SURVEY FOR THE EXPRESSION LEVELS OF DROUGHT TOLERANT GENES IN CASSAVA VARIETIES IN TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF THE SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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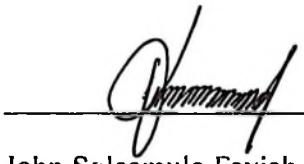
ABSTRACT

Cassava *(Manihot esculenta* Crantz*)* is an important root crop to resource-poor farmers in Sub-Sahara Africa, where the production is hampered by drought stress constraints. Given the difficulties associated with cassava breeding, a molecular understanding of drought tolerance in cassava will help in the identification of markers for use in marker-assisted selection and genes for transgenic improvement of drought tolerance. This study was to improve efficiency in breeding for drought tolerance cassava through molecular techniques by determining the expression levels for each drought tolerant genes in eight selected cassava varieties grown in screen-house environment. Results in this study indicated the presence of drought tolerant genes in Kizimbani, KBH 2006/374, KBH 2002/135, UKG 92/053, IS 30474, Kiroba, KBH 95/517 and KBH 97/212. From the four genes (*ALDH7B4, ZFP252, MSD* and *RD28*) that have previously been biologically validated as conferring or being associated with drought tolerance in other plant species; *ALDH7B4* gene was confirmed as being exclusively up-regulated in all varieties except in IS 30474. Results showed further that the four genes were exclusive up-regulated in Kiroba compared with other cassava varieties. Based on *ALDH7B4* gene, it was hypothesized that the basis of the tolerance at the cellular level in those varieties is through mitigation of the osmotic and oxidative adjustment. The *ALDH7B4* gene can now be tested in the context of cassava breeding, as possible quantitative trait loci and engineering drought tolerance in transgenic cassava, or used for introgression into other improved cassava germplasms for climate change mitigation.

DECLARATION

I, **John Soleemulo Fayiah,** do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.



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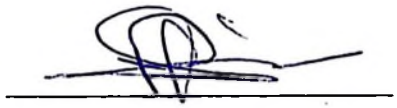

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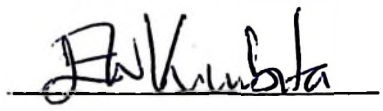

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LIST OF ABBREVIATIONS AND SYMBOLS

µg	Microgram
µl	Microliter
µM	Micromole(s)
ALDH1F	Forward primers for Aldehyde Dehydrogenase gene
ALDH1R	Reversed primers for Aldehyde Dehydrogenase gene
ALDH7B4	Aldehyde Dehydrogenase gene
BMGF	Bill and Malinda Gates Foundation
bp	Base pair
cDNA	Complementary Deoxyribonucleic Acid
CMD	Cassava Mosaic Disease
Ct	Threshold cycle
CTAB	Cetyltrimethyl ammonium bromide
Cys	Cysteine
dNTPs	Dideoxynucleotide triphosphates
DT	Drought Tolerance
EDTA	Ethylenediaminetetracetic acid
FAO	Food Agriculture Organization
His	Histidine
IITA	International Institute for Tropical Agriculture
Kb	Kilo-base pair
LiCl	Lithium Chloride
MAFSC	Ministries of Agriculture, Food security and Cooperative
MARI	Mikocheni Agricultural Research Institute
mg	Milligram

$MgCl_2$	Magnesium chloride
Ml	Milliliter
mM	Millimole
MSD	Manganese Superoxide Dismutase
MSD1F	Forward primers for Manganese Superoxide Dismutase gene
MSD1R	Reversed primers for Manganese Superoxide Dismutase gene
Ng	Nano-gram
PCR	Polymerase Chain Reaction
pH	Potency of Hydronium Ion
PVP	Polyvinyl Pyrrolidone
RD28	Responsivenessness to Desiccation gene
RD282F	Forward primers for Responsivenessness to Desiccation gene
RD282R	Reversed primers for Responsivenessness to Desiccation gene
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
rpm	Revolution per minute(s)
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
Sec	Second
SUA	Sokoine University of Agriculture
t/ha	Ton per Hectares
Taq	*Thermusaquaticus*
TBE	Tris-borate EDTA
UK	United Kingdom
ZFP 252	Zinc finger protein gene
ZFP1F	Forward primers for Zinc finger protein gene
ZFP1R	Reversed primers for Zinc finger protein gene

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Cassava (*Manihot esculenta* Crantz) serves as major food and cash crop for smallholder farmers and family in Africa. The crop is the 3rd most important source of calories in the tropics after rice and maize (El-Sharkawy, 2006: FAO, 2010). Cassava is a major part of the diet for nearly a billion people in approximately 105 countries mostly in Sub-Saharan Africa, Asia, the pacific and South America (FAO, 2008). Over 50 % of the global cassava production occurs in Africa (FAOSTAT, 2009; Alabi *et al.*, 2011). Globally, about 70 % storage roots from cassava are consumed as human food (El-Sharkawy, 2004). The greatest per capita consumption (800g per person) recorded in Sub-Saharan Africa where it is the main energy source for over 40 % of the population (Scott *et al.*, 2000; Nhassico *et al.*, 2008). A typical composition of cassava root is 70 % moisture, 24 % starch, 2 % fiber, 1 % protein and 3 % other substances including minerals (Westby, 2002; Tonukari, 2004). Cassava is an important staple crop in more than half of Tanzania and a subsistence crop, especially in the semi-arid areas (Kulembeka, 2010).

There are major challenges facing cassava production in Sub- Saharan Africa; ranging from abiotic to biotic (IITA, 2007; Reynolds and Tuberosa, 2008; Kulembeka, 2010). Abiotic stresses account for more than 50 % of potential yield losses in major crops worldwide (Rosegrant and Cline, 2003; Peters *et al.*, 2004). Drought is one of the major factors that have been reported to hamper cassava productivity among smallholder farmers in the face of climate change (MAFSC, 2009). It is currently one of the major abiotic stress, which limits the production of crops and vital food for human survive (Lokko *et al.*, 2007). Future increase and intensity of drought due to climatic changes

especially in most agriculturally productive zones around the world is anticipated (Reynolds and Ortiz, 2010; Mir *et al.*, 2012). Cassava brown streak and Cassava mosaic disease are some of the biotic constraints as well as the low yielding potential of some of the local varieties (Mkamilo, 2005).

Typically, drought and diseases for the first three to six months have the propensity to influence various plant morphological and physiological processes such as leaf area expansion, disrupts stomatal aperture, shoot growth, stem extension and root proliferation (Anjum *et al.*, 2011). It also reduces photosynthetic rates and stomatal conductance, impairs leaf chlorophyll, reduces leaf water potential, lowers turgor pressure, suppresses cell expansion and growth, shrinks plant total biomass and in extreme cases, causes plant death (Xu *et al.*, 2008). Water deficit increases leaf senescence and abscission, reduces leaf size and shorten leaf longevity (Anjum *et al.*, 2011).

At molecular levels, plants rapidly alter their gene expression in response to water stress (Shinozaki and Yamaguchi-hinozaki, 2007; Chaves *et al.*, 2009). During drought, Degenkolbe *et al.* (2009) reported a significant down- regulation of genes coding for proteins involved in photosynthetic light reactions, isoprenoid metabolism and protein synthesis, as well as concordant up-regulation of genes for amino acid and lipid degradation in rice. Dehydration threatens proteins, since diminution in cellular volume leads to macromolecular crowding and oxidative injury, which can increase the number of inactive, denatured, aggregated or oxidatively damaged proteins (Hoekstra *et al.*, 2001). The osmotic and oxidative nature of drought stress disrupts cellular homeostasis and ion distribution and denatures functional and structural proteins (Krasensky and Jonak, 2011; Vaseva *et al.*, 2012).

1.2 Problem Statement and Justification

Food insecurity is one of this millennium's serious and most shared problems throughout the world and specifically in developing countries. Many factors, including climate change and particularly drought constitute the basis of Food insecurity in the world (Passioura, 2007). Increased effect of drought stress incapacitates farmers' ability to cultivate crops and produce high yields (Sheffield and Wood, 2008). It is under these changing climatic conditions that the future food production will need to be doubled in order to feed the human population expected to plateau at nine billion by 2050 (Cassman *et al.*, 2003; Godfray *et al.*, 2010; Tilman *et al.*, 2011). This poses a serious challenge to farmers, crop breeders and the larger scientific community, especially in most food insecure regions of the world such as Sub-Saharan Africa (Rosenthal *et al.*, 2012). The impact of climatic change is expected to be very high and it double (Lobell *et al.*, 2008; Rosenthal *et al.*, 2012).

Cassava is one of the widely produced and consumed crop in Africa (Lokko *et al.*, 2007). In Tanzania according to FAOSTAT, (2009) cassava is the six most essential diets and one of the most important food crops. Cassava is important in sustaining food security and improve livelihood for the majority of the small scale farmers. In order to circumvent a paramount problem such as food insecurity, there is a need of using molecular tools to identify and characterize the genes that confer drought tolerant traits in cassava crops so as to plan how best these can be used in other drought vulnerable crops and thus alleviate food insecurity in African populations. Given the inherent challenges with cassava breeding, an understanding of the molecular basis of cassava drought responses and tolerance can help greatly in the development of appropriate varieties (Valliyodan and Nguyen, 2006; El-Sharkawy, 2007).

Plant breeders can development crops and cassava cultivars that can circumvent drought to sustainable increase in food production in drought prone or marginal areas and to feed a burgeoning human population (Okogbenin *et al.*, 2013). Clearly identifying the molecular mechanisms of drought tolerance plants has been a major goal for plant breeders and plant biologists (Cattivelli *et al.*, 2008).

Some transgenic crops have been altered to withstand being sprayed with broad-spectrum agro chemicals such as herbicides and insecticides, with the idea that one application will take care of most types of weeds or pests without killing the crop (USDA/NASS, 2005). For example, transgenic tomatoes have been designed for longer shelf life. Also, increased beta-carotene in transgenic "Golden Rice" (probably derived from the daffodil) proved usable for human nutrition, especially in the absence of dietary fats and proteins (Grains of Delusion, 2001).

1.3 Objectives of the Study

1.3.1 Overall objective

To improve efficiency in breeding for drought tolerance cassava through molecular techniques

1.3.2 Specific Objectives of the present study

i. To screen for the presence of drought tolerant genes in eight selected cassava varieties;
ii. To determine the fold difference (change in expression) for each drought tolerant genes in the selected cassava varieties

1.3.3 Hypothesis

i. Genomes of all selected cassava varieties contain drought tolerant genes

ii. There is no statistically significant difference in expression of *ALDH7B4*, *ZFP252*, *MSD* and *RD28* genes between well watered and water stressed selected cassava varieties

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Botanical Description of Cassava

Cassava (*Manihot esculenta* Crantz), is rich in carbohydrate and minerals in t tuberous roots and leaves respectively. It is a stable food for over 800 million people in Africa and Asia. The plant usually grows from one to three meters (m) high, although some cultivars may reach a height of four (4) meters. There is some variation in leaf color, from green to red which depends on the varieties (Anonymous, 2004). Cassava is grown by planting a cutting taken from the woody part of the stem (Cock, 1985).

Table 1: Taxonomy and classification of the cassava plant

Classification	Taxonomy
Class	*Diocotyledonae*
Sub-class	*Archichlamydeae*
Order	*Euphorbiales*
Family	*Euphorbiaceae*
Sub-family	*Manihotae*
Genus	*Manihot*
Species	*Manihot esculenta* Crantz

Source: IITA (2005).

2.2 Origin and Distribution of Cassava

The origin of cassava plant was in South and Central America. The Indians from the belt of the Amazon eat cassava in addition to rice, maize and potatoes (Anonymous, 2004). Portuguese explorers introduced cassava to Africa during the 16th and 17th centuries through their trade with the African coasts and nearby islands. Africa and Africans then distributed cassava further in other parts of Africa, especially in the Sub-Saharan regions (Jones, 1959). It is now in almost all parts of tropical Africa as part of their major diet. Currently, Nigeria still remains the largest producer of cassava; including Congo-

Kinshasa (IITA, 2007). Brazil and Thailand are the largest producers in the world (IITA, 2005).

2.3 Soil Requirements

Soils known for cassava growth are sandy loams, loamy sands with adequate moisture, fairly fertile and deep. This implies that cassava does not require soils of high fertility (Howeler, 1990). Applications of nitrogen, phosphorus and potassium (NPK) fertilizers were shown to significantly increase cassava yields and sustain productivity for longer periods in poor soils (Pellet and El-Sharkawy, 1997). The use of these mineral fertilizers also significantly increased cassava productivity in East Africa (Fermont *et al.*, 2009). Low temperatures (below 15°C) cannot kill cassava, but it leads to delayed stem sprouting, inhibited plant growth and leaf production rates, reduced leaf photosynthetic rates and hence low total biomass and root yields (El-Sharkawy, 2004).

2.4 Nutritional Values of Cassava

Cassava is the third most essential sources of calories in the tropical regions after rice and maize (El-Sharkawy, 2006; FAO, 2010). Cassava roots typically are composed of 70 % moisture, 24 % starch, 2 % fiber, 1 % protein and 3 % includes mineral substances (Westby, 2002; Tonukari, 2004). The carbohydrate content in root ranges from 80 % to 90 % on a dry matter basis (Montagnac *et al.*, 2009). Cassava roots are commonly processed into flour or products such as tapioca, fufu, farinha or gari, and can also be eaten fried or as boiled chips (Balagopalan, 2002). Cassava has high content of dietary fibre, magnesium, sodium, riboflavin, nicotinic acid, and citrate (Bradbury and Holloway, 1988). The iron and vitamin A levels are low (Westby, 2002), however some varieties with yellow roots contain significant amount of βeta-carotene (Ferreira *et al.*, 2008; Akinwale *et al.*, 2010; Carvalho *et al.*, 2012). The leaves contain more proteins, minerals

and vitamins than the tubers (Westby, 2002; Montagnac *et al.*, 2009). Cassava leaves are also consumed fresh or cooked (Achidi *et al.*, 2005; Lebot, 2009). The leaves and tubers can be used as animal feed (Balagopalan, 2002).

2.5 Cassava Production

2.5.1 Status of cassava production in Tanzania

Tanzania is one of the nations among the top ten largest cassava producers on the globe with an annual production of fresh cassava of 7 000 000 tones and more than 84 % of the total production is consumed as human food, 15 % is wasted and the rest is used as livestock feed (MAFSC, 2009).

Table 2: Cassava production in Tanzania

Location (Zone)	Production (%)	Estimated productions (tones)
Costal/Eastern and Southern	48.8	2 684 000
Lake	23.7	1 303 500
Western	7.9	434 500
Central	5.0	276 100

Source: MAFSC (2009).

Tanzania is the fourth largest producer of cassava in Africa and annual root production was estimated at 7 000 000 tons from 761 100 hectares in 2005. About 48.8 % of total production is produced in the coasted strip along the Indian Ocean, 23.7 % comes from Lake Victoria, 13.7 % from Lake Nyasa and 7.9 % of Lake Tanganyika area (Mkamilo, 2005).

2.5.2 Constraint with cassava production in Tanzania

There are major challenges facing cassava production in Sub- Saharan Africa; ranging from abiotic to biotic (IITA, 2007; Reynolds and Tuberosa, 2008; Kulembeka, 2010).

Abiotic stresses account for more than 50 % of potential yield losses in major crops worldwide (Rosegrant and Cline, 2003; Peters *et al.*, 2004). Drought is one of the major factors that have been reported to hamper cassava productivity among smallholder farmers in the face of climate change (MAFSC, 2009). However extreme environmental fluctuations are known to lead to significant yield reductions in the crop (El-Sharkawy, 2007). These factors include unsuitable soil types with low nutrient levels, low or high temperatures and prolonged drought sometimes caused by insufficient rainfall.

Cassava production in East Africa is constrained by both abiotic and biotic factors, which are aggravated by sub-optimal management practices (Bull *et al.*, 2011). Cassava mosaic disease (CMD) and Cassava brown streak disease (CBSD) are major viral diseases constraining cassava production (Winter *et al.*, 2010). Tanzania has recorded crop losses of up to 74 % due to CBSD, but in severely affected areas, leading to 100 % damage in susceptible varieties (Legg *et al.*, 2011). Cassava brown streak disease can cause significant reduction in both quality and quantity of cassava in all coastal areas of Tanzania, Kenya and Mozambique and in the lakeshore areas of Malawi (Legg *et al.*, 2011). Another major challenge for smallholder farmers in Tanzania, is the lack of facilities and storage which causes the cassava products to spoil (Coulson and Diyamett, 2012). Insignificant investment has been made to research, breed and improve its production when compared to major cereal crops. The crop has received little attention from government policy makers, researchers and research institutes further inhibiting the crop's production potential that should meet its growing demand (FAO, 2008).

2.5.3 The economic importance of cassava

Cassava is one of the major diets for nearly a billion people in sub-Saharan Africa, Asia, the pacific and Latin America (FAO, 2008). It is reported that over 50 % of the global

cassava production occurs in Africa (El-Sharkawy, 2006; FAOSTAT, 2009; Alabi *et al.*, 2011). Cassava production is expected to reach 290.8 million metric tons per year by 2020 (Scott *et al.*, 2000). In 2007, Africa was the world's largest producer with 118 million tons out of a global production of 22 832 million tons (FAOSTAT, 2008). In the developing world, cassava is regarded as a "drought war and famine" crop (Burns *et al.*, 2010). This is because the crop can grow in low fertility soils, is easily propagated, requires little cultivation and can tolerate sporadic and seasonally extended drought episodes (De Tafur *et al.*, 1997; El-Sharkawy, 2002; Hillocks, 2002). The highly perishable tuberous roots of cassava can be left in the soil and retrieved only when needed for up to 3-4 years after maturity (Ceballos *et al.*, 2004; El-Sharkawy, 2004; Lebot, 2009; Okogbenin *et al.*, 2013). Globally, about 70 % storage roots from cassava are consumed as human food (El-Sharkawy, 2004) particularly in sub-Saharan Africa where it is the main energy source for over 40 % of the population (Scott *et al.*, 2000; Nhassico *et al.*, 2008). Cassava can provide some form of food security during periods of climatic or agricultural instability and social unrest (Burns *et al.*, 2010; Koledoye *et al.*, 2012). More than 90 % of cassava produced in Sub-Saharan Africa is used for fresh consumption and processed foods and the remaining used for animal feed and other industrial uses such as starch (Sanni *et al.*, 2009; Okogbenin *et al.*, 2013). Cassava is used as a raw material for starch production, papermaking, as a lubricant in oil wells and in the textile industry as substrate for the production of dextrins and also glues production (Cock, 1985).

2.6 Molecular Characterization

2.6.1 Molecular characterization of response to drought stress in cassavas

The scientific world turns to carry out an evaluation of drought effects on genetic parameters in cassava and value of breeding cassava. Those parameters included yield of storage roots, the mineral content in leaves and dry matter content of cassava tubers.

Those genetics parameters within this study may be useful in the future for cassava breeding programs (Ochieng' Orek, 2014). The modern genomic tools are used in identifying the key genetic traits associated with yield-limiting factors such as drought stress in cassava (Ochieng' Orek, 2014). Cassava tissue and genotypes are valuable tools for the development of microarrays, to study genetic diversity, gene discovery and expression profiling. Those molecular instruments can create a clear understanding of molecular issues for drought tolerant crops (Zeng *et al.*, 2006; Xu *et al.*, 2008). Sequence analysis is one of the molecular evaluations used to determine cassava in response to drought; while twenty thousand full-length cDNA clones that revealed significant levels of lineage with specific expansion of genes were directly related to stress responses. This is a valuable tool that can be used by the breeding community for the improvement of cassava varieties (Cellier *et al.*, 1998; Lokko *et al.*, 2007; Sakurai *et al.*, 2007; Ochieng' Orek, 2014).

A microarray analysis conducted in three cassava genotypes and identified 168 up-regulated genes and 69 down-regulated genes (Utsumi *et al.*, 2012). The understanding of drought tolerant traits in cassava can help with the identification of molecular markers that will be used in transgenic genes improvement (Turyagyenda *et al.*, 2013). The study provided molecular insights into drought tolerance trait in cassava. There was a studied on fifty three cassava genotypes in Uganda and it indicated that MH96/0686 was tolerant to drought and leaf retention under water stress when compared to other cassava varieties (Turyagyenda *et al.*, 2013). The finding of this experiment showed that expression of Zinc finger protein (ZFP252) gene in rice increased free proline along with soluble sugars the amount by elevating the expression of stress defense genes. Tolerance to salinity was enhanced in the rice and in addition, drought stress genes were enhanced (Xu *et al.*, 2008).

2.6.2 Drought responsive genes

Given the inherent challenges with cassava breeding, an understanding of the molecular basis of cassava drought responses and tolerance can help greatly in the development of appropriate varieties (Valliyodan and Nguyen 2006; El-Sharkawy, 2007). Conventional breeding has been hindered by cassava's high heterozygosity, genotype by environment (G × E) interaction, long life cycle (Ceballos *et al.*, 2004) and limited seed production, while molecular breeding is hindered by limited information on genomic regions and genes associated with drought tolerance in cassava. Efforts to improve cassava's water use efficiency through conventional breeding have been limited in many parts of the world, including much of Sub Sahara Africa. Breeding programs in Latin America have successfully identified germplasms with increased levels of drought tolerance, with 2–3 times the yield of typical cassava genotypes in semi-arid conditions (El-Sharkawy, 2007). A range of cassava drought-tolerance levels has also been characterized in West Africa (Okogbenin *et al.*, 2003). Efforts are now under way in eastern Africa to begin breeding for drought tolerant cassava. A range of cassava drought-tolerance levels has also been characterized in West Africa (Okogbenin *et al.*, 2003). Efforts are now under way in eastern Africa to begin breeding for drought tolerant cassava.

Zinc finger protein (*ZFP252)* maintains cell membrane integrity and promotes proline synthesis (Sanchez et al., 1998; Xu *et al.*, 2008*)*. One or more "Zinc Finger" is possessed by the zinc finger protein gene, which bonds most of Zinc Ions by its residues, Histidine (His) and Cysteine (Cys). In abiotic and biotic stresses in rice plant, zinc finger protein respond significantly by regulating the plant molecular mechanism to those conditions (Li *et al.*, 2013). A cys-2/his-2-type (C2H2) zinc finger protein is a transcription factor that regulates gene expression by binding DNA in promoter regions of genes (Hardy, 2010). The C2H2 transcription factor has been associated with drought stress response in

many plant species such as *Cicer arietinum*, *Petunia hybrida*, *Oryza sativa*, and *Glycine max* (Huang *et al.*, 2009). Overexpression of *ZFP245* enhanced activity of reactive oxygen species scavenging enzymes and elevated free proline levels in rice, thus increasing drought tolerance (Huang *et al.*, 2009).

Aldehyde Dehydrogenase 7B4 (*ALDH7B4*) is a gene involved in the function as an antioxidant/reactive oxygen species (ROS)/scavenging by reducing the levels of lipid peroxidation (Kotchoni *et al.*, 2006; Ochieng' Orek, 2014*)*. Reactive Oxygen species inhibit photosynthesis and cause cellular damage to plant during salt or drought stresses (Missihoun, 2010). A model plant *Arabidopsis thaliana* was used and Aldehyde dehydrogenase gene was considered as 'aldehyde scavengers'; its main function is to eliminate toxic aldehydes in plant by causing oxidative stresses (Hou and Bartels, 2014).

The responsiveness to Desiccation (*RD28*) gene is known to have early response to dehydration in plants. It had been known for its early involvement in transporting essential molecules across the leaves and stem in plants during osmotic stress (Silva *et al.*, 2012*)*. Early response to dehydration and drought stress in the plant is enhanced by the responsiveness to Desiccation gene (Daniels *et al.*, 1994; Obidiegwu *et al.*, 2015). Abscisic acid also activates transcription factors that involved in expression of downstream-stress responsive genes such as Responsive to Dehydration (RD) and early Responsive to Dehydration (Pardo, 2010). The promoter region of *RD29A/COR78/LTI78* contains both an abscisic responsive elements and dehydration responsive element binding proteins/C-repeat binding factor, which functions in Abscisic acid-dependent and Abscisic acid-22 independent gene expression respectively in response to drought stress (Seki *et al.*, 2003).

Manganese Superoxide Dismutase (*MSD*) involves with playing a role in oxidative stress tolerance in plants. During drought stress plant can be detoxified by *MSD* (Alscher *et al.*, 2002). When plants are affected by drought stress condition, Manganese Superoxide Dismutase provides an inflammation defense subsequently causing cellular homeostasis (Li and Zhou, 2011). The *MSD* gene scavenges for reactive oxygen species (Fryer *et al.*, 2002), *ZFP252* maintains cell membrane integrity and promotes proline synthesis (Xu *et al.*, 2008). The *ALDH7B4* reduces the levels of lipid peroxidation (Kotchoni *et al.*, 2006) and *RD*28, a turgor-responsive, plasma membrane aquaporin found in plasma membranes of plant tissues and enhances drought tolerance through abscisic acid-independent pathway (Kotchoni *et al.*, 2006).

2.7 Mechanisms of Drought Tolerance in Cassava

Cassava responds to water deficit at morphological, physiological, cellular and metabolic levels just like other plants. The responses are dependent upon the duration and severity of water stress, stage of development and the cultivar (Burns *et al.*, 2010). Some of the mechanisms that make cassava innately drought tolerant is described in the following:

2.7.1 Stomatal sensitivity

Stomata are the route by which carbon dioxide enters the leaf and thus drought-induced decreases in stomatal aperture can limit the rate of carbon dioxide diffusion into the leaf hence reducing the rates of photosynthesis, transpiration and leaf or stomatal conductance (El-harkawy, 2007). The stomata have been found to partially close in low air humidity with no changes in leaf water potential and also in response to soil water shortage, hence protecting the leaf from severe dehydration (El-Sharkawy, 2004). When water is limiting, cassava confines stomatal opening to the early part of the day when temperature and vapour pressure difference are lower, thereby permitting photosynthesis to occur

(El-Sharkawy, 2007). Once cassava leaves is exposed to dry air and/or dry soils, they partially close their stomata thereby restricting water loss and partially retaining their photosynthetic capacity under prolonged water shortage (Okogbenin *et al.*, 2013; El-Sharkawy, 2004). The sensitivity of cassava stomata to incipient water deficit is associated with large increases in abscisic acid (Alves and Setter, 2000).

2.7.2 Leaf response

Cassava leaf growth is highly sensitive to drought (Alves and Setter, 2004). In cassava, drought drastically affects leaf expansion in existing leaves and rate of new leaf appearance and restricts the leaf area (Alves and Setter, 2004). Drought reduces photon interception through decline in leaf canopy (restricted new leaf formation, smaller leaf sizes and leaf fall), and adaptation that conserves water (El-Sharkawy *et al.*, 2004). This response conserves available water and maximizes water use efficiency of the crop during prolonged periods of drought (Alves and Setter, 2004). This type of response is ideal for cassava survival in environments with numerous water deficits (Setter and Fregene, 2007). Cassava stores large quantities of starch in stems and leaf petioles, which are remobilized during water deficits (Alves and Setter, 2004). While stomatal closure essentially stops photosynthesis during water deficit (Setter and Fregene, 2007).

In conjunction with stomatal sensitivity, cassava actively exhibits a strong heliotropic mechanism through which its tracks solar radiation early in the morning and late in the afternoon when the leaf-to-air water deficit is low (El-Sharkawy, 2007). The leaf movement maximizes and minimizes light interception under high and low water use efficiency respectively thereby sustaining canopy photosynthesis (El-Sharkawy, 2007). Cassava leaves droop or fold at early phases of water limitation such that the incident light flux densities are lessened and photon inhibition avoided (Calatayud *et al.*, 2000).

The final outcome of leaf drooping is reduced transpiration rates while the leaf maintains reasonable photosynthetic rates at a higher leaf conductance (El-Sharkawy, 2004). This phenomenon acts as a water stress avoidance mechanism in cassava.

2.7.3 Leaf retention/staygreen trait

Drought stress hastens leaf senescence resulting in reduced canopy size, loss of photosynthesis and low yields (Rivero *et al.*, 2007). Leaf senescence is a type of programmed plant cell death depicted by loss of chlorophyll, lipids, total protein and ribonucleic acids (Rivero *et al.*, 2007). During water shortage, staygreen genotypes are able to retain more green leaf area, potentially intercepting more solar radiation and increasing their productivity than their non-staygreen counterparts (Hortensteiner, 2009). In cassava, high leaf retention or staygreen contributes to high root yields and positively correlated with high drought tolerance, productivity and root quality during periods of water deficit, thus suggesting a sustained photosynthetic activity among the retained leaves (El-Sharkawy *et al.*, 2004). By retaining their functional leaves with high water use efficiency and reducing the production of new leaves under drought conditions, drought tolerant cassava cultivars are able to permit greater photosynthate accumulation in the roots and thus increase the harvest index (El-Sharkawy *et al.*, 2004).

2.7.4 Phytohormone and osmotic adjustment

Under drought, changes in biosynthesis, content and distribution of abscisic acid within plant organs and tissues may play an important role in sensing changes in both soil water and atmospheric humidity (Kang *et al.*, 2010; Okogbenin *et al.*, 2013). Abscisic acid is also involved in other biological functions such as expression of dehydrins and other proteins that are thought to stabilize macromolecular structures (Alves and Setter, 2004). Expanding and mature leaves of cassava genotypes accumulated high levels of abscisic

acid after 3 and 6 days of withholding irrigation (Alves and Setter, 2000). Plants also demonstrate tolerance to drought through accumulation of osmotically active solutes that maintain turgor and turgor-dependent processes during dry down episodes (Okogbenin *et al.*, 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Cassava Varieties Selection and Plant Establishment

Eight cassava varieties (Table 3) were collected from Kibaha, Dodoma and Mtwara during September to October 2015. Cassava cuttings for each were packaged in a paper envelope for the purpose of safely transporting them at Mikocheni Agriculture Research Institute (MARI); and they were washed with distilled water to disinfect them. The cassava cuttings were planted in pots filled with soil (4 kg) each in the screen house at (MARI). These known improved cassava varieties were selected for this study.

Table 3: List of Cassava varieties included in this study

Cassava varieties	Pedigree	Collection sites
Kizimbani	Kirobahalfsib	Mtwara
KBH 2006/374	Kibahahalfsib	Kibaha
KBH 2002/135	Kibahahalfsib	Kibaha
UKG 92/053	Kibahahalfsib	Dodoma
IS 30474	Kibahahalfsib	Dodoma
Kiroba	Amanihybrid	Kibaha
KBH 95/517	Kibahahalfsib	Kibaha
KBH 97/212	Kibahahalfsib	Kibaha

3.2 Soil Sampling and Analysis

Soil samples were collected at the depth of 0 – 20cm as recommended by Landan (1991) and taken to the Soil Science laboratory at SUA for Physicochemical analyses. Parameters analyzed included particle size (%), soil pH (1:2.5), organic carbon (%) and total nitrogen (%). Particle size distribution was determined by Bouyoucos Hydrometer method Gee and Bauder (1996) whereas textural classes were determined using United States Department of Agriculture (USDA) textural triangle. Soil pH was determined electrometrically in 1:2.5 soil–water suspensions as described by Thomas (1996). Organic carbon determination was done by wet digestion method of Walkley and Black

(Nelson and Sommers, 1982). Total N was determined by the micro – Kjeldahl digestion–distillation method by (Bremner and Mulvaney, 1982).

3.3 Experimental Design and Treatment

The experiment was set up in a randomized complete block design (RCBD) with three biological replicates. Three cassava cuttings (20 cm in length) for each variety were planted vertically in 4 kg of sterilized soil, in 5 liters plastic buckets. Treatment levels consisted of control (well watered) and water deficits under Screen house environment at the temperatures ranging from 40 – 45 °C during day, with humidity typically at 50 to 65 %. Both the control and treatment samples for all plants were watered with 500 ml of water every 2 days until 60 days after planting. After 60 days, plants in the stress treatment were gradually subjected to drought stress condition for additional 30 days. During the 30 days of stress, control samples received (500 ml) of water after every two days and water stress treatment samples received 250 ml of water after every four days. Irrigation was stopped 90 days after planting (DAP).

3.4 Data Collection and Laboratory Analysis

3.4.1 Sampling procedure

Three leaves of each cassava varieties (upper, middle and lower leaves) were sampled from both the control and treatment from the three biological replicates in the screen house after stressing them. Samples of cassava leaves were harvested one variety at a time, compressed into an envelope, well labeled and taken to the Molecular Biology Laboratory immediately on ice and placed in -80 °C freezer until required for ribonucleic acids extraction.

3.4.2 RNA extraction

Ribonucleic acid was extracted from 0.15-2.0 gram of fresh cassava leaf (that had been frozen at -80 °C) samples using a modified Chang *et al.* (1993) Cetyltrimethyl ammonium bromide (CTAB)-based protocol. A Modification was made to the Chang *et al.* (1993) method to reduce the time and cost of extraction without reducing quality and yield of the RNA extracted from leaves of cassava plants. In the modified protocol, all centrifugation steps were carried out at 4 °C. Before starting the extraction process, the surface area was cleaned with 70 % ethanol. The CTAB extraction buffer was prepared using 2 % CTAB, 100 mM Tris hydrochloric acid, 20 mM Ethylene diaminetetraccetic acid (EDTA), 1.4 M Sodium Chloride (NaCl), 5 % βeta-mercapto ethanol and 2 % Polyvinyl Pyrrolidone (PVP). A fresh leaf of cassava sample, weighing 0.15 – 0.2 gram was grind with 700 µl of extraction buffer. Other steps didn't change except that RNA precipitation was carried out using 2 volumes of absolute ethanol instead of Lithium Chloride (LiCl) precipitation (Appendix 1). It was incubated at -20 °C, overnight in order to completely precipitate nucleic acid from the leaves of the cassava.

3.4.3 RNase-free DNAse1 treatment

The purpose of RNase-free DNAse1 treatment of RNA was to remove contaminating DNA. Thirty five (35) µl of nucleic acid, 4 µl DNAse 1 buffer and 1µl of DNAse1 aliquots were mixed in PCR tubes and incubated at 37°C for 30 minutes. An EDTA with the stock concentration of 0.5 M (500 mM) was first diluted to 25 mM and was added after incubation to deactivate DNAse 1 enzyme. After flicking the tubes gently, it was centrifuged at 1000 rpm for 2 minutes. The supernatants which contained RNA were transferred into new tubes (Appendix 2).

3.4.4 Determination of cassava ribonucleic acid quality

The RNA quantity and quality of each of the samples was measured by Cecil CE3021 spectrophotometer (Cecil Instruments, Cambridge, UK) at the absorbance wavelengths ratios of 260 nm and 280 nm (A_{260}/A_{280}). A ratio ranging between 1.88 and 2.2 was considered an acceptable quality; and furthermore investigation on the quality of RNA was conducted by an agarose gel electrophoresis.

3.4.5 Complementary DNA (cDNA) synthesis

Master mix was prepared for cDNA synthesis using deoxynucleoside triphosphates (dNTPs), DEPC treated water and oligodT (18) to anneal to poly-A tails of the massager ribonucleic acid (mRNA) and reverse transcriptase to convert the mRNA to cDNA by reverse transcription, following manufacturer's instructions (Bioneer Corporation, Daejeon, South Korea) (Appendix 3). Two control reactions were added for each sample throughout the processed. Each of the control reactions had no RNA templates. The control reactions were to assess the quality of reagents, and the absent of contaminations.

3.4.6 Determination of complementary DNA quality and integrity

The synthesized cDNAs quantity and quality for each sample were determined using a Cecil CE3021 spectrophotometer (Cecil Instruments, Cambridge, UK). High quality cDNA was obtained at the absorbance wavelength ratio of A_{260}/A_{280}. The resulting cDNAs were standardized by diluting to a final working concentration of 50 ng/µl. The cDNAs were used during Conventional End-point PCR and RT-PCR for analyzing the following genes: *ZFP252*, *ALDH7B4*, *MSD* and *RD28*.

3.5 Screening for the Presence of ZFP252, ALDH7B4, MSD and RD28genes in Selected Cassava Varieties

3.5.1 Amplification of ZFP252, ALDH7B4, MSD and RD28 genes by end-point PCR

Amplification of the four (*ZFP252, ALDH7B4, MSD* and *RD28)* fragments was carried out using Gene Amp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA). The primers (Table 4) used were synthesized by Bioneer Corporation, (Daejeon, South Korea). The PCR reactions were carried out in 20 µl volumes containing 50 ng cDNA, x1 reaction PCR buffer (10 mM Tris-HCl at pH 8.3 and 50 mM KCl), 1 µl of each forward and reverse primer (10mM) (Table 5), 2 mM $MgCl_2$, 0.2 mM of each dNTP and 0.3 U of *Taq*DNA polymerase (Bioneer Corporation, Daejeon, South Korea). The thermal cycling profile consisted of the initial denaturation at 94 °C for 2 minutes, denaturation at 94 °C for 30 seconds, annealing at 55 °C for 1 minute and extension at 72 °C for 30 seconds and final extension at 72 °C for 10 minutes for 35 cycles. The annealing temperature of 55 °C was for all the genes and was also applied with real time PCR.

Table 4: Sequence of primers used for end-point PCR and qRT-PCR reaction

Gene symbol	Primers ID	Primer sequence (5′–3 ′)	Length (bases)	Amplicons size (bases)
ZFP252	ZFP1F	CTCTATTCTCAGCGCACATTCC	22	245
	ZFP1R	AGCATAACGAGGCAGAGAGC	20	
MSD	MSD1F	ATGAATGCAGAAGGTGCTGCA	21	269
	MSD1R	GAAGGGCATTCT TTGGCATAC	21	
RD28	RD282F	TGCACTGCTGGTATC TCAGG	20	237
	RD282R	GATCTCAGCTCCCAATCCAG	20	
ALDH7B4	ALDH1F	GGATGGAATGCATGCATTGCACTG	24	263
	ALDH1R	CTGATTCACTGTTTGTTGCACCATC	25	

Source: Turyagyenda *et al.* (2013).

3.5.1.1 Agarose gel electrophoresis of PCR products

The PCR products were verified by running samples on 1.5 % (w/v) agarose gel in one time tris-acetate EDTA buffer containing 1 % ethidium bromide for visualization.

Electrophoresis was run at 80 voltages for one hour. A one hundred bp DNA ladder was used (Bioneer Corporation, Daejeon, South Korea) to estimate sizes of *ZFP252, ALDH7B4, MSD* and *RD28* fragments.

3.6 Determining the fold change in expression for each drought tolerant genes in the selected cassava varieties

3.6.1 Amplification of ZFP252, ALDH7B4, MSD and RD28 genes by qRT-PCR

The quantitative RT-PCR for the four genes (*ZFP252, ALDH7B4, MSD* and *RD28)* was performed on a standard real time PCR System (Agilent Technologies Stratagene with Mx3000P Software version 4.10) using SYRB Green JumpStart TaqReadyMix (Sigma, USA). These reactions were run on three biological replicates for each variety with the primers in Table 4. For each of the biological replicate, there were duplicate reactions run with the total volume of 20 µl per each reaction. Each reaction consisted of: 2 µl of cDNA, 1 µl (10 pmol) each of the forward (F) and reverse (R) gene specific primers, 10 µl of 2 x SYBR Green I ready mix, 0.02 µl of passive reference dye and 5.98 µl of deionized water. There were two reactions of negative control containing only reagents with no cDNA templates. Thermal Profile conditions; initial denaturation at 94 °C for 2 minutes for cycle 1 and denaturation at 94 °C for 30 minutes, annealing at 55 °C for 1 minutes and extension at 72 °C for 30 seconds for 40 cycles. The dissociation curve was carried out on default setting to confirm the specificity of each reaction. The amplification efficiencies of the targets (genes of interest) and the endogenous control (reference gene) were determined by performing qRT-PCR on 50, 25, 10, 1 and 0.5 ng of cDNA dilutions for all experimental samples. In addition, the coefficient of determination (R^2) and standard deviations for three biological replicates were determined in order to calibrate pipetting accuracy and the reproducibility, respectively.

3.6.2 Genes expression analysis

The relative gene expression for the four genes (*ZFP252, ALDH7B4, MSD* and *RD28),* were obtained by comparing each of the gene with βeta-*Actin gene* (house-keeping gene). The ΔΔ Ct method for relative gene quantification was used to make the various comparisons from the qRT-PCR threshold (Ct) data, specially using the Relative Expression Software Tool (REST) version 2009, computed based on the analytical model by Pfaffl *et al.* (2002). The reactions for the qRT-PCR were normalized using the cassava βeta-*Actin gene* with the primers listed in Table 5. The βcta-*Actin gene* was used as reference gene for all comparison in the selected varieties (Guo *et al.*, 2009; Yang *et al.*, 2011). Expression data from control plants were used as standard calibrator or baseline for comparisons with the treatments for each variety; and Student paired sample *t-test* was applied to determine whether an up-or down regulation of a gene was significant ($P<0.05$). The expression in the control plant was taken as unity (one). A gene is significantly up-regulated or down-regulated when its expression in a treatment is higher than or lower than that of the baseline respectively, and when the *t-test statistics* is lower than 0.05 % (at 95 % significance level). Expression of more than one is up-regulation and expression less than one is down-regulation. The *t-test statistics* showed whether the up-regulation or down-regulation is significant or non-significant (NS).

Table 5: Reference gene (*βeta –Actin*) sequences used for qRT-PCR

Gene symbol	Primers ID	Primer sequence (5'–3 ')	Length (bases)
BETA-ACTIN	ACTIN1F	TGCAGACCGTATGAGCAAG	19
	ACTIN1R	CACCCTTGGAAATCCACATC	20

Source: (Guo *et al.*, 2009; Yang *et al.*, 2011).

CHAPTER FOUR

4.0 RESULTS

4.1 Soil Characteristics

Soil analysis was carried out in order to determine soil texture and soil reaction (pH) as these physical and chemical characteristics have effect on growth and development of cassava. The crop is very sensitive to soil water deficit as moisture retention is very low in sandy soils which is crucial to growth and development during the first three months after planting (Pardales *et al.*, 2001). Soil pH below 5.0 influences aluminium saturation above 80 percent (FAO, 2013). However, Nassar (2005) also reported that soil pH above 8.0 is detrimental for cassava production.

4.1.1 Physical characteristics

Soil analysis results for experiment are shown in Table 6. Soil analysis revealed that the soil texture was sandy loam with 71.00 % sand, 18.00 % clay and 11.00 % silt.

4.1.2 Chemical characteristics

Soil chemical analysis was based on classification by Landon (1991). Soil pH was moderately acidic (pH 6.2) with soil organic carbon (0.09 %), organic matter (0.05 %), total nitrogen (0.04 %) and Carbon Nitrogen (C: N) ratio (1.06:1) were within an acceptable range for cassava growth and development.

Table 6: Soil physicochemical characteristics at experimental site

Properties	Result	Unit	Remarks*
A. Physical			
Texture			Sandy Loam
Sand	71.00	%	
Clay	18.00	%	
Silt	11.00	%	
B. Chemical			
pH	6.20		Moderately acid
Organic Carbon	0.05 %		Very low
Total Nitrogen	0. 04%		Very low
Organic Matter	0.09	%	Very low
C : N ratio	1:1.06		Very low

* According to Landon (1991).

4.2 Quality and Integrity of Ribonucleic Acid and Complementary DNA

4.2.1 Quality and quantity of cassava ribonucleic acid (RNA)

In the present study, RNA was extracted from cassava leaf samples using a modified Cetyltrimethyl ammonium bromide (CTAB) - based protocol by Chang *et al.* (1993). The quality and quantity were investigated by agarose gel electrophoresis and spectrophotometry at (A_{260}/A_{280}). The RNA had acceptable quality ranging from 1.97 to 2.01 (Table 7; Figure 1).

Table 7: Ribonucleic acid (RNA) quantity and purity obtained from cassava leaves

Sample ID	Control / Treatment	Ribonucleic acid (RNA) concentration(ng/ µl)	A260/A280
Kizimbani	Control	1 106.51	1.98
	Treatment	1 058.22	1.99
KBH/2006/374	Control	1 263.53	1.98
	Treatment	1 093.22	1.99
KBH/2002/135	Control	1 082.20	1.97
	Treatment	1 299.21	2.00
UKG 92/053	Control	1 231.34	1.98
	Treatment	3 823.33	2.00
IS 30474	Control	1 150.40	2.01
	Treatment	1 130.61	2.00
Kiroba	Control	2 605.31	2.00
	Treatment	1 123.40	1.98
KBH 95/517	Control	1 522.11	1.97
	Treatment	1 632.21	1.99
KBH 97/212	Control	1 142.11	1.98
	Treatment	1 721.00	2.01

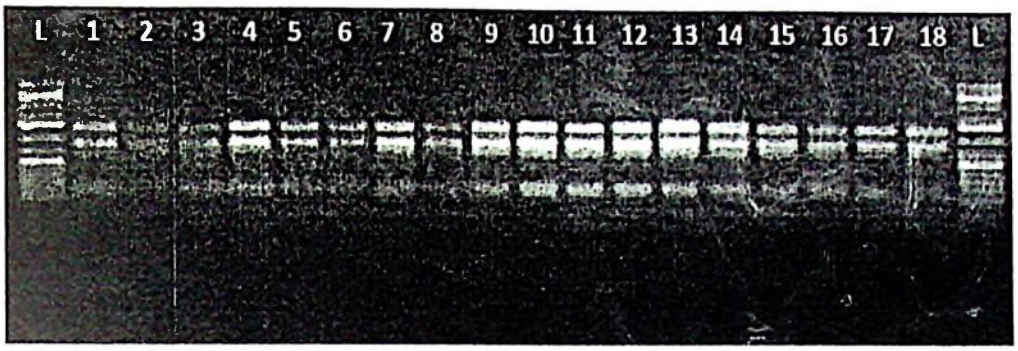

Figure 1: Quality of RNA extracted from cassava leaves. Agarose gel electrophoresis was performed to investigate the quality/integrity of RNA extracted from cassava leaves. The double clear bands (18s and 28s) of cassava RNA were observed in all samples (1 through 18) indicating good quality RNA. The letter L indicates the ladder used on both ends of the gel.

4.2.2 Complementary DNA quality and integrity

The synthesized cDNA concentration of each of the samples was determined using a Cecil CE3021 spectrophotometer (Cecil Instruments, Cambridge, UK). The quality cDNA readings were obtained at absorbance wavelength of A_{260}/A_{280} with the integrity ratio from 1.97 to 2.01, which were considered acceptable. All cDNA were synthesized from the RNA. The resulting cDNAs were standardized by diluting to a final working concentration of 50 ng/µl. The cDNAs were used during Conventional End-point PCR and RT-PCR for analyzing the following genes: *ZFP252*, *ALDH7B4*, *MSD* and *RD28*.

4.3 Screening for the presence of *ZFP252*, *ALDH7B4*, *MSD* and *RD28* genes in selected cassava varieties

The screening of genes related to drought tolerance was limited to the four genes (*ALDH7B4*, *ZFP252*, *MSD* and *RD28*) because they were previously identified as candidate cassava drought-tolerance genes, as they were exclusively up-regulated in the drought-tolerant genotype to comparable levels known to confer drought tolerance in

other species (Turyagyenda *et al.*, 2013). The screening of the four genes was limited mainly to the eight known improved cassava varieties by cassava breeding program in Tanzania.

4.4 Amplification of *ZFP252*, *ALDH7B4*, *MSD* and *RD28* by end-point PCR

Amplification was carried on a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA), using *ZFP252*-F/R, *ALDH7B4*- F/R, *RD28*- F/R and *MSD*-F/R primers in (Table 5). All of the four genes previously confirmed to have functional role in drought stress in cassava that were selected for this study gave the PCR products with a single band at the expected sizes of 245, 269, 263 and 237 base pairs for *ZFP252*, *MSD*, *ALDH7B4* and *RD28* genes, respectively. Amplification indicated the specificity in amplification of each gene. In all the varieties, the amplifications were in both the control (well-watered plants) and watered stresses plants (treatment). Each band was distinctly and clearly produced by each primer set (Figures 2 and 3).

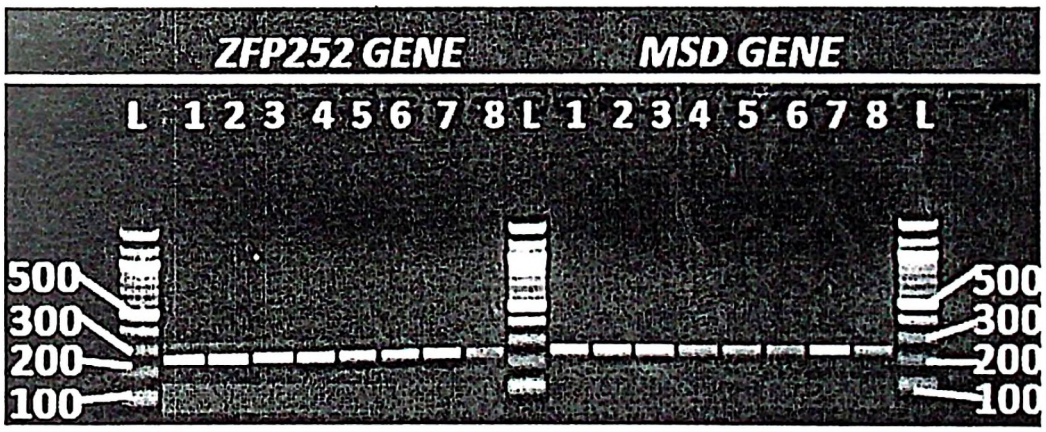

Figure 2: Agarose gel electrophoresis of PCR products of*ZFP252* and *MSD*, amplicons from cassava samples (1 through 8). The single and clear band at the expected size indicates the specificity of the primers on targeted fragments at 245 and 269 respectively. The L indicates 100 bp DNA ladders by Bioneer Corporation (Daejeon, South Korea).

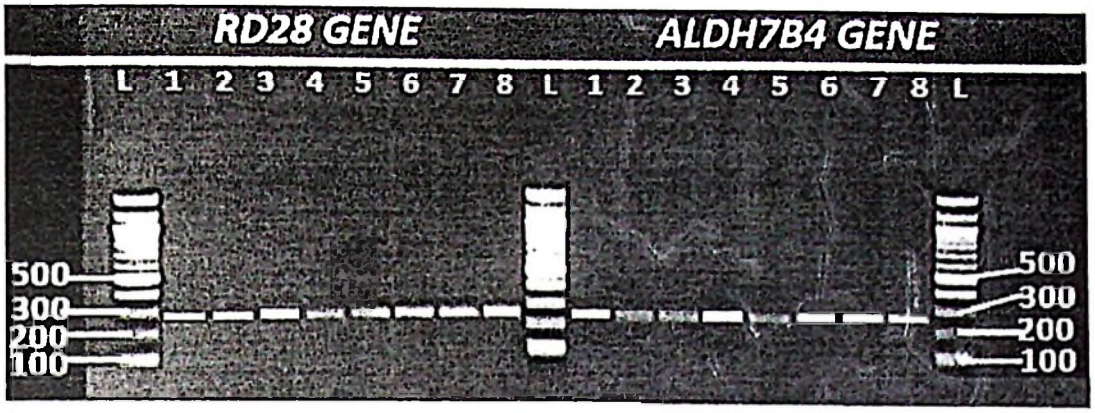

Figure 3: Gel electrophoresis of PCR products of *ALDH7B4*and *RD28* amplicons from cassava samples. A single clear band of *ALDH7B4*and *RD28* fragments was observed in all samples (1 through 8) indicating specificity of primers on targeting expected fragments. Amplification of *ALDH7B4* and *RD28* produced a band of approximately 263 and 237 base pairs (bp) respectively. The letter L indicates 100bp DNA ladders by Bioneer Corporation (Daejeon, South Korea).

4.5 Determining the Fold Change in Expression for Each Drought Tolerant Gene in the Selected Cassava Varieties

4.5.1 Amplification of *ZFP252*, ALDH7B4, MSD and *RD28* genes by RT-PCR

All the genes of interest *(ZFP252, ALDH7B4, MSD* and *RD28*genes*)* and the reference gene (*ßeta-Actin*) amplified with threshold cycles (Ct) ranging from 25 to 29 in (Figure 4). The amplification efficiencies of the reference and target genes ranged between 98.24 to 101.39 %, which is considered acceptable. The linearity for coefficient of determination (R^2) indicated the accuracy of pipetting and the threshold cycles (Ct). Standard deviations of all biological replicates were less than 0.105; which validated an acceptable reproducibility. Negative control reactions (no cDNA templates) assessed the quality of reagents, primers dimers and absent of contaminations. No amplification was detected in the negative controls.

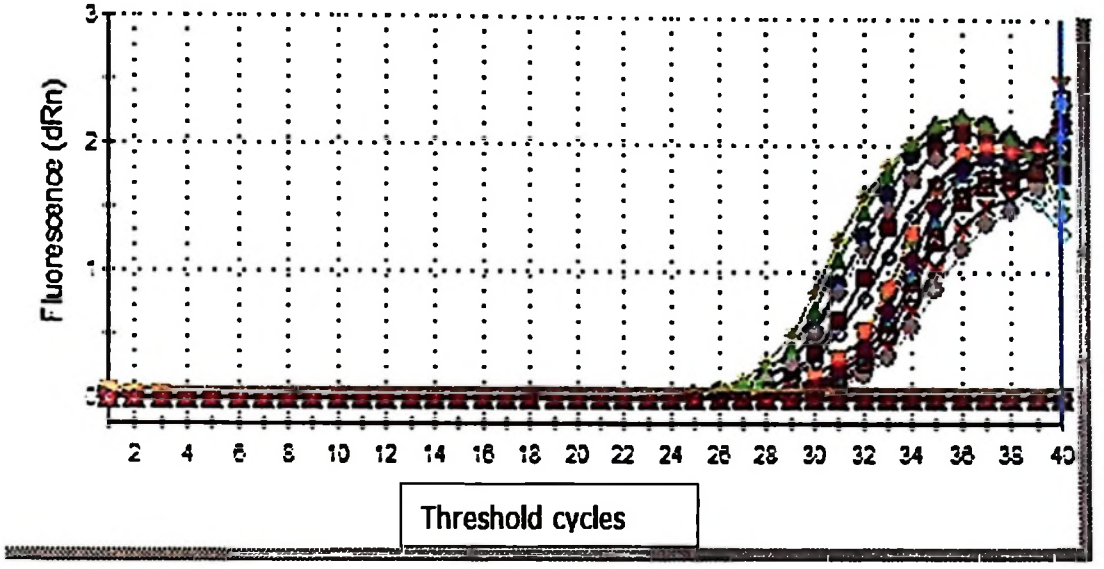

Figure 4: Amplification Plots indicate the specificity of primers within the 40 cycles of reaction. All primer pairs amplified the target genes and reference gene (*βeta-Actin*) ranging between 98.24 to 101.39 % with Ct values ranging from 25 to 29 for all samples.

4.5.2 Genes expression analysis

The relative gene expression for each of the four genes (*ZFP252, ALDH7B4, MSD* and *RD28* gene) compared with *beta-actin gene* between the controls (well watered plants) and the treatment (stressed plants) within each of the eight varieties by using the ΔΔ Ct method for quantification by Pfaffl *et al.* (2002). Results showed efficiencies of targets and reference genes were approximately equal. Results revealed that all of the four genes were significantly ($P<0.05$) up-regulated in Kiroba as shown in Table 8. The study further revealed that *ALDH7B4* gene was significantly ($P<0.05$) up-regulated in all varieties except in IS30474. The results also showed that KBH 94/774 and KBH 97/212 had the highest number of down-regulated genes in expressions. Whereas IS30747 had the highest number of non-significant gene expression as shown in Table 8: and Figure. 6.

Table 8: Fold change in expression of genes in treatment (water stress) against control (Well water) varieties

Varieties	Gene	Expression	SE	95 % CI	Probability (P = 0.05)	Result
Kizimbani	*ZFP252*	0.571	0.171 – 0.646	0.019 - 0.661	0.001	Down-regulated
	RD28	0.571	0.183 – 0.711	0.320 – 0.577	0.009	Down-regulated
	ALDH7B4	1.670	1.618 – 3.031	1.579 – 8.281	0.001	Up-regulated
	MSD	1.283	0.431 – 1.682	0.321 – 2.446	0.084	NS
KBH 2006/374	*ZFP252*	1.652	1.781 – 2.561	1.620 – 5.973	0.003	Up-regulated
	RD28	1.421	0.718 – 2.196	0.133 – 4.407	0.055	NS
	ALDH7B4	7.012	2.869 – 5.828	2.408 – 11.979	0.000	Up-regulated
	MSD	1.023	0.718 – 2.196	0.455 – 3.115	0.212	NS
KBH 2002/135	*ZFP252*	0.091	0.097 – 0.681	0.045 – 0.869	0.004	Down-regulated
	RD28	0.132	0.121 – 0.510	0.069 – 0.838	0.000	Down-regulated
	ALDH7B4	1.694	1.249 – 3.456	1.169 – 9.838	0.000	Up-regulated
	MSD	1.323	0.541 – 2.141	0.447 – 3.899	0.093	NS
UKG 92/053	*ZFP252*	1.392	0.214 – 0.998	0.149 – 1.024	0.099	NS
	RD28	1.601	1.341 – 5.182	1.226 – 9.174	0.004	Up-regulated
	ALDH7B4	6.383	1.601 – 4.127	1.409 – 8.641	0.003	Up-regulated
	MSD	0.612	0.731 – 0.873	0.018 – 0.689	0.000	Down-regulated
IS 30474	*ZFP252*	1.851	1.912 – 3.191	1.802 – 7.087	0.011	Up-regulated
	RD28	4.355	1.994 – 4.144	1.937 – 11.309	0.000	Up-regulated
	ALDH7B4	1.046	0.651 – 3.416	0.553 – 3.926	0.390	NS
	MSD	1.171	0.245 – 1.891	0.146 – 2.267	0.064	NS
Kiroba	*ZFP252*	3.233	1.301 – 4.564	1.230 – 10.976	0.003	Up-regulated
	RD28	3.927	1.716 – 4.011	1.681 – 9.496	0.002	Up-regulated
	ALDH7B4	2.991	1.513 - 3.511	1.491 – 8.789	0.001	Up-regulated
	MSD	3.972	1.991 – 4.881	1.955 – 11.058	0.000	Up-regulated
KBH 95/774	*ZFP252*	0.794	0.711 – 0.881	0.083 – 0.951	0.012	Down-regulated
	RD28	0.126	0.412 – 0.813	0.099 – 0.954	0.001	Down-regulated
	ALDH7B4	1.563	1.341 – 3.491	1.297 – 9.813	0.009	Up-regulated
	MSD	0.779	0.114 – 0.712	0.046 – 0.921	0.006	Down-regulated
KBH 97/212	*ZFP252*	0.658	0.315 – 0.331	0.085 – 0.412	0.006	Down-regulated
	RD28	0.067	0.182 – 0.561	0.097 – 0.643	0.000	Down-regulated
	ALDH7B4	1.615	1.456 – 4.516	1.373 – 10.887	0.017	Up-regulated
	MSD	0.806	0.841 – 0.612	0.422 – 0.717	0.043	Down-regulated

CI = Confidence interval at 95 %; Expression = fold change in the expression of a gene in water stress relative to control and treatment and NS = non-significant.

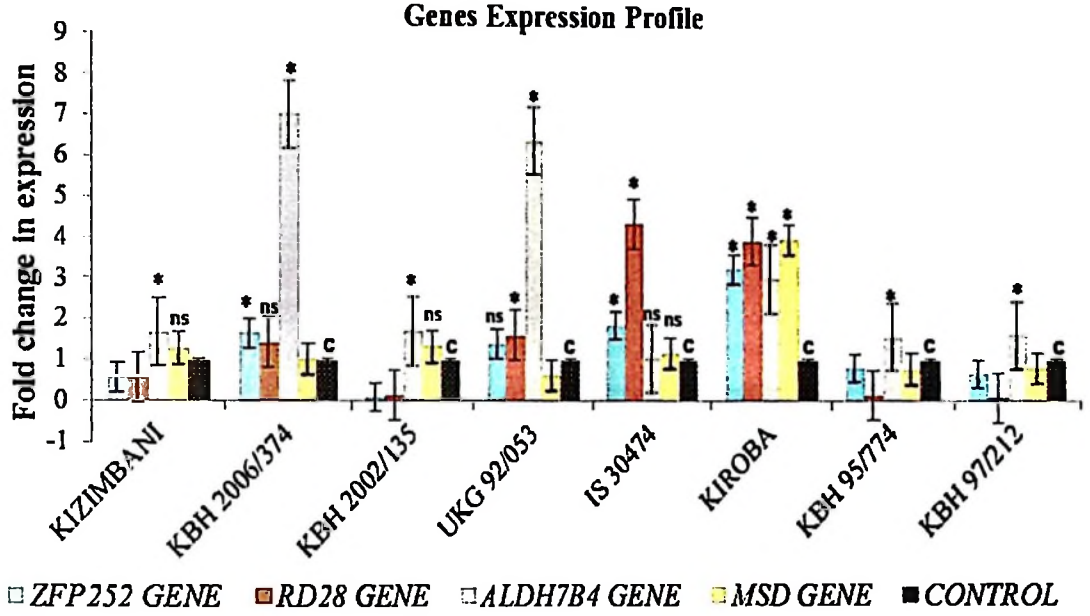

Figure 5: The fold change in expression for *ZFP2525, RD28, ALDH7B4* and *MSD* genes in eight varieties relative to the reference gene *(βeta-Actin)*.

Note: The fold change in expression for *ZFP2525, RD28, ALDH7B4* and *MSD* genes in eight cassava varieties relative to the reference *βeta-Actin* gene. Paired Sample *t-tests* significance: * = P<0.05, as up-regulated. The control (C) is the standard baseline/calibrator at unity (one), while ns = non-significant.

CHAPTER FIVE

5.0 DISCUSSION

This study was conducted in a screen house environment at Mikocheni Agriculture Research Institute, Dar es Salaam. The aim of this study was to improve efficiency in breeding for drought tolerance cassava through molecular techniques; by further determining the fold change in expression for each drought tolerant gene in eight selected improved cassava varieties (Kizimbani, KBH 2006/374, KBH 2002/135, UKG 92/053, IS 30474, Kiroba, KBH 95/517, and KBH 97/212) in Tanzania. This is the first study of its kind, to our knowledge, conducted on the eight varieties of cassava, which additional aimed at investigating molecular characteristics of gene expression levels. This study revealed and confirmed the presence of the four genes (*ZFP252, ALDH7B4, MSD* and *RD28*). They have been associated with drought adaptation, or the tolerance in improved cassava genotype (MH96/0686) from Uganda (Turyagyenda *et al.*, 2013).

Aldehyde dehydrogenase is encoded from the gene *ALDH7B4*, which was up-regulated specifically in MH96/0686 by 2.815-fold. It plausibly this gene may hence be involved in the enhancement process of drought tolerance in cassava under drought stress (Turyagyenda *et al.*, 2013). In the findings of this study, the gene was up-regulated with fold change of 1.670-fold in Kizimbani, 7.012-fold in KBH 2006/374, 6.383-fold in UKG 92/053, 2.991-fold in Kiroba, 1.563-fold in KBH 95/774 and 1.615-fold in KBH 97/212 (Table 8). This gene was over-expressed in KBH 2006/374 and UKG 92/053. The findings of this study are in strong agreement with the studies by Kotchoni *et al.* (2006), who observed that transgenic *Arabidopsis thaliana* plants with increased amounts of *ALDH7B4* were more tolerant to dehydration and salt stress than wild-type plants. They reported further that over-expression of the *ALDH7B4* gene in transgenic plants

under drought and salt stress reduced the level of lipid peroxidation, signifying that the gene confers both oxidative and osmotic stress tolerance in *Arabidopsis thaliana* through reactive oxygen species (ROS) scavenging and reducing lipid peroxidation. Additional result revealed that the gene can be induced by pathogens and might therefore be a multi-stress-responsive gene (Zimmermann *et al.*, 2004). The fold change by 2.815 over-expression of this gene in drought tolerant cassava indicated the gene may take part in the enhancement of drought tolerance in cassava, probably by reducing lipid peroxidation through ROS scavenging (Kotchoni *et al.*, 2006).

The gene *ZFP252* that translates a zinc finger protein has been conveyed during water stress to confer drought tolerance in plants by maintaining cell membrane integrity. It was revealed that the relative electrolyte leakage, an indicator of membrane injury, was lower under drought stress in *Oryza sativa ZFP252*-transformed rice plants than in non-transformed *Oryza sativa ZFP252* knock-out plants (Morsy *et al.*, 2005; Xu *et al.*, 2008). The findings suggest that *ZFP252* protects plants from stress by retaining cell membrane integrity. The higher soluble sugars and free proline contents were in transformed *Oryza sativa* plants than non-transgenic *Oryza sativa* plants (Xu *et al.*, 2008). Results suggest that enhanced stress tolerance under salt and drought stresses of *ZFP252*-transgenic plants might partially be through activation of proline synthesis and proline transference pathways by *Oryza sativa ZFP252*. Drought tolerance through osmotic adjustment was due to higher proline levels (Sanchez *et al.*, 1998; Xu *et al.* 2008). In this study, *ZFP252* gene was also exclusively up-regulated in KBH 2006/374 by 1.652-fold, 1.851-fold in IS 30474 and 3.233-fold in Kiroba. It is therefore very suggestive that this gene is among few that enhance drought tolerance in cassava and specifically in KBH 2006/374, IS 30474 and Kiroba, through increasing the free osmo-protectant proline and soluble sugars as observed in earlier studies (Sanchez *et al.*, 1998).

The gene *MSD* translates into manganese superoxide dismutase (MnSOD) enzyme that plays a role in oxidative stress tolerance in plants. Over-expression of superoxide dismutase (SOD) in transgenic plants increases oxidative stress tolerance (Basu *et al.*, 2001; Wang *et al.*, 2005). In this study, findings are in agreement with the relative expression in Kiroba by 3.972-fold. It shows a level that can believably confer increased oxidative stress and drought tolerance in cassava. Studies by Sen Gupta *et al.* (1993) showed that a 3-fold increase in total pea copper or manganese superoxide dismutase activity in transgenic tobacco resulted in an increase significantly in resistance to membrane impairment. A 1.5 to 2.5-fold increase in total (SOD) enzymes activity was reported by Basu *et al.* (2001) in transgenic *Brassica napus* plants transformed with wheat MnSOD increased oxidative stress resistance as compared with wild-type controls. Wang *et al.* (2005) reported that a 1.4-fold increase in total Superoxide dismutase enzymes activity in the MnSOD transgenic rice plants was enough to increase oxidative stress resistance and drought tolerance when the gene was fused with a chloroplast transit peptide sequence in order to target the manganese superoxide dismutase to the chloroplast. The 3.148-fold increase of expression was observed in drought tolerance genotype MH96/0686 (Turyagyenda *et al.*, 2013). Superoxide dismutase enzymes are involved in scavenging reactive oxygen species that are produced in plants during water stress (McKersie *et al.*, 1996; Fryer *et al.*, 2002). It is therefore hypothesized that this gene confers drought tolerance through ROS scavenging in cassava.

The *RD28* gene encodes the responsiveness to Desiccation. The expression of t *RD28* gene was increased1.511-fold by water stress, being exclusively up-regulated in the drought tolerant genotype, suggesting that it plays a role in enhancement of drought tolerance in cassava. In this study, findings in expression for *RD28* gene were 4.355-fold in Kiroba, 3.927-fold in KBH 95/774 and 1.601 in UKG 92/053. Daniels *et al.* (1994)

finding showed that *RD28* gene is a turgor-responsive, mercury-resistant plasma membrane aquaporin found in plasma membranes of all plant tissues except seeds. Earlier studies by Yamaguchi-Shinozaki *et al.* (1992) revealed that *RD28* gene enhances drought tolerance through an abscisic acid-independent pathway. It transport small molecules across cell membranes by protecting desiccated cells; and finding in this study believed that it enhances the cells' desiccation tolerance in drought tolerant cassava through osmotic adjustment.

The Transcription factors (TFs) interact with cis-elements in the promoter region of several stresses related genes and thus up or down-regulating the expression of many downstream genes resulting into impacting abiotic stress tolerance (Agarwal and Jha 2010). In *Arabidopsis thaliana* genome about 1500 TFs are described which are considered to be involved in expression of stress responsive genes (Riechmann *et al.*, 2000). The dehydration responsive element binding proteins/C-repeat binding factor (DREBs/CBF) are important transcription factors that induce a set of abiotic stress-related genes, thus impacting stress tolerance to plants. They play an important role in Abscisic acid (ABA)-independent pathway that activates stress response genes (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Udvardi *et al.*, 2007). These proteins specifically bind to and activate the expression of genes in the promoter of the drought response gene *RD28* (Yamaguchi-Shinozaki and Shinozaki, 1993). It is probably possible that transcription factor DREBs/CBF might not bind to the promoters of gene *RD28* during the abscisic acid-independent pathway mechanism thus resulted to down-regulation of the gene in three varieties (Kizimbani, KBH 2002/135, and KBH 95/774).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Successful amplification of *ZFP252*, *ALDH7B4*, *MSD* and *RD28* fragments by primers in this study suggests the present of these genes in the selected cassava varieties. The present study revealed at the molecular level an exclusively up-regulation of *ALDH7B4* gene with statistical significance in all varieties except IS 30474. Based on the gene known function in other species, it is likely that the tolerance to drought stress at the cellular level in these varieties consist of reduction of osmotic adjustment and oxidative stress through reactive oxygen species (ROS) scavenging and reduction of lipid peroxidation; and *ALDH7B4* gene influenced these varieties tolerance to drought. In addition, findings showed Kiroba as the only variety with all four genes exclusively up-regulated. Physiologically, the eight varieties exhibited indications of tolerance to drought stress during the entire experiment. The *ALDH7B4* gene can now be tested in the context of cassava breeding, as possible quantitative trait loci and engineering for drought tolerance in transgenic. Kiroba can also be considered for test in cassava breeding programs to engineering drought tolerance trait due to the up-regulation of the four genes. The significance of this research finding is to generate new molecular data to bridge the information gap and provide more tools for breeders to use for introgression into other improved cassava germplasms for climate change mitigation. In addition, poverty alleviation and sustainable food security are benefiting factors from this study.

6.2 Recommendations

Based on the findings of the present study, it is recommended that other studies should be conducted;

(i) To further understand the mediating signaling path-ways in response to abiotic stresses, it will be essential to identify and characterize the downstream and upstream molecules of *ZFP252, ALDH7B4, MSD* and *RD28* by microarray, yeast hybrid system and so on;

(ii) To research on Kiroba concerning its mineral contents in leaves and tuber qualities; this analysis is warranted to determine whether the up-regulation of these four genes (*ZFP252, ALDH7B4, MSD* and *RD28)* influence or affect any nutritional qualities during abiotic stresses, and

(iii) To further design field-based trials, re-evaluating experiments and compare findings with screen-house environment conditions; and provide information back to plant breeders and farmers in the field as the best realistic research design to generate sustainable or reliable results.

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APPENDICES

Appendix 1: Nucleic acid extraction

CTAB Method:

Before starting, clean working surfaces, pipettes, centrifuges and sterilize with 70% ethanol. Then wipe with RNAse zap. Use RNAse free and tubes (*filters* tips)

CTAB Extraction Buffer:

Two percent (2%) CTAB, 100mM Tri – HCl, 20mM EDTA, 1.4M NaCl. Before extraction, Add 5% βeta-mercapto ethanol (i.e. 50µl per 1ml extraction) and 2% PVP.

CTAB Extraction Buffer

Reagent	**Stock Concentration**	**Volume for 100ml extraction**	
CTAB	10%	100 x 2/10	20 ml
Tri - HCl	1 M (1000mM)	100 x 100/1000	10 ml
EDTA	0.5 M (500 mM)	100 x 20/500	4 ml
NaCl	5 M	100 x 1.4/5	28 ml
Make up with sterile distilled water			38 ml
Total volume			100 ml

1. Collect 0.15 – 0.2 gram of fresh cassava leaf sample with sterilized gloves and placed it in a sampling envelope
2. Take the sample(s) to the Laboratory and put it in -80$^{°C}$
3. Grind fresh leaf sample with pestle and mortar (sterile, autoclaved and wiped with RNAse zap, chilled in -20$^{°C}$)

4. Add 700 µl of CTAB buffer (containing βeta-mercapto ethanol) wormed at 65°C
5. Transfer the liquid mixture into1.5 ml Eppendorf tube
6. Vortex to dispense tissue in buffer
7. Incubate at 65°C for 30 minutes, mixing by inversion every 10 minutes
8. Leave at room temperature for 10 minutes

Solvent Extraction

9. Add equal volume (step 4) (700µl) of Chloroform: Isoamy alcohol (24:1) and mix by inversion for ten minutes (10 mints)
10. Centrifuge at 12,000 rpm for 10 minutes
11. Remove upper aqueous phase to a clean tube (~500 µl)
12. Repeat steps 8 – 10 (remove~490 µl) aqueous phase to a clean 1.5 Eppendorf tube

Pellet Precipitation

13. Add 0.7 volume (~343 µl) of cold (-20°C) isopropanol and shake gently
14. Spin tubes at 13,000 rpm for 10 minutes, decent the isopropanol
15. Incubate for 30 minutes in -20 °C freezer
15. Add 500 µl of 70% ethanol, wash by tapping, spin at 13,000 rpm for 10 minutes, decant ethanol, leave to air dry for about 40 minutes
16. Re- suspend in 50 µl RNAse free water and preserve your sample for further use.

Appendix 2: DNAse 1 Digestion

Combine the following in a 0.5 ml tube

Component	Amount
Total RNA	34 µl
10 x DNAse 1 buffer	4 µl
DNAse 1 enzyme	1 µl

1. Incubate at 37°C for 30 minutes
2. Add 4 µl DNAse inactivation reagent and mix well (re- suspend the DNAse inactivation agent by vortexing or flicking the tube gently before pipetting it)
3. Incubate at room temperature for 2 minutes, mixing occasionally (3-3 times) to disperse the DNAse inactivation agent
4. Centrifuge at 1000 rpm for two (2) minutes to pellet the DNAse inactivation agent
5. Carefully transfer the supernatant (RNA) to a fresh tube, avoiding the pellet.

Appendix 3: Complementary DNA (cDNA) Synthesis

Master Mix-I Preparation

Combine the following in 1.5 ml Eppendorf tube:

Component:	Amount:
OligodT (18)	1.0 µl
dNTPs (10 mM)	2.0 µl
DEPC sterile distilled water	10.5 µl
Total volume	13.5 µl

1. Aliquot 13.5 µl in each PCR tube
2. Add 2.0 µl of RNA templates to the reaction tube
3. Incubate at 65 °C for 5 minutes
4. Placed the reaction tube in ice immediately for 4 minutes

Master Mix-II preparation

Combine the following in 1.5 ml Eppendorf tube:

Component:	Amount:
DEPC sterile distilled water	2 µl
10 x RT (reverse transcriptase)	1 µl
Ribolock	0.5 µl
RT text Gen polymerase	1.0 µl
Total volume	4.5 µl

5. Aliquot 4.5 µl of Master Mix-II to the reaction tube
6. Incubate at 45 °C for 50 minutes and then 85 °C for 5 minutes
7. Remove from incubator and stored the cDNA at + 4 °C for downward reaction.