorghum genotypes

Correlation analysis to establish the relationship among the assessed traits of the sorghum genotypes under Al stress revealed existence of strong positive and negative correlations with exceptions of zero (0) and weak or non-significant associations between some traits (Table 3.8). Strong (*P <.001*) positive correlations were evident between ISRL, FSRL and NSRL. There were also strong (*P <.001*) positive correlations between FSRL and RTI, NSRL and RSRL, NSRL and RTI and RSRL and RTI. Strong (*P< .001*) negative correlations were observed between NSRL and % RESPONSE, RSRL and % RESPONSE and RTI and % RESPONSE (% reduction in root growth under Al stress) (Table 3.8).

Table 3.8: Correlation analysis among root growth traits of sorghum genotypes grown under Aluminium stress (148µM Al)

TRAITS	**ISRL**	**FSRL**	**NSRL**	**RSRL**	**RTI**	**% RESPONSE**
ISRL	-					
FSRL	0.92**	-				
NSRL	0.33**	0.68**	-			
RSRL	0.00	0.22^{ns}	0.54**	-		
RTI	0.15^{ns}	0.32**	0.46**	0.79**	-	
% RESPONSE	0.00	-0.22^{ns}	-0.54**	-1.00**	-0.79**	-

ISRL= Initial seminal root length. FSRL= Final seminal root length. NSRL= Net seminal root length. RSRL= Relative seminal root length. RTI= Root tolerance index. % RESPONSE= % Reduction in root growth under Al stress. Significance codes: **= *<.001*. ns= non-significant.

3.4.4 Root morphology

Observations made on root appearances gave a clear picture on impacts of Al toxicity on their growth and development. Contrary to tolerant genotypes, highly sensitive sorghum genotypes had their roots shorter, stubby and brittle (Figure 3.2). Genotypes *IS 8193*, *E 6518* and *E 1291* apart from exhibiting the latter characteristics had brown root coloration at the tips, an attribute also associated with Al attack among susceptible plant species.

Figure 3.2: Effects of 148µM Al on root growth and development. Tolerant genotype Gadam has longer roots compared to sensitive E 6518.

Generally, there was considerable reductions on the rate of development of lateral roots in both resistant and susceptible sorghum genotypes grown in solution culture with Aluminium (Figure 3.3). This further revealed the impacts of toxicity of Al^{3+} on root growth and development. Complete reduction in root volume among highly sensitive genotypes finally restricts plants from efficiently accessing water and mineral nutrients from the rhizosphere hence poor growth and eventually yield and quality of the produce.

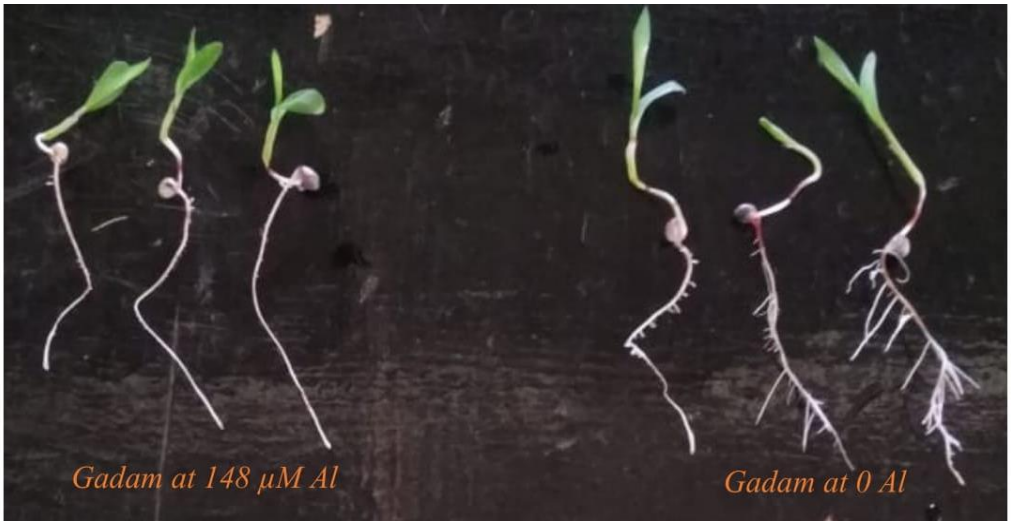


Figure 3.3: Effects of Aluminium on root lateralization/ branching. Genotype Gadam at 0 µM Al has developed lateral roots at higher rate unlike at 148µM Al.

At 0µM Al, some sensitive genotypes maintained higher root growth than a section of genotypes that were found to be tolerant to 148µM Al. For instance, genotypes *KARI Mtama 1, E 1291* and *Seredo* a sensitive check scored significantly higher root growth rates in comparison to the tolerant genotypes at the same level of Al treatment (Table 3.4 and Figure 3.4). This further enhanced the understanding on limitations of Al toxicity to sorghum growth in acidic soils.

Figure 3.4: Root growth and development performance for tolerant and sensitive genotype in solution culture without Aluminium (0µM Al). Seredo an Al sensitive genotype has longer roots compared to the tolerant genotype Gadam.

3.5 Discussion

Solution culture screening through measurements of primary root growth made it possible to classify the selected sorghum genotypes in to three phenotypic classes of Aluminium tolerance. Measurements of root length in hydroponic screens is considered the best scheme in study and identification of Aluminium tolerant lines in maize and sorghum (Hede *et al*., 2002). Parameters computed and applied in determination of genotypes' tolerance status revealed the existence of significant phenotypic variations among sorghum

genotypes for the trait. This was in agreement with earlier findings by Mariano and Keltjens (2003), Magalhaes *et al*. (2004), Caniato *et al*. (2007), Ringo *et al*. (2011) and Too (2011 & 2014) that sorghum genotypes vary significantly in tolerance to Al toxicity.

At 148µM level of Aluminium, genotypes *Gadam, Wagita, Macia* and *Kiboko local 2* scored relatively higher root growth with *Gadam* significantly (*P<.001*) outperforming the tolerant check *IS 41764.* This was an indication that the genotypes sustained root growth in presence of Aluminium stress. Hede *et al*. (2001) and Ma *et al*. (2014) stated that tolerant plant genotypes maintain relatively higher root growth in existence of root growth inhibitors such as Aluminium toxicity as they are triggered to release large volumes of citric and malic acids in response to rising levels of free Al^{3+} in the soil solution at pH<5.0.

Generally, the tolerant genotypes exhibited lower % reduction in root growth under Aluminium stress (<30% response to 148µM Al) and had their root tolerance indices >0.90. Moderately tolerant genotypes responded slightly below the grand mean of 42.46% with root tolerance indices scores of between 0.80-0.90. Root tolerance index (RTI) is an important indicator for genetic capability of a plant genotype to withstand root growth suppression (Raman and Gustafson, 2011). It is worth to note that slow root growth in presence of Aluminium toxicity should not be directly equated to sensitivity. In presence of root growth inhibitors, some genotypes have shown slow growth of roots yet have proved more tolerant than genotypes with fast root growth owing to their higher root tolerance indices (Deborah and Tesfaye, 2003). For the case of this experiment, genotype *Gadam* with highest net seminal root length was perceived to be highly tolerant as it sustained the highest root growth rate in nutrient solution with Aluminium, however, it stood out clearly that *Wagita* was more tolerant owing to its significantly (*P<.001*) higher root tolerance index compared to its tolerant counterparts including the tolerant check. The genotype posted a root tolerance index (RTI) of 1.09 translating to its genetic ability to tolerate Aluminium concentration higher than 148µM.

Root growth was notably inhibited by the Aluminium treatment among the sensitive genotypes which included *KARI Mtama 1, IS 8193, Serena, E 1291, E 6518, Makueni local,*

Nakhadabo and *Tegemeo*. Genotypes *IS 8193* and *E 6518* registered significantly (*P<.001*) lower net root growth in comparison to the sensitive check *Seredo* and showed highest % reduction in root growth under Al stress (>60% response to 148µM level of Aluminium). The two genotypes also performed significantly poor at 0µM Al indicating that they also reacted negatively to the low pH (4.3) of the screening solution. Moore (1974) and Islam *et al*. (1980) stated that not only Al^{3+} affects root growth in strongly acidic soils but also hydrogen ions (H^{+}). That apart from existing root growth inhibitors in acidic soils, acidity itself affects plant growth and development as genotypes of a given species react differently to various soil pH levels. The observed significant (*P<.001*) reduction in overall net root growth for seedlings exposed to 148 µM Al as compared to controls (seedlings at 0µM Al) was clear evidence that Al toxicity remains to be a constraint of economic importance to sorghum production in acidic soils (Too, 2014).

The study also revealed a general limitation on the rate of formation of lateral roots in both sensitive and tolerant sorghum genotypes treated with Aluminium as noted earlier by Foy (1992) and Rahman and Upadhyaya (2021). Poor root lateralization leads to inefficient utilization of water and nutrients within the rhizosphere which ultimately lowers the yield and quality of the produce as a result of poor growth and development of the crop. From these observations, Aluminium toxicity presented itself as a double tragedy to growth and development of plants in that apart from its impacts on root permeability and elongation, it also restricts root lateralization a key morphological feature of a normal and efficient root system.

Genotypic differences in tolerance to Al toxicity among sorghum accessions are attributed to variation in levels of expression of tolerance mechanisms. When tolerant sorghum genotypes encounter Al stress, they are triggered to produce considerable volumes of citric acids compared to susceptible genotypes (Ryan *et al.*, 2009; Pellet *et al*., 1995; Chauhan *et al*., 2021). The plant roots aided by cellular membrane channels are induced by Aluminium ions to release the organic acid (Ma *et al.*, 2001; Du *et al*., 2021). The organic acids bind Al^{3+} forming Alumino-carboxylate complexes that cannot be absorbed through the plant roots. This is an external mechanism employed by tolerant sorghum genotypes aimed at reducing availability or mobility of Aluminium ions (Al^{3+}) to counter the toxicity.

Sequestration or conversion of Al^{3+} that has permeated the plasmalemma into non-toxic form, a mechanism also referred to as internal tolerance is alternatively available for tolerant sorghum genotypes to cope with the stress (Hartwig *et al*., 2007; Taylor, 1995; Wei *et al*., 2021). In an event of successful entry of Aluminium ions in to the cell, Silicon accumulated in the system of sorghum plant assumes a role of detoxification through formation of Alumino-silicate complexes that are non-toxic to the cells (Hodson and Sangster, 1993).

Aluminium toxicity mainly limits root growth and development in strongly acidic soils. Restriction of root growth through interference on normal multiplication and elongation of cells at the tip of the root is the most common symptom of Aluminium attack in plants (Jones *et al.,* 2006). Aluminium ions (Al^{3+}) are known to restrict growth of roots in susceptible species and in severe conditions, roots appear brittle and very stubby (Mossor-Pietraszewska *et al*., 1997; Gupta *et al*., 2013) a phenomenon that was noted in genotypes *E 6518, E 1291*and *IS 8193*. When Al^{3+} enters into the root cells and within the membranes, it is attracted to the opposite charges of the phospholipid layers causing stiffness, altered membrane functioning and intensified oxidative tensions (Jones *et al*., 2006). Such biological root cell adjustments result into poor growth of roots, thus, limited absorption of water and nutrients, ultimately, plant's tolerance to drought and productivity are affected (Miguel *et al*., 2010).

Success in breeding programs against Aluminium toxicity in sorghum largely rely on availability of adequate genetic differences and knowledge on traits that exhibit relationship under Aluminium stress. Research findings by Roy and Bhadra (2014), Matonyei *et al*. (2020) and Richard *et al*. (2015) recommends that breeding for Al tolerance should focus on root growth traits. In this study, net root growth (NSRL) and relative root growth (RSRL) correlated positively with root tolerance index (RTI). This showed that good tolerance index will result in increase in root growth. This was reflected in genotypes *Gadam*, *Wagita* and the tolerant check *IS 41764* with high root tolerance indices and subsequent superiority in net root growth under Aluminium stress. These genotypes may be of great importance in breeding sorghum for Al toxicity tolerance.

3.6 Conclusion

Screened sorghum genotypes exhibited significant phenotypic variability in tolerance to Al toxicity. The availed level of Aluminium significantly reduced the growth of roots among the genotypes indicating the persistence of Al toxicity as a dominant constraint to production of sorghum. The study enhanced the understanding on limitations of Al toxicity to sorghum growth in acidic soils and necessity for revamped research towards arrival at sustainable interventions which includes but not limited to extensive collection of germplasm, screening and breeding against the edaphic impediment only preceded by drought in limiting crop production. Utilization of tolerant sorghum genotypes will not only contribute to improved and sustained production but also protect our soils from negative impacts of excessive liming.

CHAPTER FOUR

VALIDATION OF SORGHUM (*Sorghum bicolor* L. Moench) GENOTYPES WITH ALUMINIUM TOLERANCE GENES USING SSR MARKERS

4.1 Abstract

Genetic markers have been extensively utilized in modern crop improvement to aid in early precise detection of desirable genes within a short breeding cycle. This study aimed at validating sorghum genotypes with Aluminium (Al^{3+}) tolerance genes using specific simple sequence repeats (SSR) markers *Sb5_236*, *Sb6_342*, *Sb6_34* and *Xtxp34*. DNA from each of the 14 selected genotypes was isolated following the CTAB protocol and quantified using a nanodrop spectrophotometer before subjecting to polymerase chain reactions (PCR) and subsequent detection and analysis of patterning of products through agarose gel electrophoresis. Genotypes with DNA band pattern identical to the tolerant check were grouped as Al tolerant while those with pattern identical to the sensitive check grouped as Al sensitive. Electrophoresis of PCR amplicons showed that marker *Xtxp34* was more discriminative and specific towards revelation of sorghum genotypes with gene variants linked to Al toxicity tolerance. Basing on amplifications due to marker *Xtxp34*, cultivars *Gadam, Wagita* and *Macia* had identical band pattern to the tolerant check *IS 41764* while the remaining genotypes *KARI Mtama 1, IS 8193, Serena, E 1291, E 6518, Makueni Local, Nakhadabo, Kiboko Local 2* and *Tegemeo (2KX17/B/1)* exhibited a band pattern same to the sensitive check cultivar *Seredo*. The study showed existence of genetic potential for Aluminium tolerance in sorghum germplasm. The SSR marker *Xtxp34* was highly specific and discriminative hence could be recommended for marker assisted breeding in selection of sorghum germplasm with Aluminium toxicity tolerance genes.

4.2 Introduction

Molecular screening of sorghum (*Sorghum bicolor* L.) is necessary in order to confirm genotypes with genes responsible for Aluminium (Al) toxicity tolerance since quantitative traits are prone to influence by the environment (Anderson *et al*., 2014). Phenotyping and genotyping are paramount; however, the latter serves to either validate or invalidate the findings of the former. Through genotyping, visible or quantifiable desirable characteristics of a species or genotypes within a species can be determined to be expression of its genetic makeup. It improves chances of bringing out the true genetic variability of the test genotypes under varying environments. SSR markers have been widely used in genotyping since they are easy to work with, are codominant, specific to a given locus, highly variable, informative and reproducible (Powell *et al*.,1996; Ahmad *et al*., 2018).

Sorghum is a grass species cultivated largely for its kernel and fodder. It is native to Africa, ranked fifth among the most crucial cereal crops of the globe (Shahbandeh, 2021). In sub-Saharan Africa sorghum comes second to maize as the major source of day-to-day energy requirements for millions of people (Bhosale *et al*., 2011). The crop is cultivated throughout the tropical and subtropical environments since it is drought tolerant and stands out for its lower costs of production (Dogget, 1988; Shewale and Pandit, 2011). Regardless of the golden attributes, its productivity and production are limited by a wide range of constraints including soil acidity and salinity that are responsible for approximately 0.52 t ha^{-1} reduction in yield (FAO, 2011, Too, 2014).

Aluminium toxicity limits sorghum production in soils with pH lower than 5.0 (Shao, 2010; Rasheed *et al*., 2020). Acidic soils are widely distributed in major Kenyan sorghum growing areas that cover parts of Northern Rift Valley, Western and Nyanza regions (Kanyanjua *et al*., 2002; Osundwa, 2013). The toxic Al^{3+} in acidic soils (pH<5.5) inhibit root cell division, elongation and root membrane permeability, thus, lowering intake of water and nutrient minerals particularly Phosphorus (P). This results into poor growth leading to reduced yield and quality of sorghum grains (Samac and Tesfaye, 2003; Cicero *et al*., 2018). Application of lime to mitigate the problem has proved costly and unsustainable. This therefore calls for extensive collection, screening, identification and

utilization of genotypes with Aluminium tolerance in order to achieve crop's resilience and sustainability in production. The study aimed at validating sorghum genotypes with genes responsible for Al toxicity tolerance using specific SSR markers.

4.3 Materials and methods

4.3.1 Experimental site

Genetic screening and evaluation of the selected sorghum genotypes for Al^{3+} tolerance was conducted in the biotechnology laboratory at University of Eldoret, Kenya.

4.3.2 Genotypes used in the study

The inbred genotypes screened and evaluated for Aluminium toxicity tolerance were collected from International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Kenya. The genotypes were selected on basis of farmer and market preferences and agronomic values they are associated with. Genotypes *IS 41764* and *Seredo* were included as Al tolerant and Al sensitive checks, respectively (Table 4.1).

Table 4.1: Sorghum genotypes subjected to SSR marker screening for Al toxicity tolerance

Entry	Genotype	Description
1	KARI Mtama 1	Lowland Commercial Variety. High Yielding (4.0t/ha) & Early Maturing.
2	IS 8193	Sub-humid Commercial Variety. Early Maturing.
3	Seredo	Al Sensitive Check. Moderate Drought Tolerant. Yielding at 2.7t/ha.
4	Serena	Sub-humid Commercial Variety. Drought Tolerant.
5	Gadam	Lowland Commercial Variety. Early Maturing with High Malting Quality. Yielding stands at 3.15t/ha.
6	E 1291	Highland Dual-Purpose Variety. Good Beverage and Silage Quality.
7	E 6518	Highland Commercial Variety. Has High Forage Quality.
8	Macia	Lowland Commercial Variety. High Yielding (4.5t/ha) & Early Maturing.
9	Makueni Local	Lowland Land Race. Drought Tolerant.
10	IS 41764	Aluminium Tolerant Check.
11	Wagita	Sub-humid Local Cultivar. Early Maturing. Yields at 860kg/ha.
12	Kiboko Local 2	Lowland Local Early Maturing Variety.
13	Nakhadabo	Sub-humid Local Cultivar.
14	Tegemeo	Lowland Commercial Variety. Has High Brewing Quality.

SSR= Simple Sequence Repeats. Genotypes *Seredo* and *IS 41764* are Al sensitive and Al tolerant checks, respectively.

4.3.3 Plant tissue sampling

To acquire root material for DNA extraction, ten seeds for each of the 14 selected sorghum genotypes were planted in a labelled plastic pot measuring 25 cm x 15 cm filled with approximately 500 g of garden soil, watered to field capacity and allowed to grow for a period of 14 days in a glass house. Prior to root harvesting, seedlings were watered thoroughly which soaked the soil around the rhizosphere making it easier to uproot the seedling. Root tissues were then obtained from seedlings of each genotype, rinsed and stored at a temperature of -20 ºC to maintain the integrity of DNA.

4.3.4 Isolation of Genomic DNA

DNA from each genotype was extracted following the cetyltrimethylammonium bromide (CTAB) protocol (Mace *et al*., 2003). Approximately 0.4 g of root tissue obtained from a 14 - day old seedling was ground thoroughly using a mortar and pestle in 1 ml of extraction buffer. The extract was then transferred to 1.5 ml Eppendorf tube and 700 µl of CTAB extraction buffer and 20% sodium dodecyl sulfate (SDS) added then incubated at 65 °C in a water bath for 10 minutes and vortexed vigorously at intervals of 5 minutes. Approximately 160 µl of 5M Potassium acetate ($C_2H_3KO_2$) was added, vortexed well and centrifuged at 13,000 gravitational force for 10 minutes. Thereafter, 600 µl of the upper phase was withdrawn and to the supernatant equal volume of 24:1 ice cold chloroform: iso amyl alcohol was added, mixed gently by inversion and again centrifuged at 13,000 gravitational force for 5 minutes at room temperature (20 ºC). After centrifugation, the top aqueous layer was decanted carefully leaving the DNA pellet behind. The pellet was washed with 70% ethanol, air dried for 30 minutes and redissolved in 200 µl of 1X TE buffer and to this, approximately 2µl of RNase solution was added and incubated at 37 ºC for 1 hour before storage at -20 ºC.

4.3.5 DNA quantification

Extracted DNA was quantified using NanoDrop spectrophotometer 2000/2000c, Thermo Scientific. The Nanodrop spectrophotometer was standardized/ blanked using 2 µl of TE buffer (Tris- EDTA) then wiped using a lint free paper towel. 2 µl of each sample DNA was then quantified for its concentration in ng/µl and purity at absorbance ratio of

260/280nm. DNA with absorbance ratio of 1.8-2.0 was considered pure while any substance outside the range was re-extracted. DNA sample bearing absorbance ratio <1.8 upon quantification is considered impure as it may contain phenols, proteins or any other contaminants that absorb strongly near or at 280nm (Leninger, 1975).

4.3.6 Primer selection and optimization

The four specific simple sequence repeats (SSR) primers were selected from previous study on genetic markers linked to Al toxicity tolerance in sorghum by Too *et al.* (2018). The study recommended application of the SSR primers in screening of sorghum for tolerance to the stress. Primer optimizations were done by varying the concentrations of DNA and annealing temperatures while keeping other reagents constant. DNA concentration of 50 ng/µl in the final reaction volume and PCR conditions of denaturation temperature at 94 ºC for 5 minutes, annealing temperature at 50 ºC for 1 minute and final extension at 72 ºC for 7 minutes gave the best amplifications for all the primer pairs. Simple sequence repeats (SSRs) primer sets and their sequences for PCR reactions are provided in Table 4.1

Table 4.2: Simple sequence repeats (SSRs) primer sets used for PCR reactions

Locus Name	Primer Sequences (5′- 3′)	Repeat motif	Fragment Size (bp)	T_a (°C)
Sb5_236	F: GCC AAG AGA AAC ACA AAC AA R: AGC AAT GTA TTT AGG CAA CAC A	$(AG)_{20}$	160-208	55
Sb6_342	F: TGC TTG TGA GAG TGC CTC CCT R: GTG AAC CTG CTT TAG TCG ATG	$(AC)_{25}$	270-294	50
Sb6_34	F: AAC AGC AGT AAT GCC ACA C R: TGA CTT GGT AGA GAA CTT GTC TTC	$[(AC)/(GC)]_{15}$	188-208	55
Xtxp34	F: TGG TTC GTA TCC TTC TCT ACA G R: CAT ATA CCT CGT CGC TC	$(CT)_{29}$	365	55

Source: Too *et al.*, 2018. Bp= Base pair. $T_{a\ ^oC}$= Annealing temperature. F= Forward and R=Reverse.

4.3.7 Polymerase chain reactions

From each sample, the extracted DNA was diluted and subjected to polymerase chain reactions (PCR). Four gene specific forward and reverse specific primers namely *Xtxp34, Sb6_34, Sb6_342* and *Sb5_236* (Table 4.2) were used in amplification of DNA segments of interest from each of the fourteen sorghum genotypes. Each PCR reaction consisted 4µl

of Solis BioDyne Firepol Master mix, 1 µl forward primer, 1 µl reverse primer, 2.5 µl template DNA and 11.5 µl molecular water constituting 20 µl per reaction.

The Eppendorf Mastercycler ep gradient S Ag 22331 PCR machine program was set at 94 °C initial denaturation for 5 min, 94 °C denaturation for 30 secs, annealing temperature at 50 °C for 1 minute, 72 °C extension for 1 minute and final extension for 7 minutes for 35 cycles.

4.3.8 Fragment detection and band pattern analysis

Amplified DNA fragments were detected by running electrophoresis for 1 hour at 110 volts in 0.5X TBE buffer. DNA bands were visually analyzed on 2.5% agarose gel stained with 20µl ethidium bromide, observed under a U.V transilluminator and gel image captured using *BioDoc IT* gel documentation unit. Polymorphic band patterns were analyzed by comparing the band patterns between the test genotypes and the controls/ checks. The genotypes sharing the same band pattern with the tolerant check were considered in possession of genes responsible for Al tolerance, while those with pattern identical to the sensitive check grouped as genotypes lacking the genes conferring Al tolerance.

4.4 Results

Achievement of molecular screening and characterization of the selected sorghum genotypes for Aluminium tolerance followed the success in the preceding processes of DNA extraction, DNA quantifications, polymerase chain reactions and detection of the PCR products through agarose gel electrophoresis.

4.4.1 DNA extraction and quantification

Genomic DNA was successfully isolated from the root tissues of each genotype following the cetyltrimethylammonium bromide (CTAB) protocol (Mace *et al*., 2003). DNA quantifications carried out using a nanodrop spectrophotometer showed that the DNA samples from each genotype met the desired threshold of both quantity and quality as indicated in Table 4.3. Recommendations guided that a good quality DNA should have its score of absorbance ratio (260/280nm) not less than 1.8 (Leninger, 1975).

Table 4.3: Quantities and purity of DNA samples extracted from the selected sorghum genotypes for PCR reactions

Sample ID	DNA concentration (ng/µl)	Absorbance ratio (260nm/280nm)
KARI Mtama 1	83.9	1.94
IS 8193	166.2	2.08
Seredo	105.8	1.89
Serena	91.1	1.95
Gadam	14.4	2.03
E 1291	9.7	1.79
E 6518	10.8	1.86
Macia	158.2	1.96
Makueni Local	39.5	1.99
IS 41764	151.7	2.01
Wagita	30.5	1.80
Kiboko Local 2	96.1	2.09
Nakhadabo	39.0	1.88
Tegemeo	17.1	2.01

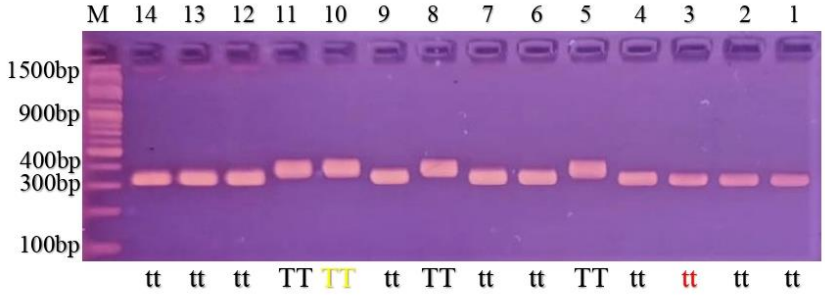
Key: ng/µl= nanograms per microliter. nm= nanometer. Recommended absorbance ratio (260nm/280nm) of good quality/pure DNA = 1.8-2.0.

4.4.2 PCR products detection and band pattern analysis

Polymorphic band patterns resulting from electrophoresis of DNA amplicons were analyzed by visually comparing the patterns between the test genotypes and the checks. The genotypes which shared the same band pattern with the tolerant check were grouped as Al tolerant (genotypes with genes responsible for Aluminium tolerance) while those with pattern identical to the susceptible check considered Al sensitive (genotypes without genes conferring Al toxicity tolerance). The specific SSR markers *Sb5_236, Sb6_342, Sb6_34* and *Xtxp34* were used for selection of Aluminium tolerant genotypes that shared the same band pattern with the tolerant check *IS 41764*.

Phenotypic evaluations of the sorghum genotypes had shown that the genotypes varied significantly (*P<.001*) on root growth in response to the Al treatment. Genotypes *Gadam* and *Wagita* were tolerant. *Macia* and *Kiboko local 2* were found to be moderately tolerant while a remainder of 8 genotypes expressed sensitivity with some performing significantly poor in comparison to the sensitive check *Seredo* (Table 3.6).

Electrophoresed amplicons of the genotypes in regard to the tolerant and susceptible controls showed that one out of the four applied SSR markers exhibited considerable polymorphism for the trait. The marker *Xtxp34* was then analyzed for band patterning between the screened genotypes and the checks to identify genotypes with gene variants responsible for Aluminium tolerance. Band pattern analysis of the genotypes with respect to the checks showed that genotypes *Gadam*, *Wagita* and *Macia* had the same band pattern to the tolerant check *IS 41764* while genotypes *KARI Mtama 1, IS 8193, Serena, E 1291, E6518, Makueni Local, Nakhadabo, Kiboko Local 2 and Tegemeo (2KX17/B/1)* had identical band pattern to the sensitive control *Seredo* (Figure 4.1).



1 – 14: Genotypes subjected to SSR marker analysis

TT: Genotypes tolerant to Al toxicity

tt: Genotypes sensitive to Al toxicity

TT: Tolerant check *IS 41764*

tt: Sensitive check *Seredo*

M: 100bp marker

Genotype 1-14: **1**. KARI Mtama 1, **2**. IS 8193, **3**. Seredo, **4**. Serena, **5**. Gadam, **6**. E 1291, **7**. E 6518, **8**. Macia, **9**. Makueni local, **10**. IS 41764, **11**. Wagita, **12**. Kiboko local 2, **13**. Nakhadabo and **14**. Tegemeo.

Figure 4.1: Gel electrophoresis of amplicons due to primer *Xtxp34*. Al tolerant genotypes have identical band pattern to tolerant check at 400bp while Al sensitive genotypes shared the same band pattern with sensitive check at about 330bp.

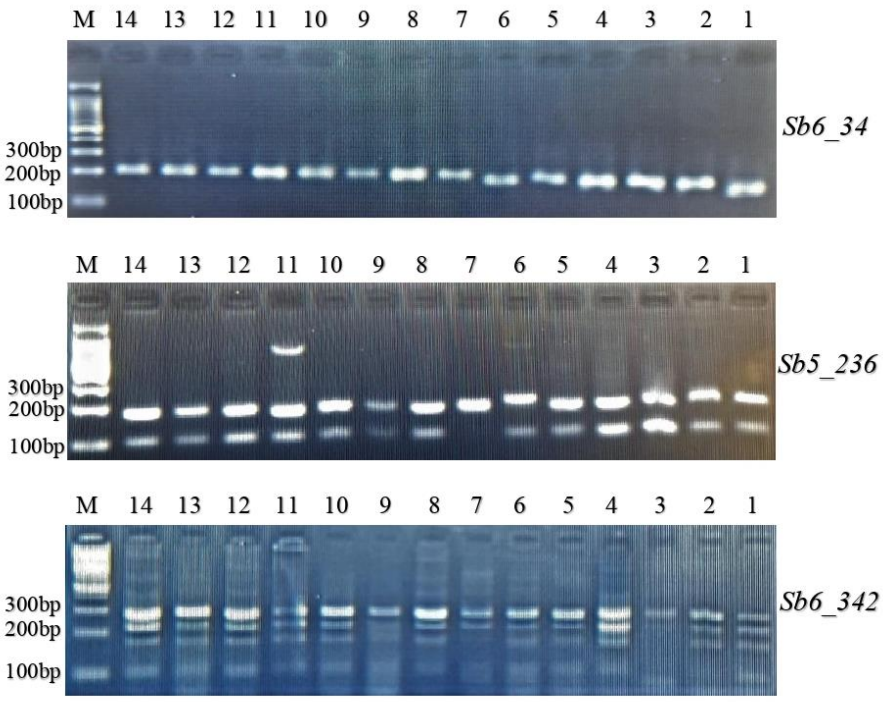
Genotype *Macia* that was noted to be moderately tolerant to Aluminium through phenotypic evaluations shared the same band pattern with the highly tolerant genotypes *Gadam, Wagita* and the tolerant check *IS 41764* (Figure 4.1). This indicated that the genotype contains Al tolerance gene, however, the level of expression of the gene may be

low or the highly tolerant genotypes could be in possession of multiple alleles otherwise referred to as additive gene effect contributing to the high tolerance to Al stress.

The results for the study revealed marker *Xtxp34* as most specific and discriminative in distinguishing between genotypes with and without gene variants responsible for Aluminium tolerance (Figure 4.1). The banding pattern due to this marker strongly associated the genotypes' morphological Al tolerance data with SSR allelic diversity making it highly recommendable for marker assisted selection (MAS) of sorghum for Al tolerance. The tolerant check *IS 41764* together with *Gadam*, *Macia* and *Wagita* amplified the expected tolerance loci at 400bp while sensitive genotypes as guided by sensitive check *Seredo* had their bands aligned at about 330bp (Figure 4.1).

Band pattern analysis also indicated that genotype *Gadam* had identical band pattern to the tolerant check *IS 41764* along the four markers. On the other hand, genotypes *KARI Mtama 1* and *IS 8193* presented banding pattern same as that for sensitive check *Seredo* across the markers. Contrary to results for nutrient solution screening, *Kiboko local 2* which was noted to be moderately tolerant genotype had its expected tolerance loci not amplified in respect to the markers.

Markers *Sb6_34*, *Sb5_236* and *Sb6_342* were not specific and discriminative enough to draw substantial conclusion on genetic tolerance status of the genotypes. The markers did not show considerable polymorphism in respect to the targeted band sizes. The banding patterns due to these markers (Figure 4.2) unlike marker *Xtxp34* did not relate with Aluminium toxicity tolerance status of the test genotypes determined through phenotypic evaluations.



1-14: Sorghum genotypes subjected to Aluminum tolerance SSR marker analysis

M-100bp marker

Figure 4.2: Agarose gel electrophoresis images for primers *Sb6_34*, *Sb5_236* and *Sb6_342*, respectively. Genotypes 1-14 are KARI Mtama 1, IS 8193, Seredo (Al sensitive check), Serena, Gadam, E 1291, E 6518, Macia, Makueni local, IS 41764 (Al tolerant check), Wagita, Kiboko local 2, Nakhadabo and Tegemeo, respectively.

4.5 Discussion

Deployment of tolerant genotypes is considered the most feasible strategy of managing Al stress in soils of low pH (Abebe, 2007). It is of essence therefore to extensively and continually evaluate the available genetic resources for tolerance to the edaphic stress.

Results for this study revealed the presence of genetic variation and potential for Al toxicity tolerance in sorghum. This was in accordance with earlier findings on sorghum genetic variability to Al tolerance by Magalhaes *et al*. (2007). Based on marker *Xtxp34* (Kong *et al*., 2000; Too *et al*., 2018) clear distinction was made between genotypes that are tolerant and sensitive to Aluminium toxicity. Genotypes *Gadam*, *Macia* and *Wagita* had identical band pattern with the tolerant check *IS41764*, suggesting that they possess alleles

responsible for Al tolerance. Nugroho *et al.* (2019) indicated that visualized amplified band is regarded as an allele and that DNA bands that have travelled the same distance are assumed as identical loci. Contrary to findings by Too *et al.* (2018), the study established that tolerant sorghum genotypes had their expected tolerance locus amplified at 400bp instead of 365bp.

Contrary to expectation, genotype *Kiboko local 2* that was noted to be moderately tolerant through solution culture screening shared the same band pattern with *Seredo* the sensitive check. This indicated that there could be other genes for Al tolerance in this genotype that are yet to be ascertained as it is not known whether sorghum populations solely rely on the already identified *Alt SB* locus as stated by Caniato *et al*. (2007 & 2014) and Magalhaes *et al*. (2007).

Genotype *Gadam* had identical band pattern to the tolerant check *IS 41764* along the four markers. This indicated presence of multiple genes otherwise known as additive gene effect contributing to Aluminium tolerance in the genotype. Nguyen *et al.*, (2002) when researching on Al tolerance in rice stated that a group of Al tolerant plants of a species may host many Al tolerance genes. This was also in agreement with reports by Caniato *et al*. (2007 & 2011) who detected a multiple allelic locus *Alt SB* responsible for Aluminium tolerance within sorghum accessions.

Genotypes *KARI Mtama 1, IS 8193, Serena, E 1291, E 6518, Makueni Local, Nakhadabo, Kiboko local 2 and Tegemeo (2KX17/B/1)* had identical band pattern to the sensitive control *Seredo* at about 330bp. *KARI Mtama 1* and *IS 8193* exhibited identical band patterns to the sensitive check along the four markers translating to absence of gene variants for Al tolerance. These genotypes maintained their Aluminium sensitivity status as earlier noted through solution culture screening at 148µM Al.

Al tolerance locus identified as *Alt SB* underlying the *SbMATE* gene in *Sorghum bicolor* has been ascertained and mapped on the third chromosome (Magalhaes *et al*., 2004; Caniato *et al*., 2014). However, it is not known whether sorghum populations solely rely on this locus or variant Aluminium tolerance genes as noted among members of grass family possessing conserved genomic areas hosting Aluminium tolerance genes. The

multiple allelic locus *Alt SB* is responsible for the remarkable range of variations in sorghum towards Al stress tolerance and normally segregates together with Al^{3+} dependent efflux of citrate from the roots (Magalhaes *et al*., 2007; Caniato *et al*., 2007 & 2014).

The *SbMATE* gene encoding Aluminium activated citrate transporter (AACT) is mostly expressed in root tips of sorghum genotypes tolerant to Al toxicity. On expression, the gene leads to synthesis of membrane transporter proteins also known as cellular membrane anionic channels responsible for citric acid exudation (Magalhaes *et al.,* 2007) which in turn binds/chelate Al^{3+} into Alumino-carboxylate complexes that cannot be absorbed by plant roots. This constitutes the external physiological mechanism of Al^{3+} tolerance in cereal species. Caniato *et al.* (2014) identified molecular markers in respect to the gene *SbMATE* that are applicable for marker enabled selection for Al toxicity tolerance in sorghum.

The SSR genetic markers in linkage with genes responsible for Aluminium tolerance in sorghum have been located and recognized by QTL analysis, genetic linkage and association mapping which have promised speedy generation of varieties that are high yielding and tolerant to Aluminium stress (Caniato *et al*., 2007; Magalhaes *et al*., 2007; Too *et al*., 2018). In regard to the markers deployed in this study, *Xtxp34* and *Sb5_236* are located on the third (3) chromosome where as *Sb6_342* and *Sb6_34* are located on the seventh (7) and eighth (8) chromosomes, respectively (Menz *et al*., 2002; Magalhaes *et al*., 2004). One out of the four specific SSR markers used in the study exhibited a remarkable association with Al toxicity tolerance and could be useful in further screening and selection of parental lines for breeding sorghum against the edaphic stress. Band pattern due to marker *Xtxp34* strongly associated with Al tolerance status of the genotypes deduced from phenotypic evaluations making it highly recommendable for marker assisted selection (MAS) for Al toxicity in sorghum as suggested by Too *et al.* (2018) and confirmed from this study.

4.6 Conclusion

This study revealed the existing genetic variability for Aluminium toxicity tolerance in sorghum. Genotypes, *Gadam*, *Wagita* and *Macia* were validated to be in possession of Al tolerance genes as their DNA amplified band 365 bp identical band pattern to the tolerant check *IS 41764*. Marker *Xtxp34* proved more specific and discriminative in distinguishing between genotypes with and without gene variants conferring Al tolerance. The banding pattern due to this marker strongly associated with the genotypes' Al tolerance status established through phenotypic evaluations thus most ideal for marker assisted selection (MAS) of sorghum for Al tolerance.

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 General discussion

Sorghum genotypes varied in tolerance to Aluminium toxicity. Phenotypic evaluations through solution culture screening revealed existence of significant (*P<.001*) variability in sorghum germplasm on tolerance to Al stress. Genotypes exhibited significant variabilities on all the root growth parameters computed and applied in establishing their tolerance status. On net seminal root growth, there was a significant decline in overall mean for seedlings exposed to Aluminium treatment in reference to seedlings grown in nutrient solution without Aluminium otherwise referred to as control. This indicated the persistence of Al toxicity as an important edaphic factor affecting growth and development of plant roots in soils of low pH (<5.0). Analysis of variance on root growth related parameters showed that 4 genotypes were tolerant to 148µM Al while a remainder of 8 were sensitive with some performing poorly compared to the sensitive check *Seredo*. Genotypes *Gadam* and *Wagita* were found to be highly tolerant whereas *Macia* and *Kiboko local 2* were moderately tolerant. Tolerant genotypes maintained relatively high growth of roots in existence of Aluminium in the solution culture. Generally, tolerant genotypes had their net seminal root growth and relative seminal root growth above 1.70 cm and 70%, respectively and responded lowly (<30%) to Aluminium treatment. Low response to the treatment among the tolerant genotypes translates to their low % reduction in root growth under Al stress. Genotype *Gadam* significantly outperformed its tolerant counterparts including the tolerant check *IS 41764* on net root growth, however, *Wagita* having scored significantly higher root tolerance index (RTI) indicated its ability to tolerate Aluminium concentration higher than 148µM.

Tolerance to Aluminium toxicity in cereals has been attributed to two major physiological mechanisms commonly referred to as external and internal mechanisms. External mechanism operates to ensure that Al^{3+} is excluded from plant roots. In strongly acidic soils where Aluminium ions are highly solubilized, tolerant sorghum genotypes are

induced to produce large volumes of organic acids mainly citric acids. These organic acids bind/ chelate toxic Aluminium ions leading to formation of Alumino-carboxylate compounds that cannot be taken up by the plant roots. On the other hand, internal mechanism functions to detoxify Al^{3+} that has gained entry in to the root cell. Ability to accumulate Silicon in its system is the main enabler of internal mechanism for Aluminium resistance in sorghum. The mechanism solely relies on the reaction involving Silicon and the Al trivalent cation. In the cell, Silicon binds Aluminium ions (Al^{3+}) resulting into formation of Alumino-silicate complexes that are non-toxic hence cannot affect the normal functioning of cells.

The physiological mechanisms responsible for Aluminium tolerance in cereals are under control of genes from MATE and ALMT gene families. In sorghum, Aluminium tolerance locus identified as *Alt* $_{SB}$ underlying the *SbMATE* gene has been mapped on the third chromosome (Caniato *et al*. (2007); Magalhaes *et al*. (2007). The locus normally co-segregates with Al^{3+} dependent efflux of citrate from the roots. The gene encodes synthesis of membrane transporter proteins also known as cellular membrane anionic channels that aid in secretion of malate and citrate acids by root cells on encountering solubilized Aluminium in acidic soils (Sasaki *et al*., 2004; Magalhaes *et al*.,2007). Genotypes lacking such genes cannot produce the organic acids hence their root growth and development are restricted by Aluminium toxicity in acidic soils.

Molecular screening and characterization carried out on the selected sorghum genotypes showed that *Gadam*, *Wagita* and *Macia* possessed alleles responsible for Aluminium tolerance. One out of four applied SSR markers was specific and polymorphic to the targeted locus. Marker *Xtxp34* clearly discriminated between genotypes with and those without Al tolerance genes. Based on the band pattern resulting from electrophoresis of PCR products due to the marker, genotypes *Gadam*, *Wagita* and *Macia* had identical band pattern to the tolerant check *IS 41764*. A remainder of 9 genotypes inclusive of *Kiboko local 2* which had earlier been noted to be moderately tolerant through nutrient solution screening shared an identical band pattern to the sensitive check *Seredo*. Conclusion was

then drawn that the genotype *Kiboko local 2* may be in possession of a variant Aluminium tolerance locus that is yet to be ascertained.

Genotype *Macia* that was noted to be moderately tolerant to Aluminium through phenotypic evaluations shared the same band pattern with the highly tolerant genotypes *Gadam, Wagita* and the tolerant check *IS 41764*. This indicated that the genotype contains Al tolerance gene, however, the expression of the gene may be low or the highly tolerant genotypes may be in possession of multiple alleles otherwise referred to as additive gene effect contributing to the high tolerance to Al stress.

The band pattern analysis of marker *Xtxp34* strongly associated with the results obtained from phenotypic evaluations of the genotypes making it highly recommendable for marker assisted selection (MAS) otherwise marker assisted breeding (MAB) in improving sorghum against Al toxicity.

5.2 Conclusion

Generally, the study findings confirmed the existence of genetic variability and potential for Al tolerance in sorghum. The availed level of Aluminium treatment significantly reduced the overall mean for net root growth among the genotypes. This confirmed that Aluminum toxicity restricts sorghum growth and development in acidic soils.

Both phenotypic and molecular screening revealed genotypes *Gadam, Wagita* and *Macia* as tolerant to Aluminium toxicity. The genotypes amplified band 365bp identical band pattern to the tolerant check *IS 41764*. Marker *Xtxp34* was highly specific and discriminative in differentiating between genotypes with and without genes responsible for Al tolerance. The banding pattern due to the marker strongly associated with the Al tolerance status of the genotypes established through phenotypic evaluations hence highly valuable in marker assisted selection (MAS) for Al tolerance in sorghum.

5.3 Recommendations

Based on the findings of the study, it is hereby recommended that:

i. Genotypes that proved tolerant to Aluminium toxicity through laboratory screening may be rescreened in a production area with acidic soil for further validation of their performance under natural conditions.

ii. Tolerant genotypes be evaluated for yielding capacity in strongly acidic soil. This will help to appreciate their performance in such environments also characterized by deficiency of Phosphorus, Magnesium and Molybdenum and presence of Manganese (Mn^{2+}) and Hydrogen (H^{+}) toxicities in addition to Al toxicity.

iii. Extensive collection and screening of available sorghum germplasm is encouraged in order to identify more tolerant genotypes to serve as potential donors for Aluminium tolerance genes for breeding against the edaphic stress.

iv. The identified genotypes can potentially be used in breeding programs against Al toxicity as sources of genes for introgression into elite cultivars for future deployment to farmers.

v. Genotype *Wagita* having scored significantly higher root tolerance (RTI) be given priority during selection of parental donors for Aluminium tolerance genes. High root tolerance index score by the genotype indicates its genetic ability to tolerate Aluminium level higher than 148µM.

vi. There is necessity to employ more and variety of molecular markers in screening of sorghum in attempts to identify additional/ conserved loci responsible for Aluminium tolerance.

vii. The identified genes can potentially be used in a marker-enabled breeding program to accelerate genetic gain and increase turnover of varieties for commercialization thus contributing to the efforts towards achieving food security and protection of soils against negative impacts of excessive liming.

viii. The level of variability noted among the screened sorghum genotypes could be employed in generation of population of hybrids for quantitative trait loci (QTL) mapping for Aluminium tolerance.

ix. Tolerant and high yielding varieties for instance *Gadam* and *Macia* be availed to farmers for production in areas dominated by acidic soils.

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