ANALYSIS OF 'STAYGREEN' CHARACTERISTICS ASSOCIATED WITH DROUGHT TOLERANCE IN CASSAVA (*Manihot esculenta* Crantz) UNDER GREENHOUSE CONDITIONS

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DECLARATION

I declare that this is my original work and has not been presented for an award of a degree in any other university.

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DEDICATION

God my creator gets the entire Praise and Thanksgiving! for giving me the ability, strength and focus to complete this study. I dedicate this work to my dear sister Rita Atieno Orek. Because of you I have an Education. To my dear mother Cornelia Akinyi Orek, for my birth, upbringing as well as nurturing and instilling in me the spirit of discipline and hard work. You have been a pillar of strength to our family and I owe you a great deal of appreciation. May God bless all the days of your life and may you live to see my great great grand-daughter!

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) ranks as the fourth most important staple food cultivated and consumed mainly in the tropics and other developing countries. The crop yields well in areas with uncertain rainfall patterns and prolonged drought. Leaf retention or longevity (staygreen) has been associated with sustained cassava production under drought. This study analyzed and screened for this characteristic in both transgenic and non transgenic cassava genotypes under greenhouse conditions.

Significant ($P \leq 0.05$) genotypic variations in internode growth, leaf abscission, photosynthetic rates, and stomatal conductance under drought stress treatments were observed between transgenic and non-transgenic cassava genotypes. Based on these results, provisional levels of staygreen or drought stress tolerance were assigned to the genotypes. Non-transgenic genotypes 98-0002, 98-2226 and transgenic line 529-48 showed high levels of staygreen, non transgenic genotypes 91-02322, TME-3 and transgenic line 529-28 expressed moderate levels of staygreen and non transgenic genotype 95-0306 and wild type TMS 60444 showed drought stress susceptibility.

Expression of staygreen trait was clearly marked out under 30 and 60% levels of drought stress as well as in fully irrigated or control treatment. Photosynthetic rates positively correlated with stomatal conductance and negatively correlated with leaf abscission. Leaf abscission and stomatal conductance correlated negatively.

The initial stages of characterization of cassava transcriptome upon drought treatment, (for the identification of up- and down regulated genes), involves RNA purification. It is a pre-requisite that for successful procedure of microarray-based gene expression profiling, the RNA extracted should be of high integrity, quality and quantity.

In this study, the following five protocols were tested, optimized and used in the extraction of RNA from the leaves of cassava genotype TMS 604444: modified CTAB based method, RNeasy Plant Minikit, Total Nucleic acid and DNase treatment, protocol described by Reilly et al. (2001) and Trizol Reagent-based method.

The modified CTAB method produced RNA of high concentration (more than 1 microgram), high quality (A_{260}:A_{280} and A_{260}:A_{230} ratios more than 2.0) and high integrity (distinct and visible 28S and 18S rRNA bands) from young and old cassava leaves, compared to RNA (from the same leaf tissues) generated by the other four methods.

CHAPTER ONE:

1.1 General introduction

Agricultural drought has been described as an inadequacy of water availability (including precipitation and soil moisture capacity) in quantity and distribution during the life cycle of a crop, which restricts the expression of its full genetic yield potential (Sinha, 1996; Rauf, 2008). This is often indicated by impaired crop growth and reduced crop yields. Drought affects agriculture in 45% of the world geographical area that is occupied by 38% of the world's population (Bot et al., 2000).

Areas within the global savannah zone, which extends through much of Africa, India, China, and the middle of South America, are increasingly prone to poor soil moisture conditions for plant growth and development (Jury and Vaux, 2005). Studies on drought distribution have shown that over 50% of Sub-Saharan Africa, Asia and the Pacific possess significant hazards associated with drought and dryland conditions (Bot et al., 2000). Compounding this is the current outlook on global climatic changes, which suggests future increase in the frequency and severity of drought in many other parts of the world (Salinger et al., 2005).

Drought poses a significant challenge to sustainability in food production since agricultural production is highly dependent on the amount and distribution of rainfall during the crop cycle (Ceccarelli et al., 2007). Compared to other abiotic stresses, drought imposes a severe limitation on food, fodder and fiber as well as fuel production (Viswanathan et al., 2007).

Bray et al., (2000) estimated that between 51 to 82% of the potential yield of annual crops losses were attributed to abiotic stresses with erratic and insufficient rainfall recorded as a major factor. An estimated 24 million tons of maize grain are lost on a yearly basis in the tropics, with losses reaching up to 60% in well-watered production (Bänziger et al., 2000; Tuberosa et al., 2003). Sorghum is well adapted to dry environments and is considered a drought resistant crop, but a severe post-flowering drought can result in a reduction of its grain yield as well as a decrease in its seed grain weight relative to the fully irrigated environment (Tuinstra et al., 1997).

Late drought treatment has also produced a large mass of small or empty barley grains (Jamieson et al., 1995). Upland rice, especially in Africa is prone to varying degrees and duration of drought stress during the crop cycle, causing yield reduction and sometimes total crop failure (Saxena and O'toole, 2002). Cassava (*Manihot esculenta* Crantz) poses a number of traits that allow it to cope with drought stress better than other crops. These includes high stomatal sensitivity to environmental humidity (Oguntunde, 2005), a deep rooting capacity and quick recovery after stress (El-Sharkawy and Cock, 1987).

Although these features classify cassava as drought tolerant (Alves, 2002), when exposed to extended water shortage, the crop's productivity not only remain Sub-optimal and unstable, but most of the cultivars also show increased cyanide contents within their storage roots (El-Sharkawy, 2006; Alves, 2002; Rosling, 1994; Essers, 1995; Bokanga, 1994).

Other studies indicate that the storage root yield reduction in cassava depends on the duration of the water deficit as well as the sensitivity of a particular growth stage to the drought stress (Alves, 2002). Connor et al. (1981) found out that water deficit during at least 2 months of the stages of rapid leaf growth, root initiation and tuberization, reduced cassava's storage root yield from 32 to 60%.

1.2 Research Justification, Hypotheses and Study Objectives

It has since been established that tolerance to drought stress in cassava is genotype-dependent (Bray, 1994; Alves, 2002; Lokko et al., 2007), with particular cultivars exhibiting a high degree of drought tolerance especially in areas characterized by low and erratic precipitation, coupled with dry air during a great part of the growth cycle, high air temperatures, high potential evapo-transpiration and low fertility soils (El-Sharkawy, 2006).

Under these conditions, other staple food crops such as grain cereals and legumes rarely survive and produce, compared to the drought tolerant cassava clones, which once established, are able to survive the drought stress and produce reasonably well (El-Sharkawy, 2006). These improved cassava cultivars have yielded over 3 tons ha^{-1} oven-dried storage roots when grown in semi-arid areas receiving less than 700 mm annual rainfall (El-Sharkawy, 2006). A number of traits contribute to this success with the most recent one being a leaf retention trait referred to as 'staygreen'.

Staygreen cassava cultivars show enhanced ability to retain their leaves during drought. Lenis et al. (2006) characterized some cassava clones for this trait and found out that clones expressing leaf retention trait produced more total fresh biomass and their root dry matter were 33% higher than the clones without the trait.

The importance of a broadened study on leaf retention or staygreen trait and its role in cassava drought tolerance cannot be under-estimated. In an effort to improve the efficiency of conventional cassava breeding methods, Setter and Fregene (2007) called for the use of molecular markers and marker assisted selection (MAS) to breed for drought tolerance. This approach was further supported by Lokko and colleagues (2007), who suggested an investigation into the molecular basis for tolerance to water stress, as well as identification of possible controlling genes using contrasting cassava germplasm.

Once the molecular markers for drought tolerance in cassava are developed, they could be used both in mapping populations and in released cultivars to assess their staygreen potential under varying (but limited) drought stress regimes. Therefore, the broad objective of this study was to establish a suitable drought stress regime or level (s) that can be effectively applied in the screening or characterization of staygreen characteristics in cassava genotypes under greenhouse conditions.

In addition, the study sought to understand some of the cassava's morphological and physiological responses to drought stress as well as initiate a ribonucleic acid purification method that could aid in future understanding of the genetic and biochemical basis of the staygreen trait.

Since no study has been carried out on differential staygreen phenotypic expression in cassava under greenhouse conditions, this study hypothesizes that an effective drought stress level (s) can be optimized and efficiently used to screen for staygreen characteristics in transgenic and non-transgenic cassava genotypes under greenhouse conditions. In addition to leaf retention, measurement of parameters such as inter-nodal growth, photosynthetic rates and stomatal conductance can further be used to describe or characterize staygreen phenotype or drought tolerance in cassava.

In order to characterize staygreen cassava transcriptome upon drought treatment, accessing RNA of high quality, integrity and quantity is a pre-requisite step. Although cassava leaves contain high concentration of polysaccharides, polyphenols and proteins that inhibits isolation of RNA, an efficient, simple, and reproducible protocol or method can be tested, optimized and used to extract RNA of high quality, integrity and in sufficient concentration from both young and old cassava leaves.

1.3 Specific objectives

The specific objectives of the research were to:

1) Determine leaf retention, elongation of the last internodes, photosynthetic rates and stomatal conductance in transgenic and non transgenic cassava genotypes subjected to different levels of drought stress under greenhouse conditions.

2) Test, optimize and recommend the most efficient protocol (s) for purification of RNA from cassava leaves in order to aid in the establishment of a cassava-leafy spurge cDNA microarray procedure.

CHAPTER TWO:

2.0 Review of the Literature

2.1 Cassava production and utilization

Cassava (*Manihot esculenta* Crantz) is a perennial shrub widely cultivated for its starchy roots within the tropical and Sub-tropical countries of Africa, Asia and Latin America, between latitudes 30^0N and 30^0S, from sea level to just above 2 300 m above sea level and in areas receiving less than 600 mm rainfall i.e. in semi arids to more than 1 500 mm in the sub-humid and humids (Alves, 2002; El-Sharkawy, 2006; Setter and Fregene, 2007).

Cassava is grown mostly by small scale, resource-limited farmers on marginal and highly eroded low fertility acidic soils and virtually without application of agrochemicals (Ruppenthal et al., 1997; El-Sharkawy and Cadavid, 2000; El-Sharkawy, 1993, 2004). The crop is the only species in its genus that is cultivated as a food crop (Fauqet and Fargette, 1990). It is a staple food for over six hundred million people, ranks as the third source of calories, after rice and maize, in the tropics and sub-tropics (FAO, 2008) and fourth most important source of food globally after rice, sugarcane and maize (Balagopalan, 2002; El-Sharkawy, 2006; Lokko et al., 2007).

Total world cassava use is expected to increase from 172.7 million to 291 million tons in the period 1993-2020, with use in Africa equivalent to 62% (Scott et al., 2000; Westby, 2002). Perhaps as a possible indicator to this expected trend, the global production of cassava increased to 203 million metric tons in 2005, cultivated on 19 million hectares, with an average yield of 9.9 tonnes per/ha (FAO, 2005; Alves et al., 2006).

Though the world's largest cassava producer, the yield potential of an African cassava is still low, at a value of 8.9 t/ha, representing a paltry 70% and 61% of production in South America and Asia respectively (Legg and Thresh, 2004). Eighty eight percent of cassava in Africa is for human food (Westby, 2002). In Kenya, cassava is grown for both food and income on approximately 77,502 ha with an annual output of 841,196 tons (FAO, 2007). Approximately 60% of this production is in Western Kenya, 10% in Eastern province and 30% in Coast province (Crop Crisis Control Project, 2006; Mware et al., 2009).

2.2 Breeding for drought tolerance in cassava

As water resources and arable land become limiting, the use of marginal lands for agriculture as well as production and development of drought tolerant crops is increasingly becoming important (Lokko et al., 2007). The drought tolerance mechanisms enable drought tolerant crops to cope with the physiological effects associated with drought (Blum, 1988), which sustainably benefits agricultural production through reduced use of groundwater resources, expanded dryland farming and increased yield stability (Tuberosa et al., 2003).

Since cassava production is also carried out in environments defined by various degrees of stress and with little or no production inputs by resource-limited small scale farmers, Jennings and Iglesias (2002) and Kawano (2003) concluded that the aims of most recent breeding strategies have been shifted to selection and development of cassava cultivars with reasonable and stable yields, as well as to those that are easily adaptable to a wide range of abiotic and biotic stresses.

Attainment of these breeding strategies and goals are further stimulated by the inherent capacity of cassava to tolerate adverse edapho-climatic stresses, producing yields where other main staple food crops such as cereals and legumes would fail (El-Sharkawy, 2006). This is one of the reasons why the cassava has recently been expanding into more marginal lands and semi-arid regions, particularly in sub-Saharan Africa and Latin America (Romanoff and Lynam, 1992; El-Sharkawy, 2007).

This trend has beefed up the importance of improving the genetic base of cassava, and of breeding new cultivars, more adapted to the severe drought stress conditions prevailing in these regions. Therefore, the identification of cassava characteristics that can be or have been used to breed the crop, especially for drought tolerance, is of utmost importance. Obviously, cassava's adaptation response to drought stress is genotype dependent (Okogbenin and Fregene, 2003; Lokko et al., 2007).

This genetic variability in tolerances to stress should be exploited in breeding and improvement of cassava germplasm for dry environment production (Hershey and Jennings, 1992; El-Sharkawy, 1993; El-Sharkawy et al., 1992). El-Sharkawy (2006) noted that selection of parental materials for tolerance to drought stress and infertile soils has resulted in breeding improved germplasm adapted to both favorable and stressful environments. Stomatal sensitivity to changes in atmospheric humidity that is directly translated into the crop canopy and hence productivity, has been observed among cassava cultivars (El-Sharkawy, 2004; El-Sharkawy, 2006).

The stomata partially closes in response to water stress, conferring the beneficial effect of preventing severe leaf dehydration and consequently preventing impairment of photosynthetic capacity of the leaf as well as reducing water loss through transpiration, further maximizing water use efficiency (El-Sharkawy, 2007). Therefore selection and breeding of more stomata sensitive cultivars is thus considered suitable for sub-humid or seasonally dry and semi-arid zones where there are prolonged periods of water deficits (El-Sharkawy, 2006).

Research has shown that new leaf formation in cassava is often restricted under prolonged drought (El-Sharkawy and Cock, 1987; El-Sharkawy, et al., 1992), and therefore selection for higher stomatal sensitivity should be combined with longer leaf retention (El-Sharkawy, 1993; El-Sharkawy, 2004), as the latter trait has been found to be positively correlated with productivity under naturally extended water deficits (Lenis et al., 2006). Cassava should be bred for longer leaf span as this saves up the dry matter invested in leaf canopy formation, and therefore more assimilates translocated to the storage roots for higher harvestable index and yield (El-Sharkawy, 1993).

Traits which cassava can also be bred for include early bulking, medium or short-stemmed and deep rooting capacities, which have notable advantages under semi-arid conditions (El-Sharkawy, 2006). Cassava has a long breeding cycle (18-24 months) and the production of its recombinant seeds is difficult (Ceballos et al., 2004; Setter and Fregene, 2007), rendering the use of conventional breeding methods in cassava inefficient.

Application of genetic engineering (Setter and Fregene, 2007; Lokko et al., 2007) to avert some of the breeding difficulties has been gaining momentum, especially after transformation protocols for cassava that use either particle bombardment or *Agrobacterium* systems having been established (Schöpke et al., 1997; Taylor et al., 2004). For example, an accelerated leaf senescence and leaf abscission is a drought response strategy that decreases canopy size and enables perennial plants to survive and complete their life cycle under drought stress, but the strategy reduces the yields of most annual crops (Rivero et al., 2007).

Leaf senescence has been delayed or inhibited in transgenic cassava plants over-expressing a cytokinin synthesis gene, *isopentenyl transferase,(ipt),* from *Agrobacterium tumefasciens* under the control of senescence associated gene promoter *PSAG12* (Gan and Amasino, 1995; Zhang and Gruissem, 2004). The resultant drought tolerant transgenic plants showed repressed chlorophyll degradation, improved total leaf harvest (40% more than the wild type), early root bulking, reduced excessive leaf abscission and improved leaf retention of green color in response to drought stress under greenhouse conditions (Zhang and Gruissem, 2004; 2005).

Traits in cassava that are related to productivity and tolerance to water stress, such as high leaf photosynthetic capacity, longer leaf life span, extensive fine rooting systems and stomatal control of water losses, have been identified. The cultivars with such traits have been selected and used as parental materials to breed improved and more adaptable genotypes (El-Sharkawy, 2007).

2.3 Cassava response to drought stress

Although grown in a wide range of climates, cassava is also cultivated in areas receiving less than 800 mm annual rainfall with a dry season of 4-6 months, where tolerance to water deficit is an important attribute (Alves, 2002; Setter and Fregene, 2007). Generally, most studies have concluded that cassava is tolerant to prolonged drought due to a multiple and inherent morphological and physiological traits that allow the crop to obviate the negative effects of severe drought stress (El-Sharkawy, 2003; El-Sharkawy, 2007). A number of these traits are briefly described below:

2.3.1 The stomatal system

When measured using the photosystem infra-red gas analyzer (IRGA, Oguntunde, 2005; El-Sharkawy, 2007), cassava's net leaf photosynthetic rate under high humidity, wet soil, high leaf temperature and high solar radiation exceeds 40 umolCO_2 m^{-2} s^{-1} (El-Sharkawy et al., 1990; El-Sharkawy et al., 1992). This groups the crop with the other highly productive C_3 and C_4 plants types (El-Sharkawy et al., 1993; El-Sharkawy, 2004).

Photosynthesis is however one of the most affected processes under drought stress environments, due largely to cassava's stomatal sensitivity to lowered water status (Palta, 1984). Calatayud et al., (2000) demonstrated this in cassava plants subjected to 45 days of water limitation, where the photosynthetic rates of young intact leaves exposed to intermediate irradiance was decreased by 66%. In the early phases of water limitation, cassava closes its stomata and the leaves droop such that incident light flux densities are lessened and photo-inhibition avoided (Calatayud et al., 2000).

The stomatal opening tends to be limited to the early part of the day when temperatures and vapor pressure differences are low, thereby permitting photosynthesis to occur with higher water use efficiency (Itani et al., 1999). Stomatal sensitivity has also been noted in slight decreases to leaf water potential, such that during initial periods of the water deficit, the leaves drastically limit water loss and maintain leaf water potential at values near those of well-watered control plants for relatively extended periods of drought (Ike, 1982; Itani et al., 1999).

The tendency to limit water loss to the extent that leaf water potential in water stressed plants is almost maintained about at the same values as of well watered plants (Wood, 2005), places cassava in the category of plants that stabilize their leaf water contents by adjusting their stomatal aperture, a phenomenon described as being isohydric (Chaerle and Van der Straeten, 2007), along with maize, cowpea and poplar (Tardieu and Simonneau, 1998).

Cassava leaves maintain high stomatal conductance (Gs, mmol m^{-2} s^{-1}, measured by IRGA as the rate of passage of water vapor through the stomata) and can keep internal CO_2 (umolCO_2 m^{-2} s^{-1}) concentration high when water is available, but drought stress induces reduction in stomatal aperture limiting rates of CO_2 diffusion into the leaf and subsequently reducing photosynthetic rates (Alves, 2002). The stomatal closure also reduces transpiration rate, lessens the decrease in leaf water potential and soil water depletion, thereby protecting leaf tissues from desiccation (Palta, 1984; El-Sharkawy and Cock, 1984).

Although cassava's high stomatal sensitivity to water status is potentially advantageous, there is evidence that suggests its excessive conservative use of water tends to limit its optimal performance (Setter and Fregene, 2007). For example, in a field trial of irrigated cassava plots, Cock and colleagues (1985) reported that when the relative humidity of the crop canopy was increased by artificial misting, the crop biomass and production increased by 27%, and the storage root yield by 91%; an effect that was associated with enhanced photosynthetic rate per unit leaf area of the misted plants due to stomatal opening.

2.3.2 The leaf system

Cassava conserves water under extended stress by reducing photon interception through a decline in leaf canopy mainly resulting from restricted new leaf formation, smaller leaf sizes, and leaf fall (Palta, 1984; El-Sharkawy and Cock, 1987; El-Sharkawy et al., 1992). As drought stress progresses, the crop not only droop its leaves to lessen radiant heating and water loss (Calatayud et al., 2000), but also a substantial fraction of the leaves abscises thereby decreasing transpirational surface area (Ramanujam, 1990; Lenis et al., 2006).

Leaf growth in cassava is highly sensitive to drought stress (Yao et al., 1988; Alves and Setter, 2000; Alves and Setter, 2004b). During periods of water scarcity, the loss of leaf area is dominated by restricted leaf area development (Connor and Cock, 1981). The rate of leaf expansion in existing leaves is also affected (Yao et al., 1988; Alves and Setter, 2004b), as well as cell expansion, cell proliferation and the rate of new leaf appearance that are essentially halted.

The development of the plant transpirational surface is further limited by a decrease in leaf area growth (Palta, 1984; El-Sharkawy and Cock, 1987), thus keeping the sink and demand well balanced with plant assimilatory capacity (Alves and Setter, 2000). The response further lead to conservation of available water and maximize water use efficiency of the crop during prolonged periods of drought (Alves and Setter, 2000; Alves and Setter, 2004b).

Other studies have shown that while leaves of well watered cassava plants grow and advance beyond their development window for growth, leaves in water stressed plants remain arrested at a young developmental stage, but are able to rapidly resume growth when rainfall returns (El-Sharkawy and Cadavid, 2000; Alves and Setter, 2004b). This is ideal for environments with numerous water deficit episodes interspersed with brief rainfalls (Setter and Fregene, 2007).

Extended and severe drought stress often characterized by leaf senescence and abscission, causes leaf buds to grow slowly and also leads to the development of numerous young shoots in the vicinity of abscised leaf scars (Duque and Setter, 2005). The recovery of the leaf growth is further much slower and this limits storage root bulking and yield (Baker et al., 1989). The damage is however avoided, especially at later stages, when most cassava storage root growth is sustained relatively better than leaf re-growth, as the proportion of assimilates imported by storage roots is higher at this phase, perhaps due to the utilization of stored carbohydrates in stems and other vegetative organs by roots (Yao et al., 1988).

Generally, reduction of leaf area increases water conservation during water stress, but it may also lead to reduction in total biomass and yield (El-Sharkawy et al., 1998; El-Sharkawy and Cadavid, 2002). However, cassava recovers rapidly once released from stress, by forming new leaves, which increase photon interception and canopy photosynthesis, thus compensating for previous losses in biomass, particularly root yield (El-Sharkawy, 1993; El-Sharkawy, et al., 1998; El-Sharkawy and Cadavid, 2002).

The newly developed leaves not only exhibit higher photosynthetic rates as compared to the non-stressed plants (El-Sharkawy, 1993; De Tafur et al., 1997; El-Sharkawy, 2006), but they are also associated with higher leaf conductance, higher nutrient contents, as well as with stronger sinks for carbohydrate in storage roots (Cayón et al., 1997). Finally, coupled with stomatal sensitivity to air humidity, cassava shows a strong heliotropic response which allows its leaves to track solar radiation early in the morning and late in the afternoon when the leaf-to-air water deficit is low (El-Sharkawy, 2007).

The movement however, changes at midday when solar elevation is high and leaf-to-air water deficit is greatest forcing the leaves to droop irrespective of soil water content and leaf turgor pressure (El-Sharkawy and Cock, 1984). These two leaf movements are beneficial in two ways; it maximizes light interception and hence total canopy photosynthesis when water use efficiency is greatest, and minimizes light interception when water use efficiency is least (El-Sharkawy, 2007).

2.3.3 Leaf retention (staygreen) in cassava

Leaf senescence is a type of programmed cell death characterized by loss of chlorophyll, lipids, total protein, and RNA (Smart, 1994; Gan and Amasino, 1997). Delayed leaf senescence is referred to as staygreen (Thomas and Howarth, 2000). Staygreen is also referred to as leaf longevity or leaf retention. Drought stress hastens leaf senescence resulting in a reduced canopy size, loss of photosynthesis and reduced yields (Rivero et al., 2007). However, staygreen genotypes are able to retain more green leaf area compared to genotypes without staygreen trait during water deficits (Thomas and Howarth, 2000; Mathews et al., 2001; Lenis et al., 2006).

Staygreen characteristic is more likely to be effective when the source rather than the sink tends to be limiting, a situation likely to occur when a plant is stressed (Lenis et al., 2006). The trait is also effective under high plant densities (Edmeades et al., 1996), or when the crop is planted late (De la Vega and Hall, 2002). Advantages of staygreen trait have been described in crop species such as *Capsicum annum* (Moser and Matile, 1997), *Zea mays* (Bölanos and Edmeades, 1996; Rajcan and Tollenaar, 1999), *Helianthus annuus* (de la Vega and Hall, 2002), *Sorghum bicolor* Moench. (Crasta et al., 1999; Borrell et al., 2000), as well as cassava (*Manihot esculenta* Crantz) (El-Sharkawy et al., 1992; Lenis et al., 2006).

In sorghum, for example, some staygreen genotypes not only remain leafy and green during drought (Duncan et al., 1981), but they also contain significantly more carbohydrates in the stem at all maturity stages than the go-brown types and have a higher grain weight (McBee et al., 1983).

This suggests that the staygreen leaves remain photosynthetically active (Thomas and Howarth, 2000). In addition, staygreen in sorghum has also been associated with a reduction in the incidences of charcoal rot disease, increased resistance to stem rots and stalk lodging (Tuinstra et al., 1997). Potential benefits associated with staygreen trait in cassava have also been cited. El-Sharkawy and colleagues (1992) showed that staygreen trait contributed to high root yields in cassava and was positively correlated with a high drought tolerance, productivity and root quality during water deficit. This suggests that the staygreen leaves remain photosynthetically active (Thomas and Howarth, 2000).

Cassava's storage root growth is often limited by the source or leaf area index than by the sink strength of the roots. This particular limitation to storage root growth can be circumvented by increased leaf longevity, with the leaves maintaining a higher photosynthetic rates, making it possible to maintain a given leaf area index with less distribution of assimilate to leaf development, and hence more assimilate made available for cassava root growth (Lenis et al., 2006).

Recent studies on leaf retention suggests that staygreen combined with other drought tolerance mechanisms commonly found in cassava, is advantageous in terms of total biomass production under growing conditions that include a long drought period. 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., 1992).

Increased biomass production and the higher harvest index of cassava clones with leaf retention trait, when grown under a prolonged period of drought, did result in a 32% greater average root yield when harvested towards the end of the dry season and 39% at the beginning of subsequent wet season (Lenis et al., 2006).

2.3.4 Other drought response mechanisms

Other drought tolerance mechanisms in cassava include an increase in abscissic acid (ABA) concentration and osmotic adjustment (OA). The rapid accumulation of ABA by cassava leaves and the halting of leaf area growth in response to drought stress (Alves and Setter, 2000; Alves and Setter, 2004ba), does indicate the extent to which the plant has gained access to water through deep rooting and has also conserved water via stomatal closure. Studies have shown that accumulation of osmotically active solutes during episodes of dry down, allows for cell enlargement, the stomata to remain partially open and CO_2 assimilation to continue at low water potentials that are otherwise inhibitory (Pugnaire et al., 1994; Alves, 2002).

However, in cassava, the leaf water potential remains relatively unchanged during water deficit episodes suggesting little or no role of osmotic adjustment (Ike and Thurtell, 1981; El-Sharkawy et al., 1992; Sundaresan and Sudhakaran, 1995; Itani et al., 1999; Alves and Setter, 2004a). The described morphological as well as physiological mechanisms underlying cassava's productivity and tolerance to prolonged drought should not only help to develop better crop management strategies in both favorable and stressful environments, but should also be used to identify cassava cultivars able to maximize yield under drought stress conditions.

2.4 Genetic engineering for drought tolerance

Conventional breeding has been very successful in constantly raising the yield potential of crops (Campos et al., 2004; Duvick, 2005). However, this approach may now be insufficient, because of the pressure to provide crop improvements at a rapid pace in a mounting global climate change that is increasing the frequency and severity of abiotic constraints (Collins et al., 2008).

Genetic engineering can be used to complement traditional breeding techniques. Ortiz et al., (2007) stated that transgenic-based breeding is attractive for the single, dominant nature of the transgene that makes transfer and maintenance of the system in any cultivar much easier, compared to conventional sources based on polygenes. Hoisington and Ortiz (2006) had predicted that genetically engineered cultivars containing various gene constructs that enhance crop performance under drought stress would be an important future strategy for facilitating the production of crops in drought-prone environments.

Research has shown that several genes with distinct functions are induced by environmental stresses, including drought stress, in plants (Agarwal et al., 2006). Development of transformation protocols has enabled the transfer of these genes across species and indeed enhanced an understanding of their functions and networks that contribute to improved plant drought resistance under water-limited conditions. Late embryogenesis abundant (LEA) proteins were first characterized in cotton as a set of proteins that highly accumulated in embryos at the late stage of seed development as well as in vegetative tissues during periods of water deficit (Dure et al., 1989; Cellier et al., 1998).

Over-expression of the barley *HVA1* gene (LEA protein gene) improved the performance of transgenic rice plants by protecting their cell membranes from injury under drought stress (Babu et al., 2004). Transgenic rice plants that over-expressed the wheat LEA gene, *PMA80,* showed enhanced tolerance of water-deficit conditions (Sivamani et al., 2000; Cheng et al., 2002). Transgenic wheat plants, over-expressing the barley *HVA1* gene, also showed improved biomass productivity and water-use efficiency under water-deficit conditions (Sivamani et al., 2000).

Abscisic acid (ABA), a phytohormone, plays important roles in seed maturation and dormancy as well as in the adaptation of vegetative tissues to abiotic environmental stresses such as drought and high salinity (Ortiz et al., 2007). The induction of the dehydration-responsive *Arabidopsis* gene, *rd29B*, is mediated mainly by ABA (Uno et al. 2000), whereas the stress-induced gene *rd29A* is induced through the ABA-independent pathway (Liu et al. 1998).

The Dehydration-Responsive Element Binding gene 1 (*DREB1*) and *DREB2* are transcription factors that bind to the promoter of genes such as *rd29A*, thereby inducing expression in response to drought, salt and cold stress (Dubouzet et al. 2003). Transgenic rice plants expressing *AtDREB1A* (or its ortholog *OsDREB1A) gene,* were tested in pots, where they showed improved tolerance to simulated drought and other stresses (Yamaguchi-Shinozaki and Shinozaki, 2004). Transgenic groundnut (*Arachis hypogea*) lines containing *AtDREB1A* gene construct under the control of various promoters, also showed delayed wilting under simulated drought by 20 to 25 days compared with non-transgenic controls (Bhatnagar et al., 2007).

Under the control of a stress-inducible promoter from the *rd29A* gene, *DREB1A* gene construct has also been inserted into bread wheat (*Triticum aestivum*) via biolistic transformation and transgenic lines expressing *DREB1A* gene manifested in this crop resistance to drought stress by a 10-day delay in wilting when water was withheld in comparison to control plants (Ortiz et al., 2007). Drought accelerates leaf senescence, leading to a decrease in canopy size, loss in photosynthesis and reduced yields. Since, senescence is a type of cell death program in-appropriately activated during drought, it is possible to enhance drought tolerance by delaying drought induced leaf senescence (Rivero et al., 2007).

In vitro and greenhouse cassava lines transformed with the *isopentenyl transferase (ipt)* gene, from *Agrobacterium tumefaciens*, under the control of the *PSAG12* promoter, expressed significant stay-greenness or resistant to leaf senescence after drought treatment, repressed degradation of chlorophyll, total protein and Rubisco in mature leaves, as well as early storage root bulking in comparison with wild-type plants (Zhang and Gruissem, 2004).

2.5 Statistical methods to screen for drought tolerance

Mechanisms of leaf maintenance in cassava under drought stress have been statistically analyzed using different methods (Itani et al., 1999). For example, determining significant leaf retention variation in cassava cultivars by counting their total leaf fall or leaf abscission method was first used by Rosas et al. (1976).

Similarly, El-Sharkawy and Cock (1987) as well as El-Sharkawy et al. (1992) evaluated leaf retention in cassava by counting the total number of leaves falling per hectare under drought stress. Okogbenin et al. (2003) measured cumulative leaf scars to determine leaf retention; while recently, Lenis et al. (2006) statistically quantified leaf retention using a 1 – 5 visual score, with five corresponding to excellent leaf retention.

The photosystem Infra-Red Gas Analyzer (IRGA) equipped with a modulation Fluorometer (LI-COR 6400 Photosynthesis System, Lincoln, NE, USA) utilizes gas and vapor exchange principles to measure the rates of photosynthesis and stomatal conductance in of plants. Photosynthetic rates are therefore expressed as rates of CO_2 uptake (umolCO_2 m-^{2} s-^{1}), and stomatal conductance as rate of passage of water vapor through the stomata (mmol m^{-2} s^{-1}).

Following manufacturer's instructions, the IRGA/LI-COR based photosystem method has been previously used to measure and therefore evaluate statistical significant differences in rates of CO_2 uptake and passage of water vapor in different cassava cultivars and in other plants (Oguntunde, 2005; El-Sharkawy, 2007).

2.6 References (Chapter one and two)

Agarwal, P.K., Agarwal, P., Reddy, M.K., and Sopory, S.K., 2006: Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Report, Vol. 25, pp.1263-1274.

Alves, A., Fregene, M., Ceballos, H., Setter, T., Ferguson, M., Mkamilo, G., Osei, C., 2006: Identifying the physiological and genetic traits that make cassava one of the most drought tolerant crops. Generation Challenge Program - Annual Review Meeting, São Paulo, September, 2006.

Alves, A.A.C., and Setter, T.L., 2004a: Abscisic acid accumulation and osmotic adjustment in cassava under water deficit. Environmental and Experimental Botany, Vol. 51, pp. 259-271.

Alves, A. A.C., and Setter, T.L., 2004b: Response of cassava leaf area expansion to water deficit: Cell proliferation, cell expansion and delayed development. Annals of Botany, Vol. 94, pp. 605 - 613.

Alves, A.A.C., 2002: Cassava botany and physiology. In: Hillocks, R.J., Thresh, J.M. and Bellotti, A.C. (Eds.). Cassava: Biology, production and utilization, CABI Publishing (2002), pp. 67-89.

Alves, A.A.C., and Setter, T.L., 2000: Response of Cassava to Water Deficit: Leaf Area Growth and Abscisic Acid. Crop Science, Vol. 40, pp. 131–137.

Babu, C., Zhang, J., Blum, A., Ho T-HD, Wu, R., and Nguyen, H.T., 2004: HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. Plant Sciences, Vol. 166, pp.855-862.

Baker, G.R., Fukai, S., and Wilson, G.L., 1989: The response of cassava to water deficits at various stages of growth in the sub-tropics. Australian Journal of Agricultural Research, Vol.40, pp. 517-528.

Balagopalan, C., 2002: Cassava utilization in food, feed and industry. In *CAB International*, 2002. Cassava: Biology, Production, and Utilization (Eds R.J. Hillocks, J.M., Thresh and A.C., Bellotti). pp. 301 - 318

Bänziger, M., Edmeades, G.O., Beck, D., and Bellon, M., 2000: Breeding for Drought and Nitrogen Stress Tolerance in Maize: From Theory to Practice. CIMMYT, Mexico, 68p.

Bhatnagar-Mathur, P.B., Devi, M.J., Srinivas, R.D., Lavanya, M., Vadez, V., Serraj, R., Yamaguchi-Shinozaki, K., and Sharma, K.K., 2007: Stress-inducible expression of *At*DREB1A in transgenic peanut (*Arachis hypogea* L.) increases transpiration efficiency under water-limiting conditions. Plant Cell Report, DOI 10.1007/s00299-007-0406-8.

Blum, A., 1988: Drought Resistance: Plant Breeding for Stress Environments. CRC Press, Boca Raton, FL, pp 43–77.

Bokanga, M., 1994: The cyanogenic potential of cassava. In: Root Crops and Food Security: Proceedings of the Fifth Triennial Symposium of the International Society for Tropical Root Crops-Africa, (Eds) Akoroda, M.O., IITA, Ibadan, Nigeria. pp 336-339.

Bölanos, J., and Edmeades, G.O., 1996: The importance of the anthesis-silking interval in breeding for drought tolerance in tropical maize. Field Crops Research, Vol. 48, pp. 65-80.

Borrell, A.K., Hammer, G.L., and Robert, G. H., 2000: Does Maintaining Green Leaf Area in Sorghum Improve Yield under Drought? II. Dry Matter Production and Yield. Crop Sciences, Vol. 40, pp. 1037 - 1048.

Bot, A.J., Nachtergaele, F.O., and Young, A., 2000: Land resource potential and constraints at regional and country levels. World Soil Resources Report, FAO, Rome (2000), pp. 28-49.

Bray E.A., Bailey-Serres, J., and Weretilnyk, E., 2000: Responses to abiotic stresses. In: Buchanan, B.B., Gruissem, W., and Jones, R.L., (Eds) Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, pp 1158-1203.

Bray E.A, 1994: Alterations in gene expression in response to water deficit. In: Basra, A.S (Eds) Stressed-Induced Gene expression in plants. Hardwood Academy Publishers, Chur, Switzerland, pp. 1-23.

Calatayud, P.A, Llovera, E., Bois, J.F, and Lamaze, T., 2000: Photosynthesis in drought-adapted cassava. Photosynthetica, Vol.38, pp.97-104.

Campos, H., Cooper, A., Habben, E., Edmeades, G., and Schussler, J., 2004: Improving drought tolerance in maize: a view from industry. Field Crops Research, Vol. 90, pp. 19–34.

Cayón, M.G., El-Sharkawy, M.A., Cadavid, L.F., 1997: Leaf gas exchange of cassava as affected by quality of planting material and water stress. Photosynthetica, Vol. 34, pp. 409 - 418.

Ceballos, H., Iglesias, C. A., Perez, J. C. and Dixon, A. G. O., 2004: Cassava breeding: opportunities and challenges, Plant Molecular Biology, Vol. 56, pp. 503 – 516.

Ceccarelli, S., Grando, S., and Baum, M., 2007: Participatory plant breeding in water-limited environments. Experimental Agriculture, Vol.43, Pp. 411–435.

Cellier, F., Genevieve, C., Jean-Christophe, B., and Francine, C., 1998: Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower; accumulation of dehydrins transcripts correlates with tolerance. Plant physiology, Vol. 116, pp. 319-328.

Chaerle, L., and Van Der Straeten, D., 2007: Regulating plant water status by stomatal control. In: M.A. Jenks et al. (eds.), Advances in Molecular Breeding toward drought and salt Tolerant Crops (Springer, 2007), pp. 73-90.

Cheng, Z., Targolli, J., Huang, X., and Wu, R., 2002: Wheat LEA genes, PMA80 and *PMA*1959, enhance dehydration tolerance of transgenic rice (*Oryza sativa* L.). Molecular Breeding, Vol. 10, pp. 71-82.

Cock, J.H., Porto, M.C., and El-Sharkawy, M.A., 1985: Water use efficiency of cassava: Influence of air humidity and drought stress on gas exchange of field grown cassava. Crop Sciences, Vol. 25, pp. 265-272.

Collins, N., Francxois T., and Tuberosa, R., 2008: Quantitative Trait Loci and Crop Performance under Abiotic Stress: Where Do We Stand? Plant Physiology, Vol. 147, pp. 469-486.

Connor, D.J., and Cock, J.H., 1981: Response of cassava to water shortage. II. Canopy Dynamics. Field Crops Research, Vol.4, pp. 285-296.

Connor, D.J., Cock, J.H., and Parra, G.E., 1981: Response of cassava to water shortage. Growth and Yield. Field Crops Research, Vol. 4, pp. 181-200.

Crasta, O.R., Xu, W.W., Rosenow, D.T., Mullet, J., and Nguyen, H.T., 1999: Mapping of post-flowering drought resistance traits in grain sorghum: association between QTLs influencing premature senescence and maturity. Molecular Genetics, Vol. 262: 579-588.

Crop Crisis Control Project, 2006: Cassava Mosaic Disease pandemic management in western Kenya. Quarterly report, November 2006.(USAID, IITA, CRS, ASARECA.http://c3project.iita.org.

De la Vega, A.J., and Hall, A.J., 2002: Effects of planting date, genotype, and their interactions on sunflower yield. I. Determinants of oil corrected grain yield. Crop Sciences, Vol. 42, pp. 1191–1201.

De Tafur, S.M., El-Sharkawy, M.A., and Calle, F., 1997: Photosynthesis and yield performance of cassava in seasonally dry and semi-arid environments. Photosynthetica, Vol. 33, pp. 249-257.

Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K., 2003: *OsDREB* genes in rice, (*Oryza sativa* L.) encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. The Plant Journal, Vol. 33, pp. 751-763.

Duncan, R.R., Bockholt, A.J., and Miller, F.R., 1981: Descriptive comparison of senescent and non-senescent sorghum genotypes. Crop Agronomy, Vol.73, pp.849-853.

Dure, L., Crouch, M., Harada, J., Ho T-HD., and Mundy, J., 1989: Common amino acid sequence domains among the LEA proteins of higher plants. Plant Molecular Biology, Vol. 12, pp. 475-486.

Duvick, D.N., 2005: The contribution of breeding to yield advances in maize (*Zea mays L.*). Advanced Agronomy, Vol. 86, pp 83-145.

Duque, L., and Setter, T.L., 2005: In Inter-drought II, the 2nd International conference on Integrated approaches to sustain and improve plant production under drought stress. Rome, Italy, pp.L5.09.

Edmeades, G.O., Bolanos, J., and Chapman, S.C., 1996: Value of secondary traits in selecting for drought tolerance in tropical maize. In: Edmeades, G.O., Bänziger, M., Mickelson, H.R., and Pena-Valdivia, C.B., (Eds.), Developing Drought and low N-tolerant Maize. CIMMYT (Mexico)

El-Sharkawy, M.A., 2007: Physiological characteristics of cassava tolerance to prolonged drought in the tropics: Implications for breeding cultivars adapted to seasonally dry and semiarid environments. Plant Physiology, Vol. 19 pp. 257-286.

El-Sharkawy M.A, 2006: International research on cassava photosynthesis, productivity, eco-physiology, and responses to environmental stresses in the tropics. Photosynthetica, Vol. 44 (4) pp. 481-512.

El-Sharkawy, M.A., 2004: Cassava biology and physiology. Plant Molecular Biology, Vol. 56 (4), pp. 481-501.

El-Sharkawy M.A, 2003: Cassava biology and physiology. Plant Molecular Biology, Vol.53, pp.621-641.

El-Sharkawy M.A. and Cadavid L.F, 2002: Response of cassava to prolonged drought stress imposed at different stages of growth. Experimental Agriculture, Vol. 38, pp. 333-350.

El-Sharkawy, M. A., and Cadavid, L. F., 2000: Genetic variation within cassava germplasm in response to potassium. Experimental Agriculture, Vol. 36, pp. 323 - 334.

El-Sharkawy, M.A., Cadavid, L.F., De Tafur, S.M., and Caicedo, J.A., 1998: Genotypic differences in productivity and nutrient uptake and use efficiency of cassava as influenced by prolonged water stress. Acta Agronomy, Vol. 48, pp. 9-22.

El-Sharkawy, M.A, 1993: Drought tolerant cassava for Africa, Asia and Latin America. Biosciences, Vol. 43, pp. 441-451.

El-Sharkawy, M. A., De-Tafur, S. M. and Cadavid, L. F., 1993: Photosynthesis of cassava and its relation to crop productivity. Photosynthetica, Vol. 28, pp.431-438.

El-Sharkawy, M.A., Hernandez, A.D.P., and Hershey, C., 1992: Yield stability of cassava during prolonged mid-season water stress. Experimental Agriculture, Vol. 28, pp.165-174.

El-Sharkawy, M.A., Cock, J.H., Lynam, J.K., Hernandez, A.P., and Cadavid, L., 1990: Relationships between biomass, root-yield and single-leaf photosynthesis in field-grown cassava. Field Crops Research, Vol. 25, pp. 183-201.

El-Sharkawy, M A., and Cock, J.H., 1987: Response of cassava to water stress. Plant Soil, Vol. 100, pp. 345–360.

El-Sharkawy M.A and Cock J.H, 1984: Water use efficiency of cassava. Effects of air humidity and drought stress on stomatal conductance and gas exchange. Crop Science, Vol.24, pp. 497-502.

Essers, J.A., 1995: Removal of Cyanogens from Cassava Roots: Studies on Domestic Sun drying and Solid-substrate Fermentation in Rural Africa. PhD Thesis; Wageningen Agricultural University, Wageningen pp. 114-143.

FAO, 2008: Investing in Sustainable Agricultural Intensification: The Role of Conservation Agriculture. FAO, Rome.

FAO, 2007: Cassava production statistics 2006, *http://www.fao.org.2007*; November, 2007.

FAO, 2005: *FAOSTAT agricultural* database. Rome: [www document] http://faostat.fao.org/faostat/collections?version=ext&hasbulk=0&subset=agriculture.

Fauguet, C., and Fargette, D., 1990: African cassava mosaic virus. Etiology, Epidemiology and Control. Plant Diseases, Vol. 74, pp. 404 – 411.

Gan, S.S., and Amasino, R.M., 1997: Making sense of senescence: molecular genetics regulation of leaf senescence. Plant Physiology, Vol. 113, pp. 313-319.

Gan, S.S., and Amasino, R.M., 1995: Inhibition of Leaf Senescence by Auto-regulated Production of Cytokinins. Plant Sciences, Vol. 270. (5244), pp. 1986 – 1988.

Hershey, C.H., and Jennings, D.L., 1992: Progress in breeding cassava for adaptation to stress. Plant Breeding Abstract, Vol. 62, pp. 823-831.

Hoisington, D., and Ortiz, R., 2006: Experience with monitoring and GM-crops in CIMMYT. In: Genetically Modified Organisms in Crop Production and their Effects on the Environment: Methodologies for Monitoring and the Way Ahead. (Ghosh, K., and Jepson, P.C., Eds, Rome, Italy, FAO /UN). pp. 106-113.

Ike, I. F., 1982: Effect of water deficits on transpiration, photosynthesis and leaf conductance in cassava (*Manihot esculenta* Crantz). Physiologia Plantarum, Vol. 55, pp.411- 414.

Ike, I.F., and Thurtell, G.W., 1981: Osmotic adjustments in indoor grown cassava (Manihot esculenta Crantz) in response to water stress. Physiologia Plantarum, Vol.52, pp.257-262.

Itani, J., Oda, T., and Numao, T., 1999: Studies on mechanisms of dehydration postponement in cassava leaves under short-term soil water deficits. Plant Production Science, Vol. 2, pp.184-189.

Jamieson, P.D., Martin, R.J., And Francis, G.S., 1995: Drought influences on grain yield of barley, wheat, and maize. New Zealand Journal of Crop and Horticultural Science, Vol. 23, pp. 55-66.

Jennings, D.L., and Iglesias, C., 2002: Breeding for crop improvement. In: Hillocks, R.J., Thresh, J.M., Bellotti, A.C. (Eds.): Cassava: Biology, Production and Utilization. CABI Publications (pp. 149-166).

Jury, W.A., and Vaux, H., 2005: The role of science in solving the world's emerging water problems. PNAS, Vol. 3 (44), pp. 15715–15720.

Kawano, K., 2003: Thirty Years of Cassava Breeding for Productivity: Biological and Social Factors for Success. Crop Science, Vol. 43, pp. 1325-1335.

Legg, J. P., and Thresh, J. M., 2004: Cassava virus diseases in Africa. In: Hughes, Jd'A., Adu, B.O., (Eds) Plant Virology in Sub-Saharan Africa Conference Proceedings, Ibadan, Nigeria, IITA, pp. 517 – 552.

Lenis, I.J., Calle, F., Jaramillo, G., Perez, J.C., Ceballos, H., and Cock, J.H., 2006: Leaf retention and cassava productivity. Field Crops Research, Vol. 95, pp. 126–134.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K., 1998: Two transcription factors, *DREB1* and *DREB*2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis thaliana*. Plant Cell, Vol. 10, pp. 1391–1406.

Lokko, Y., Anderson, J., Rudd, S., Raji, A., Horvath, D., Mikel, M., Kim, R., Liu, L., Hernandez, A., Dixon, G., and Ingelbrecht, L., 2007: Characterization of an 18,166 EST dataset for cassava (*Manihot esculenta* Crantz) enriched for drought-responsive genes. Plant Cell Reporter, Vol. 26, pp.1605–1618.

Mathews, M.S., Lee, C.G., Frank, S., Wilco, R.M., Geert, M. S., Evert, D., Hans, J., Brian, J.P., and Michael, R.D., 2001: Effects of *PSA12-IPT* on development and senescence in transgenic lettuce. Plant Physiology, Vol.127, pp.505-516.

McBee, G.G., Waskom, R.M., Miller, F.R., and Creelman, R.A., 1983: Effect of senescence and non-senescence on carbohydrates in sorghum during late kernel maturity states. Crop Sciences, Vol. 23, pp.372-376.

Moser, D., and Matile, P., 1997: Chlorophyll breakdown in ripening fruit of *Capsicum annuum*. Journal of Plant Physiology, Vol.150, pp. 759-761.

Mware, B.O., Ateka, E.M., Songa, J.M., Narla, R.D., Olubayo, F., Amata, R., 2009: Transmission and distribution of cassava brown-streak virus disease in cassava growing areas of Kenya. Journal of Applied Biosciences, pp. 864 – 870.

Oguntunde, G.P., 2005: Whole-plant water use and canopy conductance of cassava under limited available soil water and varying evaporative demand. Plant and Soil Sciences, Vol. 278, pp.371-383.

Okogbenin, E., and Fregene, M., 2003: Genetic mapping of QTLs affecting productivity and plant architecture in a full-sib cross from non-inbred parents in cassava (*Manihot esculenta* Crantz). Theoretical and applied genetics, Vol.107, pp.1452-1462.

Ortiz, R., Iwanaga, M., Matthew, P.R., Huixia, W., and Jonathan, H.C., 2007: Overview on Crop Genetic Engineering for Drought-prone Environments. An Open Access Journal (ICRISAT), Vol. 4 (1), pp. 1-30.

Palta, J. A., 1984: Influence of water deficits on gas-exchange and the leaf area development of cassava (Manihot esculenta Crantz) cultivars, Journal of Experimental Botany, Vol. 35, pp. 1441–1449.

Pugnaire, F.I., Endolz, L.S., and Pardos, J., 1994: Constraints by drought stress on plant growth. In: Pessarakli, M. (Eds.), Handbook of Plant and Crop Stress. Marcel Dekker, New York, pp. 247-259.

Rajcan, I., and Tollenaar, M., 1999: Source: sink ratio and leaf senescence in maize. I. Dry matter accumulation and partitioning during grain filling. Field Crops Research, Vol. 60, pp. 245-253.

Ramanujam, T., 1990: Effect of moisture stress on photosynthesis and productivity of cassava. Photosynthetica, Vol. 24, pp. 217-224.

Rauf, S., 2008: Breeding sunflower (*Helianthus annuus* L.) for drought tolerance. Biometry and Crop Science, Vol. 3 (1) pp. 29-44.

Rivero, M., Kojima, M., Gepstein, A., Hitoshi, S., Mittler, R., Gepstein, S., and Blumwald, E., 2007: Delayed leaf senescence induces extreme drought tolerance in a flowering plant. PNAS, Vol.104 (49), pp. 19631–19636.

Romanoff, S., and Lynam, J., 1992: Cassava and African food security: some ethnographic examples. Ecology and Food Nutrition, Vol. 27, pp. 29-41.

Rosling, H., 1994: Measuring effects in humans of dietary cyanide exposure from cassava. Acta horticulture, Vol. 375, pp. 271-283.

Salinger, M.J., Sivakumar, M.V., and Motha, R., 2005: Reducing vulnerability of agriculture and forestry to climate variability and change: workshop summary and recommendations. Climatic Change, Vol. 70, pp.341–362.

Saxena, N.P., and O'Toole, J.C., 2002: Field Screening for Drought Tolerance in Crop Plants with Emphasis on Rice: Proceedings of an International Workshop on Field Screening for Drought Tolerance in Rice, ICRISAT, Patancheru, India, pp. 60-63.

Schöpke, C., Taylor, N. J., Carcamo, R., Beachy, R. N., and Fauquet, C., 1997: Optimization of parameters for particle bombardment of embryogenic suspension cultures of cassava (*Manihot esculenta* Crantz) using computer image analysis. Plant cell reports, Vol. 16, pp.526–530.

Scott, G.J., Rosegrant, M.W., and Ringler, M.W., 2000: Roots and Tubers for the 21st century: Trends, projections, and policy options. Food, Agriculture and the Environment Discussion Paper 31. International Food Policy and Research Institute (IFPRI).

Setter, T.L., and Fregene, M.A., 2007: Recent advances in molecular breeding of cassava for improved drought stress tolerance. In: Jenks, M.A., Hasegawa, P.M., and Jain, S.M., (Eds), Advances in Molecular Breeding toward Drought and Salt Tolerant Crops, pp.701-711. Springer, Netherlands.

Sinha, H.A., 1996: Breeding for abiotic resistance. In: Singh, B.D. (Ed.) Plant Breeding-Principles and Methods. Kalyani Publishers, Ludhyana, Punjab, India.

Sivamani, E., Bahieldin, A., Wraith, J., Thamir A., Dyer, E., Tuan-Hua, D., and Rongda, Q., 2000: Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley *HVA*1 gene. Plant Science, Vol. 155, pp. 1-9.

Smart, C., 1994: Gene expression during leaf senescence. New phytologist. Vol. 126, pp. 419-448.

Sundaresan, S., and Sudhakaran, P.R., 1995: Water stress-induced alterations in the proline metabolism f drought-susceptible and tolerant cassava cultivars. Physiologia plantarum, Vol.94, pp.635-642.

Tardieu, F., and Simmoneau, T., 1998: Variability among species of stomatal control under fluctuating soil water status and evaporative demand. Journal of Experimental Botany, Vol. 49, pp. 419-432.

Taylor, N., Kent, L., and Fauquet, C., 2004: Progress and Challenges for the Deployment of Transgenic Technologies in Cassava. Agri.Bio-Forum, Vol. 7, (1&2), pp. 51-56.

Thomas, H., and Howarth, C.J., 2000: Five ways to staygreen. Journal of Experimental Botany, Vol. 51, pp.329-337.

Tuberosa, R., Salvi, S., Sanguineti, M.C., Maccaferri, M., Giuliani, S., and Landi, P., 2003: Searching for QTLs controlling root traits in maize: A critical appraisal. Plant Soil sciences, Vol.255, pp.35–54.

Tuinstra, M.R., Edwin, M.G., Goldsborough, P.B., and Ejeta, G., 1997: Genetic analysis of post-flowering drought tolerance and components of grain development in *Sorghum bicolor* (L.) Moench. Molecular Breeding, Vol.3, pp. 439 – 448.

Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., and Yamaguchi-Shinozaki, K., 2000: *Arabidopsis thaliana* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proceedings of the National Academy of Sciences (USA), Issue, 97, pp. 11632-11637.

Viswanathan, C., Jianjun, Z., Tao, Z., and Jian-Kang, Z., 2007: Small RNAs: Big role in abiotic stress tolerance of plants. In: M.A. Jenks and Colleagues (Eds.), Advances in Molecular Breeding toward Drought and Salt Tolerant Crops, Springer (2007), pp. 223-260.

Westby, A., 2002: Cassava utilization, storage, and small-scale processing. In *CAB International*, 2002. Cassava: Biology, Production, and Utilization (Eds R.J. Hillocks, J.M., Thresh and A.C., Bellotti). pp. 281 - 300

Wood, A.J., 2005: Eco-physiological adaptations to limited water environments. In: Plant Abiotic Stress, M.A. Jenks and P.M. Hasegawa (eds.), Blackwell Publishing, Oxford, UK, pp. 1-13.

Yamaguchi-Shinozaki, K., and Shinozaki, K., 2004: Improving drought and cold stress tolerance in transgenic rice. In: Proceedings of World Rice Research Conference 'Rice is life' scientific perspectives for the 21st century" Tsukuba, Japan (2004), pp. 94-97.

Yao, N.R., Goue, B., Zeller, B., and Monteny, B., 1988: Effect of drought on leaf development and dry matter production of the cassava plant. Tropical agriculture, Vol.65, pp.84-88.

Zhang, P., and Gruissem, W., 2005: Leaf senescence-inducible expression of isopentenyl transferase in cassava rendering it resistant to drought stress. Deutscher Tropentag, Abstract, p.1

Zhang, P., and Gruissem, W., 2004: Leaf Senescence-Inducible Expression of *isopentenyl transferase* in Cassava Rendering it Resistant to Drought Stress. In Sixth International Scientific Meeting of the Cassava Biotechnology Network. CIAT/CBN, Cali, Colombia (2004), pp. 99.

CHAPTER THREE:

Evaluation of Staygreen Trait through Measurement of Internode Elongation, Leaf Abscission, Photosynthetic Rates, and Stomatal Conductance Response in Drought Stressed Cassava Genotypes

Abstract

Staygreen trait or increased longevity of leaves has been associated with drought tolerance and hence yield in cassava. This study evaluated staygreen trait in two transgenic cassava genotypes (transformed with *isopentenyl transferase (ipt)* gene for reduced leaf senescence and abscission under drought stress) and six non-transgenic cassava genotypes. The experiment was carried out under greenhouse conditions located at the Swiss Federal Institute of Technology (ETH) Lindau-Eschikon, Switzerland (47°27'N, 8°41' E, and 560 m above sea level) between November, 2007 and November, 2008.

In order to determine drought tolerance or to characterize staygreen phenotype expression under drought stress, data on leaf abscission (for leaf retention), elongation of the last internodes, photosynthetic rates, and stomatal conductance were taken from the eight cassava genotypes submitted to three levels of drought stress treatments (0, 30, and 60 %) and a positive control or fully irrigated plants. Two non transgenic genotypes (98-0002 and 98-2226) and one transgenic line (529-48) that expressed significantly ($P \leq 0.05$) high level of staygreen or leaf retention, also exhibited significantly ($P \leq 0.05$) higher photosynthetic rates, internode elongation and low stomatal conductance compared to other genotypes.

Non transgenic genotypes 91-02322 and TME-3 and transgenic line 529-28, expressed moderate levels of staygreen, and non transgenic genotype 95-0306 and wild type TMS 60444 (for the transgenic lines) were significantly ($P \leq 0.05$) susceptible to drought stress treatments.

Expression of staygreen trait was clearly marked out under 30 and 60% levels of drought stress as well as in fully irrigated or control treatment. Results further showed a significant ($P \leq 0.01$) positive correlation between photosynthetic rates, stomatal conductance, and internode elongation and a negative correlation between photosynthetic rates and leaf abscission. Significant ($P \leq 0.01$) negative correlation between leaf abscission, stomatal conductance and internode growth were also observed.

3.2 Abbreviations

ELI: elongation of the last internodes; LA: leaf abscission; P_N: net photosynthetic rates; Gs: stomatal conductance; *ipt*: *isopentenyl transferase (ipt)* gene; *PSAG*: senescence associated gene promoter; Rubisco: Ribulose-1,5-bisphosphate carboxylase oxygenase; °C: degree celcius; µM: micro-mole; RH: relative humidity; CBM: cassava basic media; $CuSO_4$: copper sulphate; DAP: days after planting; FC: field capacity; RCD: randomized complete design; IRGA: infrared gas analyzer; ANOVA: analysis of variance; LSD: least significant difference.

3.3 Introduction

Cassava (*Manihot esculenta* Crantz), cultivated for its starchy roots, is ranked as the fourth most important food source for saccharides after rice, sugarcane and maize for over 600 million people in developing countries of tropical and sub-tropical Africa, Asia and Latin America (Balagopalan, 2002; Nweke et al., 2002; Uzo and Sayre, 2006; El-Sharkawy, 1993; 2006; Lokko et al., 2007). The crop is mostly grown by small scale, resource-limited farmers on marginal, highly eroded and low fertility soils and in areas with uncertain rainfall patterns and prolonged dry periods (El-Sharkawy, 1993; 2004; Ruppenthal et al., 1997).

Although cassava has a high growth rate under optimal conditions, the crop also performs well in drought-prone areas (Sakai et al., 1994; Lokko et al., 2007) and almost never fails to produce due to drought when compared to other staple crops (Lenis et al., 2006). The inherent ability of cassava to yield under stressful environments has increased its importance especially in areas considered difficult to cultivate other crops.

A list of morpho-physiological traits contributes to the ability of cassava to produce under drought conditions. One such trait is staygreen, also referred to leaf retention or leaf longevity. Staygreen has been described as delayed leaf senescence (Thomas and Howarth, 2000). Drought stress often hastens senescence resulting in a reduced canopy size, loss of photosynthesis and reduced yields (Rivero et al., 2007). However, staygreen genotypes are able to retain more green leaf area compared to genotypes without staygreen trait during water deficits (Thomas and Howarth, 2000).

Staygreen cassava clones show enhanced ability to retain leaves that remain photosynthetically active under drought stress (El-Sharkawy, 2004). These clones produce more total fresh biomass, yield, dry matter and express high drought tolerance (El-Sharkawy et al., 1992; Lenis et al., 2006). Drought tolerance or staygreen expression can been enhanced by delaying drought induced leaf senescence. For example, *in vitro* and greenhouse cassava lines transformed with the *isopentenyl transferase (ipt)* gene, from *Agrobacterium tumefaciens*, under the control of the *PSAG12* promoter, expressed significant stay-greenness or resistant to leaf senescence after drought treatment compared to wild-type plants (Zhang and Gruissem, 2004).

Although advantages of long leaf life in drought stressed and unstressed cassava clones at field level has been evaluated by Lenis et al. (2006), the main objective in this chapter or study was to evaluate variations in staygreen characteristics in transgenic cassava genotypes (transformed with *ipt* gene for improved reduction in leaf senescence) and non transgenic cassava genotypes (selected from farmer fields) submitted to varying drought stress treatments under greenhouse conditions.

In addition, the study compared the relationship between leaf retention or staygreen trait with photosynthetic rates, stomatal conductance and internode growth or elongation among the genotypes. The measurement of these parameters for the identification of drought-tolerant or staygreen genotypes of cassava is essential to optimize cassava productivity in drought-prone environments (Okogbenin et al., 2003).

3.4 Materials and Methods

3.4.1 Experimental site

The study was carried out at two sites: (a) the Swiss Federal Institute of Technology (ETH); Institute of Plant Science, Plant Biotech Laboratory located at ETH-Zentrum, LFW E38, Zurich, Switzerland; latitude 47°21'N, longitude, 8°31' E, and altitude of 490 m above sea level and (b) the Swiss Federal Institute of Technology (ETH); Institute of Plant Science greenhouse located at Lindau-Eschikon, 20 km North-East of Zurich, Switzerland; at latitude 47°27'N, longitude, 8°41' E, and altitude of 560 m above sea level.

3.4.2 Growth conditions

The growth chamber conditions in the laboratory, where the genotypes were first *in vitro* multiplied, included a 12 hour light exposure and temperature set at 26°C (day/night), while in the greenhouse, the temperature was set at 17°C/26°C (day/night), the relative humidity (RH) was 60%/50% (day/night), 14 hours of light at an intensity of 35 Klux and an average air ventilation rate of 84.7%. The cassava basic media (CBM) used for *in vitro* multiplication of the cassava plants, consisted of Murashige & Skooge salts with vitamins, 2 μM $CuSO_4$, 2% Sucrose, 0.3% gelrite and the pH set at 5.8. The media was then autoclaved for 1 hour before being used.

For establishment in the greenhouse, a 1:1 mixture of Soil (Topf und Pikiererde, 140, Ricoter, Aarberg, Switzerland) and Perlite (GS-Forma-SA, Mezzovico-Vira, Lugano, Switzerland) was used as a substrate. The substrate contained 50% soil (mixture of sand, compost, small pieces of plant debris and other organic materials) and 50% perlite.

3.4.3 Cassava genotype selection and establishment

Two transgenic cassava lines, a wild type and five non transgenic cassava genotypes were selected for establishment in the greenhouse. The non transgenic genotypes which included: 91-02322, 98-0002, 98-2226, TME-3 and 95-0306, were provided by the International Institute of Tropical Agriculture (IITA, PMB, 5320, Ibadan, Oyo state, Nigeria). Although farmers widely cultivated the five genotypes, they provided conflicting assessment on their levels of drought tolerance.

The transgenic lines 529-28 and 529-48 (transgenic staygreen cassava lines expressing *isopentenyl transferase (ipt)* gene in TMS 60444 genotype) and TMS 60444 (used as a control or wild type), were obtained from Swiss Federal Institute of Technology (ETH-Zentrum); Institute of Plant Sciences, Zürich, Switzerland. The two lines (529-28 and 529-48) had been transformed with *ipt* gene for improved leaf retention or reduced leaf senescence under drought conditions.

The cassava plants were first multiplied *in vitro* in the growth chamber for 45 days after which they were transplanted to 0.45 litre pots (one plantlet per pot) filled with the substrate in the greenhouse. The plants were grown (hardened) for 30 days and then transferred to 1 litre pots (one plant per pot) where they were grown. All the potted plants were irrigated once a day until 135 DAP (days after planting), when the drought stress treatments were applied.

3.4.4 Drought stress treatments

Before screening for staygreen trait, four drought stress treatments were formulated for application to the established cassava plants. The method for establishing the different water deficit treatments were adopted as described by Alves and Setter, (2004b). The drought stress levels were based on percent water supplied to control plants. The treatments included well watered treatment, here referred to as fully irrigated (100% irrigation) which was used as a positive control, 60% drought stress treatment (i.e. cassava plants were irrigated with 60% of water supplied to control plants. The, 30% drought stress treatment was achieved by irrigating the plants with 30% of water supplied to control plants, while No-irrigation treatment or 0% irrigation was implemented by totally withholding water supply from the plants.

Each pot was filled with the same amount of substrate (50% soil + 50% perlite) of 500g. The pots were then fully watered and allowed to drain freely to determine their weight at field capacity (FC). The mean initial weight of the pots with soil at FC (PS_{FC}) was then determined in grams. On day 0, the weight of Pot + Soil at FC + Plant ($PS_{FC}P_0$) was taken and the plant (s) weight at day 0 (P_0) estimated by: $P_0 = PS_{FC}P_0 - PS_{FC}$.

The water content at time *t* relative to the field capacity ($\%FC_t$) was then calculated using the equation: $\%FC_t = [(PSP_t - P_0)/PS_{FC}] \times 100$. The PSP_t represented the weight of the Pot + Soil at FC + Plant at time, *t*, after the onset of treatments. The pots were then allowed to lose soil water until they weighed about 60 % of FC (for plants to be submitted to 60% drought stress treatment) and 30% of FC (for plants to be submitted to 30% drought stress treatment).

Irrigation was totally withheld for plants submitted to 0% drought stress treatment and normal or full irrigation was maintained for the well watered plants (positive control treatment). At 3 pm each day, the 'Pot + Soil + Plant' (PSC_t) was weighed and water added to the 60% and 30 % FC set points (water deficit) or 100 % FC (well-watered control), according to the equation above. Total irrigation was withheld to achieve 0% irrigation or non-irrigated treatment.

3.4.5 Screening for drought response or staygreen trait in cassava plants

At 135 days after planting (DAP, here referred to as day 0), cassava plants were randomly assigned to the 4 treatments. Twelve plants of uniform height or stature were selected from each genotype (96 plants in total). Out of these, each treatment contained 3 plants (replicates) from each cassava genotype (a total of 24 plants for each treatment).

The four drought stress regimes were carried out concurrently for 20 days during which measurement of photosynthetic rates, stomatal conductance, elongation of the last internodes and leaf abscission were screened and analyzed for staygreen trait among cassava genotypes. The plants and treatments were arranged in a randomized complete design (RCD) and the entire experiment was replicated three times.

3.4.6 Parameters used to evaluate staygreen trait in cassava genotypes

Characterization of staygreen phenotype in cassava genotypes submitted to different drought stress regimes, was carried out by measuring the following parameters: leaf abscission, elongation of the last internodes, rates of photosynthesis and stomatal conductance.

All the measurements were taken from Day 0 (135 DAP) and subsequently after every 2 days for the entire period of drought stress treatments. All the measurements were taken between 9 am and 2 pm.

3.4.6.1 Elongation of the Last Internode (ELI)

The base of a petiole of the last expanding internode from each plant was tagged by marking with a black felt-tip pen and the initial internode length measured and recorded using a centimeter rule.

Subsequent internode length was measured after every 2 days for the 20 days of drought stress treatment. Internode growth or elongation (increase in length) was determined by subtracting the new length from the previous length. The original lengths were covariates during data analysis.

3.4.6.2 Leaf Abscission (LA) for leaf retention

The highest leaf scar or petiole (from the soil level) and the oldest folded leaf scar or the last fully expanded leaf (top most) were tagged by marking a red felt-tip pen and the total number of leaves in between counted and recorded from each plant on Day 0 of the treatments. The tagged leaf scar close to the soil level was henceforth used as a baseline and leaf retention was determined by counting the number of leaves abscising above this leaf scar. The new highest leaf scar was subsequently tagged upon recording a leaf drop and the number of leaves dropping was then counted and recorded on every 2nd day of the 20 days of drought stress treatments.

3.4.6.3 Rate of Photosynthesis (P_N) and Stomatal Conductance (Gs)

The P_N (in umolCO_2 m-^{2} s-^{1}) and Gs (in mmolm-^{2} s-^{1}) were both measured using an Infrared Gas Analyzer (IRGA) equipped with a modulation Fluorometer (LI-COR 6400 Photosynthesis System, Lincoln, NE, USA) following manufacturer's instructions. The IRGA/LI-COR based photosystem has been previously used to measure both P_N and Gs in cassava other plants (Oguntunde, 2005; El-Sharkawy, 2007). The three last fully expanded or unfolded leaves (top most), and one leaf at mid-stem of each plant were tagged by marking their petioles with a blue felt-tip pen for easy location.

The P_N and Gs were measured from these four leaves from Day 0 and subsequently after every 2 days for the 20 days of drought stress treatments. The four P_N and Gs values for each day were pooled by calculating their averages, which were then used in the subsequent analysis of variances.

3.4.7 Data analysis

All the data collected for each parameter were subjected to analysis of variance (ANOVA) using the PROC ANOVA procedure of Genstat Discovery Edition 3 (Lawes Agricultural Trust Rothamsted Experimental Station, UK). The differences among the treatment means were compared or separated using the Fisher's protected LSD test at 5% probability level (Payne et al., 2007). Correlation analyses among the parameters were also done using the same program.

3.5 Results

3.5.1 Elongation of the last internodes (ELI) in cassava genotypes

3.5.1.1 Effect of different levels of water deficit on internode growth or elongation

Significant differences ($P \leq 0.05$) in internode growth were observed within drought stress treatments as well as between cassava genotypes, while the genotype x treatment interaction produced no significant ($P > 0.05$) variations (Appendix, 1; Table 1). Among treatments, control plants (fully irrigated) showed significantly higher ($P \leq 0.05$) internode elongation than cassava plants in all other drought stress regimes. Plants that were not irrigated grew or elongated the least. Internode growth in plants subjected to 60 and 30% water deficit levels varied significantly ($P \leq 0.05$) (Table 1).

3.5.1.2 Effect of full irrigation or control treatment on internode growth or elongation of cassava genotypes

Internode growth response significantly varied in control transgenic cassava genotypes; with 529-48 elongating significantly higher ($P \leq 0.05$) than the internodes of wild type TMS 60444 and transgenic line 529-28, while 529-28 elongated the least (Table 1). Among non-transgenic genotypes, internode elongation of genotype 98-0002 and 98-2226 did not significantly vary ($P > 0.05$), although both their internodes elongated more significantly ($P \leq 0.05$) than other genotypes (Table 1). Internode of genotype 95-0306 elongated the least. There was also significant variation ($P \leq 0.05$) in internode growth of genotypes TME-3 and 91-02322 (Table 1). When the internode growth of transgenic and non-transgenic genotypes were compared, internode of transgenic line 529-48 lengthened more significantly ($P \leq 0.05$) than internode of all non-transgenic genotypes (Table 1).

The internodes of line 529-28, 529-48 and their wild type TMS 60444 significantly ($P \leq 0.05$) outgrew non-transgenic genotype 95-0306, although the genotype 98-0002, 98-2226 and wild type TMS 60444 did not significantly vary ($P > 0.05$) in their internode growth (Table 1). Significant variation in internode elongation was also noted between transgenic line 529-28, its wild type TMS 60444 and non-transgenic genotypes TME-3 and 91-02322 (Table 1).

3.5.1.3 Effect of withholding total irrigation (0%) on internode growth or elongation of cassava genotypes

When irrigation was totally withheld, there was no significant ($P > 0.05$) internode growth differences between transgenic lines 529-28 and wild type TMS 60444 (Table 1). However, the internode of transgenic line 529-48 lengthened significantly ($P \leq 0.05$) more than the internodes of transgenic line 529-28 and their wild type TMS 60444 (Table 1). Significant variation in internode elongation among non-transgenic genotypes were also observed with genotype TME-3 showing significantly ($P \leq 0.05$) higher elongation than the rest of the genotypes (Table 1).

Although the least internode growth was observed in genotypes 98-0002 and 95-0306, (the two genotypes did not significantly vary ($P > 0.05$), their internode elongation were significantly ($P \leq 0.05$) outgrown by internode of genotypes 98-2226 and 91-02322. Internode growth between genotypes 98-2226 and 91-02322 also significantly ($P \leq 0.05$) differed (Table 1). When comparison between the two sets of cassava plants (transgenic vs. non-transgenic) were made, genotype 98-0002, 95-0306, and transgenic line 529-28 did not significantly vary in their internode elongation, although they grew the least compared to other genotypes (Table 1).

Internodes of transgenic line 529-48 and genotype TME-3 did not significantly vary ($P>0.05$), but the two grew significantly ($P\leq0.05$) higher than all the genotypes (Table 1). Inter-nodal growth of wild type TMS 60444 and genotype 91-02322 did not significantly ($P>0.05$) vary, although the wild type significantly ($P\leq0.05$) out-grew genotype 95-0306 (Table 1).

3.5.1.4 Effect of 30% water deficit on internode growth or elongation of cassava genotypes

There was significant variation in internode elongation across all cassava genotypes that received 30% water deficit (Table 1). Internode of transgenic line 529-48 significantly ($P\leq0.05$) lengthened the most compared to wild type TMS 60444 and transgenic line 529-28. Internode of transgenic line 529-28 grew the least. The internode of line 529-28 significantly ($P\leq0.05$) varied with the wild type TMS 60444 (Table 1). Internode growth of non-transgenic genotype 98-2226 significantly ($P\leq0.05$) grew more than the internode of other non-transgenic genotypes.

Internode of genotype 95-0306 elongated the least, while the inter-nodal growth of genotypes TME-3, 91-02322, and 98-0002 were not significantly ($P>0.05$) varied (Table 1). Comparing transgenic and non-transgenic genotypes indicated that internode of transgenic line 529-48 significantly ($P\leq0.05$) outgrew all the internodes of genotypes within the two classes of cassava plants (Table 1). The internode of genotype 95-0306 grew the least. There was no significant differences ($P>0.05$) in internode growth of wild type TMS 60444, genotypes TME-3, 98-0002, and 91-02322, as well as between transgenic line 529-28 and genotypes 98-0002 and 91-02322 (Table 1).

Genotype 98-2226 internode significantly ($P \leq 0.05$) out-grew internodes of transgenic line 529-28 and its wild type TMS 60444 (Table 1).

3.5.1.5 Effect of 60% water deficit on internode growth or elongation of cassava genotypes

When cassava plants under 60% drought stress regime were analyzed, highly significant ($P \leq 0.05$) internode growth was observed in transgenic line 529-48 compared with wild type TMS 60444 and transgenic line 529-28 (Table 1). Significant variation ($P \leq 0.05$) in inter-nodal elongation between wild type TMS 60444 and transgenic line 529-28 was also observed with internode of line 529-28 lengthening the least (Table 1).

Non-transgenic genotypes also showed variation in their internode growth, with internode of genotype 98-2226 significantly ($P \leq 0.05$) growing the most compared to other genotypes, while internode of genotype 95-0306 increased the least (Table 1). The internode elongation in genotypes 98-0002, TME-3 and 91-02322 did not significantly vary ($P > 0.05$), although they significantly ($P \leq 0.05$) differed with internode growth of genotype 95-0306 (Table 1).

The internode of transgenic line 529-48 significantly ($P \leq 0.05$) grew more than the internodes of all non-transgenic genotypes. Although the internode growth of transgenic line 529-28 and variety 95-0306 were not significantly different ($P > 0.05$), they nevertheless grew the least (Table 1). There was no significant differences ($P > 0.05$) in internode growth of genotype 98-2226 and wild type TMS 60444, although the internode of the wild type still elongated significantly more ($P \leq 0.05$) than the internode of variety 98-0002, TME-3 and 91-02322 (Table 1).

3.5.1.6 General effects of drought stress treatment on internode growth variation among cassava genotypes

Significant variation in internode elongation was observed in transgenic cassava lines 529-28, 529-48 and their wild type TMS 60444 (Table 1). Transgenic line 529-48 internode elongated significantly ($P \leq 0.05$) higher than its wild type TMS 6044 and transgenic 529-28 (Table 2.4.1). Although line 529-28 internode grew the least, the elongation was still significantly varied ($P \leq 0.05$) with wild type TMS 60444 (Table 1). Internode growth in non-transgenic cassava genotypes were also significantly varied with internode of genotype 98-2226 lengthening the most and genotype 95-0306 internodes elongating the least (Table 1). There was no significant variation ($P > 0.05$) in internode growth between genotypes TME-3 and 98-0002, although their internode significantly ($P \leq 0.05$) grew more than internode of genotypes 91-02322 and 95-0306 (Table 1).

Internodes of genotypes 91-02322 and 95-0306 also varied significantly ($P \leq 0.05$). Despite inter-nodal growth of non-transgenic cassava genotypes showing significantly ($P \leq 0.05$) less lengthening compared to transgenic line 529-48, the inter-nodal growth of most of these genotypes (non-transgenic) were significantly higher ($P \leq 0.05$) than internode growth of transgenic line 529-28 (Table 1). The only exception was the internode elongation of genotype 95-0306, which did not significantly ($P > 0.05$) vary with internode growth of transgenic line 529-28 (Table 1). The inter-nodal elongation of wild type TMS 60444 was significantly ($P \leq 0.05$) more than those of genotypes 91-02322 and 95-0306, but did not differ significantly ($P > 0.05$) with the internode elongation of genotypes TME-3 and 98-0002 (Table 1). The internode of genotype 98-2226 elongated significantly ($P \leq 0.05$) more than the wild type TMS 60444 (Table 1).

Table 1: Means of internode growth or elongation (in cm) of transgenic and non-transgenic cassava genotypes subjected to different levels of drought stress treatments under greenhouse conditions

		Treatments				
Source	Genotype	Not Irrigated	30% Irrigated	60% Irrigated	Fully-Irrigated	**Mean (Genotype)**
ETH-Zurich Transgenic	529-28	0.16^{i}	0.34^{n}	0.38^{t}	0.56^{s}	**0.36^{e}**
	529-48	0.29^{fg}	0.56^{j}	0.65^{q}	0.78^{u}	**0.57^{a}**
	TMS 60444	0.20^{hi}	0.40^{m}	0.56^{r}	0.70^{v}	**0.47^{c}**
IITA-Ibadan Non Transgenic	91-02322	0.21^{h}	0.37^{mn}	0.48^{s}	0.60^{g}	**0.41^{d}**
	98-0002	0.17^{i}	0.37^{mn}	0.51^{s}	0.73^{v}	**0.44^{c}**
	98-2226	0.27^{g}	0.47^{k}	0.59^{r}	0.73^{v}	**0.52^{b}**
	95-0306	0.15^{i}	0.26^{p}	0.36^{t}	0.51^{o}	**0.32^{e}**
	TME-3	0.31^{f}	0.39^{m}	0.49^{s}	0.65^{i}	**0.46^{c}**
Mean (Treatment)		**0.22^{z}**	**0.40^{y}**	**0.50^{x}**	**0.66^{w}**	

LSD ($P \leq 0.05$) Treatment = 0.03 LSD ($P \leq 0.05$) Genotype = 0.04

Mean internode lengths followed by the same letter in specific drought stress treatment column, overall genotype column (in bold) and the overall treatment row (in bold) of table 1 (above) are not significantly different at 5% significant level.

3.5.2 Leaf abscission (LA) for leaf retention in cassava genotypes

3.5.2.1 Effects of different levels of water deficit on leaf abscission or leaf retention

There was significant ($P \leq 0.05$) variation between treatments in terms of number of leaves dropped from cassava plants and within cassava genotypes, but the genotype x treatment interaction produced no significant ($P > 0.05$) differences (Appendix, 2; Table 2). . An observed trend was that significantly ($P \leq 0.05$) more leaves were dropped from non-irrigated plants compared to plants that received the other three treatments (Table 2). The fully irrigated cassava plants lost the least number of leaves; while the number of leaves dropped from the 30 and 60% irrigated cassava plants also significantly ($P \leq 0.05$) varied (Table 2).

3.5.2.2 Effect of full irrigation or control treatment on leaf abscission of cassava genotypes

Total number of leaves lost by control (or fully irrigated) cassava genotypes were not significantly ($P > 0.05$) different (Table 2). The total leaves abscised from each genotype under this treatment were few and varied little.

3.5.2.3 Effect of withholding total irrigation (0%) on leaf abscission of cassava genotypes

Although not significantly varied ($P > 0.05$) in leaf abscission themselves, transgenic line 529-28 and wild type TMS 60444 had a significantly higher ($P \leq 0.05$) leaf loss than transgenic line 529-48 (Table 2). Genotype 95-0306 expressed significantly ($P \leq 0.05$) higher leaf abscission compared to other non-transgenic cassava genotypes (Table 2).

Leaf abscission in genotype TME-3 significantly varied ($P \leq 0.05$) with other genotypes and although total leaves abscised by genotypes 98-2226, 98-0002 and 91-02322 were not significantly different ($P > 0.05$), the three genotypes however lost the least number of leaves (Table 2). Leaf abscission in transgenic line 529-28, wild type TMS 60444 and genotype 95-0306 did not significantly vary ($P > 0.05$), although the two genotypes that showed higher number of leaves dropped. Transgenic line 529-48, genotypes 98-2226 and 98-0002 did not significantly vary ($P > 0.05$) in their leaf abscission. The three genotypes however expressed the least number of leaves drop i.e. no change (Table 2).

3.5.2.4 Effect of 30% water deficit on leaf abscission of cassava genotypes

Under 30% irrigation, both transgenic line 529-28 and wild type TMS 60444 did not significantly vary ($P > 0.05$) in their leaf abscission, although the two genotypes lost significantly ($P \leq 0.05$) more leaves than transgenic line 529-48 (Table 2). Significant variation in leaf abscission was also observed in non-transgenic cassava genotypes; with genotype 95-0306 showing significantly higher ($P \leq 0.05$) leaf abscission than other genotypes and genotype 98-0002 showing the least leaf drop.

Leaf abscission in genotypes TME-3, 98-2226 and 91-02322 were not significantly ($P > 0.05$) different (Table 2). Total number of leaves dropped by wild type TMS 60444, genotype 95-0306, and transgenic line 529-28 were not significantly different ($P > 0.05$), although leaf abscission in the three genotypes were the highest (Table 2). Transgenic line 529-48 lost significantly ($P \leq 0.05$) more leaves than genotype 98-0002, although line 529-48 leaf abscission mean did not significantly vary ($P > 0.05$) with those of genotypes 91-02322, 98-2226 and TME-3 (Table 2).

3.5.2.5 Effect of 60% water deficit on leaf abscission of cassava genotypes

Analyzing leaf abscission across cassava genotypes subjected to 60% drought stress showed significant variations in the means of total number leaves lost. Transgenic line 529-28 and wild type TMS 60444 did not significantly vary ($P>0.05$) in the number of leaves they each dropped, although the two genotypes lost significantly ($P\leq0.05$) more leaves than transgenic line 529-48 (Table 2).

Analyzing non-transgenic cassava genotypes indicated wide variations in leaf abscission. Significantly higher ($P\leq0.05$) leaf abscission or leaves lost was observed in genotype 95-0306 compared to other genotypes, while genotype 98-0002 showed the least number of leaves dropped (Table 2). The total number of leaves dropped by genotypes TME-3, 98-2226 and 91-02322 were not significantly ($P>0.05$) different (Table 2).

Comparing leaf abscission in transgenic and non-transgenic cassava genotypes showed genotype 95-0306, wild type TMS 60444 and transgenic line 529-28 not significantly varying ($P>0.05$) in number of leaves they lost, although their leaf abscission means still represented the highest number of leaves dropped (Table 2).

Transgenic line 529-48 and genotype 91-02322 did not significantly vary ($P>0.05$) in number of leaves they lost (Table 2). Significant variation in leaf abscission was however observed between genotypes TME-3, 98-2226, 98-0002 and transgenic line 529-48 (Table 2). Genotype 98-0002 lost significantly ($P\leq0.05$) few number of leaves than either of the transgenic lines (529-28 and 529-48) and their wild type TMS 60444 (Table 2).

3.4.2.6 General effects of drought stress treatment on leaf abscission or leaf retention variation among cassava genotypes

There were no significant differences ($P \leq 0.05$) on the number of leaves dropped by transgenic line 529-28 and wild type TMS 60444, although the two genotypes lost more leaves (significantly; $P \leq 0.05$) compared to transgenic line 529-48. Line 529-48 lost the least number of leaves (Table 2). When all non-transgenic cassava genotypes were compared, genotype 95-0306 lost significantly ($P \leq 0.05$) more leaves, while the total leaves dropped by genotype 98-0002 (though not significantly different ($P > 0.05$) from genotypes 91-02322, and 98-2226), were the least (Table 2).

There were also no significant differences ($P > 0.05$) in total leaves lost by genotypes TME-3, 98-2226, and 91-02322 (Table 2). When transgenic cassava lines and their wild type were compared with non-transgenic cassava genotypes, there were no significant differences ($P > 0.05$) in total number of leaves lost by wild type TMS 60444, transgenic line 529-28 and genotype 95-0306 (Table 2). However, the trend showed that the three genotypes lost more leaves than the rest of the cassava genotypes. Transgenic line 529-48 and genotype 98-0002 dropped the least total number of leaves (Table 2).

Table 2: Means of total number of leaves abscised (to determine leaf retention) from transgenic and non-transgenic cassava genotypes subjected to different levels of drought stress treatments under greenhouse conditions

		Treatments				
Source	Genotype	Not Irrigated	30% Irrigated	60% Irrigated	Fully-Irrigated	**Mean (Genotype)**
ETH-Zurich Transgenic	529-28	1.89^{p}	1.81^{m}	1.47^{b}	0.01^{g}	**1.29^{a}**
	529-48	1.54^{u}	1.40^{n}	1.14^{i}	0.02^{g}	**1.03^{bd}**
	TMS 60444	1.83^{pr}	1.93^{m}	1.57^{b}	0.09^{g}	**1.36^{a}**
IITA-Ibadan Non Transgenic	91-02322	1.62^{su}	1.33^{nk}	1.24^{hi}	0.06^{g}	**1.06^{bd}**
	98-0002	1.54^{u}	1.21^{k}	0.92^{j}	0.04^{g}	**0.93^{cd}**
	98-2226	1.56^{tu}	1.44^{n}	1.16^{h}	0.05^{g}	**1.05^{bd}**
	95-0306	1.79^{pr}	1.87^{m}	1.60^{b}	0.03^{g}	**1.32^{a}**
	TME-3	1.72^{qrs}	1.47^{n}	1.30^{h}	0.03^{g}	**1.12^{b}**
Mean (Treatment)		**1.69^{w}**	**1.56^{x}**	**1.30^{y}**	**0.04^{z}**	

LSD ($P \leq 0.05$) Treatment = 0.10 LSD ($P \leq 0.05$) Genotype = 0.15

Means of total number of leaves dropped followed by the same letter in specific drought stress treatment column, overall genotype column (in bold) and the overall treatment row (in bold) of table 2 (above) are not significantly different at 5% significant level.

3.5.3 Rates of photosynthesis (P_N) of cassava genotypes

3.5.3.1 Effects of different levels of water deficit on rates of photosynthesis

Significant variations ($P \leq 0.05$) in rates of photosynthesis (P_N) were observed between the drought stress treatments as well as within cassava genotypes, while analysis of genotype x treatment interaction produced no significant ($P > 0.05$) variations (Appendix, 3; Table 3). Among the treatments, control (fully irrigated) plants expressed higher P_N and those where water was completely withheld showing the least P_N (Table 3). Rates of photosynthesis in cassava plants subjected to 30 and 60% water deficit also differed significantly ($P \leq 0.05$) (Table 3).

3.5.3.2 Effects of full irrigation or control treatment on photosynthetic rates of cassava genotypes

Significant differences in P_N were observed among cassava genotypes subjected to this treatment. Transgenic line 529-28 expressed the least P_N value, although its P_N did not differ significantly ($P > 0.05$) with the P_N of its wild type TMS 60444 (Table 3). Transgenic line 529-48 expressed significantly ($P \leq 0.05$) higher P_N compared to the wild type TMS 60444 and transgenic line 529-28 (Table 3).

Genotype 98-2226 expressed significantly higher ($P \leq 0.05$) P_N, while genotype 95-0306 expressed significantly ($P \leq 0.05$) lower P_N when all non-transgenic genotypes were compared (Table 3). The P_N in genotypes 98-0002, TME-3, and 91-02322 were also significantly ($P \leq 0.05$) different (Table 3).

Although P_N in transgenic line 529-48 and non transgenic genotype 98-2226 were not significantly different (P>0.05), the P_N of the two genotypes were still significantly (P≤0.05) higher than other transgenic and non-transgenic cassava genotypes (Table 3).

The P_N of all non-transgenic varieties (with the exception of variety 95-0306), were significantly higher (P≤0.05) than the P_N of transgenic line 529-28 as well as the P_N its wild type TMS 60444 (Table 3).

3.5.3.3 Effects of withholding total irrigation (0%) on photosynthetic rates of cassava genotypes

When subjected to complete drought stress (irrigation totally withheld), transgenic line 529-48 expressed significantly more (P≤0.05) P_N than either transgenic line 529-28 or wild type TMS 60444 (Table 3). Transgenic line 529-28 expressed significantly (P≤0.05) the least P_N (Table 3). Most of the non-transgenic cassava genotypes showed no significant differences in their P_N when total irrigation was withheld. The only exception was genotype 98-0002 that expressed significantly higher (P≤0.05) P_N compared to the other genotypes (Table 3).

Although P_N of genotypes 98-2226, 91-02322, TME-3 and 95-0306 were not significantly different (P>0.05), the general trend observed showed their P_N values declining respectively (in the order of arrangement) such that genotype 95-0306 had the lowest P_N value (Table 3). When the P_N responses of transgenic and non-transgenic cassava genotypes were compared, genotype 98-0002 expressed significantly higher (P≤0.05) P_N than both transgenic line 529-48, 529-28 and their wild type TMS 60444 (Table 3).

All non-transgenic cassava genotypes expressed significantly ($P \leq 0.05$) more P_N than transgenic line 529-28 (Table 3). The P_N of wild type TMS 60444 significantly differed ($P \leq 0.05$) with P_N of genotype 91-02322, but did not significantly vary ($P > 0.05$) with the P_N of genotype TME-3 and 95-0306 (Table 3).

3.5.3.4 Effects of 30% water deficit on photosynthetic rates of cassava genotypes

Transgenic line 529-48 expressed significantly higher P_N ($P \leq 0.05$) than either transgenic line 529-28 or their wild type TMS 60444 (Table 3). There was no significant differences ($P > 0.05$) in the P_N of transgenic line 529-28 and wild type TMS 60444, although line 529-28 showed relatively lower photosynthetic rates (Table 3).

Comparing non-transgenic cassava genotypes revealed that although the P_N of genotype 98-0002, did not significantly vary ($P > 0.05$) with the P_N of genotypes 98-2226 and TME-3, the P_N of the three genotypes were significantly ($P \leq 0.05$) more compared to other genotypes (Table 3). The P_N of genotypes 91-02322 and 95-0306 (though not significantly different ($P > 0.05$), were the least (Table 3).

When the comparisons included transgenic genotypes, the P_N of transgenic line 529-48 and genotype 98-0002 did not significantly vary ($P > 0.05$) although the two genotypes expressed higher P_N than other genotypes (Table 3). The least significant ($P \leq 0.05$) values of P_N were observed in genotypes 91-02322, line 529-28 and genotype 95-0306 (Table 3). The P_N of wild type TMS 60444 also significantly varied ($P \leq 0.05$) with the P_N of non-transgenic genotypes (Table 3).

3.5.3.5 Effects of 60% water deficit on photosynthetic rates of cassava genotypes

Under this drought stress regime, significant variations in P_N were observed in both transgenic and non-transgenic cassava plants. Transgenic line 529-48 showed significantly higher ($P \leq 0.05$) P_N than line 529-28 and wild type TMS 60444 (Table 3). The P_N of wild type TMS 60444 and transgenic line 529-28 did not significantly ($P > 0.05$) vary (Table 3).

Although the P_N of genotypes 98-0002 and 98-2226 did no significantly vary ($P > 0.05$), the P_N of the two genotypes nevertheless were significantly higher ($P \leq 0.05$) compared to other non-transgenic genotypes (Table 3). Genotypes 91-02322 and 95-0306 expressed lower P_N. Significant variation ($P \leq 0.05$) in P_N was also observed between genotype TME-3 and other genotypes (Table 3). With the exception of genotype 95-0306, the P_N of non-transgenic genotypes was significantly more ($P \leq 0.05$) than P_N of transgenic line 529-28 (Table 3).

Although the P_N of wild type TMS 60444 did not significantly vary ($P > 0.05$) with the P_N of the genotypes 91-02322 and 95-0306, P_N of TMS 60444 however varied significantly ($P \leq 0.05$) with the P_N of genotypes TME-3, 98-2226 and 98-0002 (Table 3). The P_N of transgenic line 529-48 was not significantly different ($P > 0.05$) from P_N of genotypes 98-0002 and 98-2226 (Table 3).

3.5.3.6 General effects of drought stress treatment on photosynthetic rates variation among cassava genotypes

Analysis of transgenic cassava genotypes, showed transgenic line 529-48 expressing significantly higher ($P \leq 0.05$) P_N than its wild type TMS 60444 and transgenic line 529-28 (Table 3). Although there was no significant difference ($P > 0.05$) in P_N between wild type TMS 60444 and line 529-28, wild type TMS 60444 still expressed higher P_N (Table 3). Among non-transgenic cassava materials, genotypes 98-0002 and 98-2226 did not significantly vary ($P > 0.05$) in P_N, although the two genotypes expressed higher P_N when compared to other genotypes (Table 3).

There was no significant difference ($P > 0.05$) in the P_N of genotypes TME-3 and 91-02322. Although genotype 95-0306 showed the least P_N value compared to all genotypes, its P_N did not significantly ($P > 0.05$) differ with the P_N of genotype TME-3 (Table 3). Comparing transgenic and non-transgenic cassava genotypes also revealed significant variation in rates of photosynthesis. The P_N in transgenic line 529-48, non-transgenic genotypes 98-0002 and 98-2226 did not significantly vary ($P > 0.05$), although the three genotypes expressed higher P_N than other genotypes (Table 3).

Genotype 95-0306 and transgenic line 529-28 expressed the least P_N, although the P_N of the two genotypes did not significantly vary ($P > 0.05$) with the P_N of wild type TMS 60444 and genotype TME-3 (Table 3). With the exception of P_N of wild type TMS 60444, which did not significantly vary ($P > 0.05$) with the P_N of genotype 91-02322, genotype 91-02322 expressed significantly ($P \leq 0.05$) higher and lower P_N than transgenic line 529-28 and 529-48 respectively (Table 3).

Table 3: Mean rates of photosynthesis (P_N; umolCO_2 m^{-2} s^{-1}) of transgenic and non-transgenic cassava genotypes subjected to different levels of drought stress treatments under greenhouse conditions

		Treatments				
Source	Genotype	Not Irrigated	30% Irrigated	60% Irrigated	Fully-Irrigated	**Mean (Genotype)**
ETH-Zurich Transgenic	529-28	2.13^{p}	4.09^{wy}	4.40^{iz}	6.10^{fv}	**4.18^{d}**
	529-48	3.01^{m}	5.42^{r}	5.84^{e}	8.27^{p}	**5.64^{a}**
	TMS 60444	2.56^{no}	4.30^{vxy}	4.59^{hz}	6.29^{t}	**4.43^{bd}**
IITA-Ibadan Non Transgenic	91-02322	3.00^{m}	4.37^{uvxy}	4.88^{fh}	7.04^{s}	**4.82^{b}**
	98-0002	3.90^{k}	5.12^{rt}	6.20^{e}	7.12^{rs}	**5.58^{a}**
	98-2226	3.10^{m}	4.73^{stv}	5.89^{e}	8.21^{p}	**5.48^{a}**
	95-0306	2.68^{mo}	4.05^{y}	4.63^{ghz}	5.74^{uv}	**4.28^{cd}**
	TME-3	2.76^{mo}	4.70^{tx}	5.10^{f}	7.74^{q}	**4.43^{bd}**
Mean (Treatment)		**2.89^{z}**	**4.60^{y}**	**5.19^{x}**	**7.06^{w}**	

LSD ($P \leq 0.05$) Treatment = 0.31 LSD ($P \leq 0.05$) Genotype = 0. 44

Means of photosynthetic rates (P_N) followed by the same letter in specific drought stress treatment column, overall genotype column (in bold) and the overall treatment row (in bold) of table 3 (above) are not significantly different at 5% significant level.

3.5.4 Stomatal Conductance (Gs) of cassava genotypes

3.5.4.1 Effects of different levels of water deficits on stomatal conductance

Variations in stomatal conductance within cassava genotypes and the genotype x treatment were not significant ($P>0.05$), while significant ($P\leq0.05$) variation in stomatal conductance were observed between the drought treatments (Appendix, 4). Cassava plants grown under full irrigation showed significantly higher ($P\leq0.05$) Gs than plants grown under other drought stress treatments (Table 4). Plants where water was completely withheld showed the least amount of water vapor, while there was no significant difference ($P>0.05$) in Gs between 30% and 60% water stressed plants (Table 4).

3.5.4.2 Effects of full irrigation on stomatal conductance of cassava genotypes

There were no significant differences ($P>0.05$) in the amount of water vapor conducted by transgenic line 529-28, 529-48 and their wild type TMS 60444 (Table 4). Non transgenic genotypes 98-0002, TME-3 and 95-0306 did not significantly vary ($P>0.05$) in Gs, although the three genotypes conducted significantly more ($P\leq0.05$) amount of water vapor than genotypes 98-2226 and 91-02322, while genotype 91-02322 conducted the least amount of water vapor (Table 4).

Genotype 98-0002 conducted significantly ($P\leq0.05$) more amount of water vapor than both transgenic lines (529-28 and 529-48) and their wild type TMS 60444 (Table 4). Stomatal conductance of transgenic line 529-48, wild type TMS 60444, genotypes 95-0306 and TME-3 were not significantly ($P>0.05$) varied, while genotype 91-02322 conducted significantly lower ($P\leq0.05$) amount of water vapor than both transgenic lines and their wild type TMS 60444 (Table 4).

3.5.4.3 Effects of withholding total irrigation (0%) on stomatal conductance of cassava genotypes

Wild type TMS 60444 conducted significantly ($P \leq 0.05$) more amount of water vapor compared to transgenic lines 529-28 and 529-48 and the Gs of the two transgenic lines did not significantly ($P > 0.05$) vary (Table 4). Although non-transgenic genotype 95-0306 expressed higher Gs, the amount of water vapor it conducted was not significantly different ($P > 0.05$) from the amount of water vapor conducted by genotypes 91-02322, 98-0002, and TME-3. Genotype 98-2226 conducted the least amount of water vapor (Table 4).

Wild type TMS 60444 and non transgenic variety 95-0306 showed higher Gs, while transgenic line 529-48 and genotype 98-2226 conducted the least amount of water vapor (Table 4). The amount of water vapor conducted varied significantly ($P \leq 0.05$) between transgenic line 529-28 and all non-transgenic cassava genotypes with the exception of genotype 98-2226 (Table 4).

3.5.4.4 Effects of 30% water deficit on stomatal conductance of cassava genotypes

Although the Gs of transgenic line 529-48 and wild type TMS 60444 did not significantly vary ($P > 0.05$), the two genotypes nevertheless conducted significantly ($P \leq 0.05$) less amount of water vapor than transgenic line 529-28 (Table 4). Non transgenic genotypes 98-0002 and 95-0306 conducted significantly higher ($P \leq 0.05$) amount of water vapor compared to other non-transgenic cassava genotypes.

Genotype 98-2226 conducted the least amount of water vapor although this did not significantly vary (P>0.05) with Gs of genotypes 91-02322 and TME-3 (Table 4). Stomatal conductance of transgenic line 529-28, wild type TMS 60444, genotypes 98-0002, and 95-0306 did not significantly vary (P>0.05), although they conducted the most amount of water vapor than other genotypes (Table 4).

The amount of water vapor conducted by transgenic line 529-48 was not significantly different (P>0.05) from the amount of water vapor conducted by genotype TME-3, but line 529-48 conducted significantly more (P≤0.05) water vapor than genotypes 91-02322 and 98-2226 (Table 4).

3.5.4.5 Effects of 60% water deficit on stomatal conductance of cassava genotypes

There was no significant differences (P>0.05) in the amount of water vapor conducted by transgenic line 529-28, 529-48 and their wild type TMS 60444 (Table 4). All the non-transgenic cassava varieties (with the exception of variety 91-02322 and TME-3 which conducted the least amount of water vapor) did not significantly vary (P>0.05) in Gs (Table 4).

The amount of water vapor conducted by transgenic lines 529-28, 529-48 and their wild type TMS 60444 did not significantly vary (P>0.05) with stomatal conductance in non-transgenic genotypes 98-0002, 98-2226 and 95-0306 (Table 4). However the transgenic genotypes conducted more water vapor than genotype TME-3 and 91-02322 (Table 4).

3.5.4.6 General effects of drought stress treatment on variation in amount of water vapor conducted by cassava genotypes

Transgenic line 529-28, 529-48 and their wild type TMS 60444 showed no significant differences ($P>0.05$) in Gs (Table 4). There were also no significant differences ($P>0.05$) in the amount of water vapor conducted by non-transgenic cassava genotypes 98-0002, 95-0306 and TME-3 (Table 4). The three genotypes however conducted significantly ($P\leq0.05$) more water vapor than genotypes 98-2226 and 91-02322 (Table 4).

Although stomatal conductance in genotypes 98-2226 and 91-02322 did not significantly vary ($P>0.05$), the two conducted the least amount of water vapor (Table 4). When Gs was compared between transgenic and non-transgenic cassava genotypes, transgenic lines 529-28, 529-48 and their wild type TMS 60444 did not significantly vary ($P>0.05$) with the amount of water vapor conducted by non transgenic genotypes 98-0002, 95-0306 and TME-3. The Gs of the six genotypes significantly varied ($P\leq0.05$) with Gs of 98-2226 and 91-02322 (Table 4).

Table 4: Mean Stomatal Conductance (Gs; mmol m^{-2} s^{-1}) or amount of water vapor conducted by transgenic and non-transgenic cassava genotypes subjected to different levels drought stress treatments under greenhouse conditions

		Treatments				
Source	Genotype	Not Irrigated	30% Irrigated	60% Irrigated	Fully-Irrigated	**Mean (Genotype)**
ETH-Zurich Transgenic	529-28	$0.025d^{eg}$	0.052^{h}	0.043^{qv}	0.051^{jk}	**0.043^{a}**
	529-48	0.021^{fg}	0.046^{ijm}	0.047^{q}	0.053^{hik}	**0.042^{ad}**
	TMS 60444	0.035^{a}	0.049^{hj}	0.046^{qs}	0.053^{hik}	**0.046^{a}**
IITA-Ibadan Non Transgenic	91-02322	0.029^{bce}	0.039^{np}	0.037^{tw}	0.042^{m}	**0.037^{cd}**
	98-0002	0.029^{bce}	0.052^{h}	0.043^{qv}	0.060^{g}	**0.046^{a}**
	98-2226	0.025^{deg}	0.037^{p}	0.044^{qu}	0.051^{jk}	**0.039^{bd}**
	95-0306	0.032^{ac}	0.050^{hj}	0.044^{qu}	0.057^{gi}	**0.046^{a}**
	TME-3	0.029^{bce}	0.041^{kmp}	0.041^{rsuvw}	0.057^{gi}	**0.042^{ad}**
Mean (Treatment)		**0.028^{y}**	**0.046^{x}**	**0.043^{x}**	**0.053^{w}**	

LSD ($P \leq 0.05$) Treatment = 0.004 LSD ($P \leq 0.05$) Genotype = 0.006

Means of stomatal conductance (Gs) followed by the same letter in specific drought stress treatment column, overall genotype column (in bold) and the overall treatment row (in bold) of table 4 (above) are not significantly different at 5% significant level.

3.5.5 Correlations between rates of photosynthesis (P_N), stomatal conductance (Gs) and leaf abscission (LA)

There was a small but significant ($P \leq 0.01$) positive correlation between P_N and Gs (Table 5; Figure 1), a large and significant ($P \leq 0.01$) negative correlation between P_N and LA (Table 5; Figure 2) and a small but significant negative correlation between LA and Gs (Table 3.5; Figure 3).

Cassava genotypic variations in terms of correlations between the above stated parameters were also observed. For example, among transgenic genotypes, line 529-48 expressed high P_N (Table 3) and low LA (Table 2) compared to both line 529-28 and wild type TMS 60444 that expressed high LA (Table 2) and low P_N (Table 2). Among non transgenic plants, both genotypes 98-0002 and 98-2226 expressed high P_N (Table 3) and low LA (Table 2), compared to genotype 95-0306 that exhibited high LA (Table 2) and low P_N (Table 3).

The negative correlation between P_N and LA were also consistent in genotypes 91-02322 and TME-3 (Table 3 and Table 2) and LA (Table 2). Although Gs of transgenic genotypes 529-28, 529-48 and wild type TMS 60444 were not significantly ($P>0.05$) different (Table 4), line 529-48 still exhibited a high P_N (Table 3), and lower LA (Table 2). Among non transgenic plants, genotype 98-0002 expressed high P_N, high Gs and low LA compared to other genotypes (Table 2; Table 3 and Table 4).

Table 5: Correlation matrices of parameters: Stomatal Conductance, Leaf Abscission and Rates of Photosynthesis of water stressed cassava genotypes

		Stomatal conductance	Leaf abscission	Photosynthetic rate
Stomatal conductance	Pearson Correlation	1	-.099(**)	+.476(**)
	Sig. (2-tailed)		.000	.000
Leaf abscission	Pearson Correlation	-.099(**)	1	-.252(**)
	Sig. (2-tailed)	.000		.000
Photosynthetic rate	Pearson Correlation	+.476(**)	-.252(**)	1
	Sig. (2-tailed)	.000	.000	

** Correlation is significant at the (P≤ 0.01) level using a 2-tailed test.

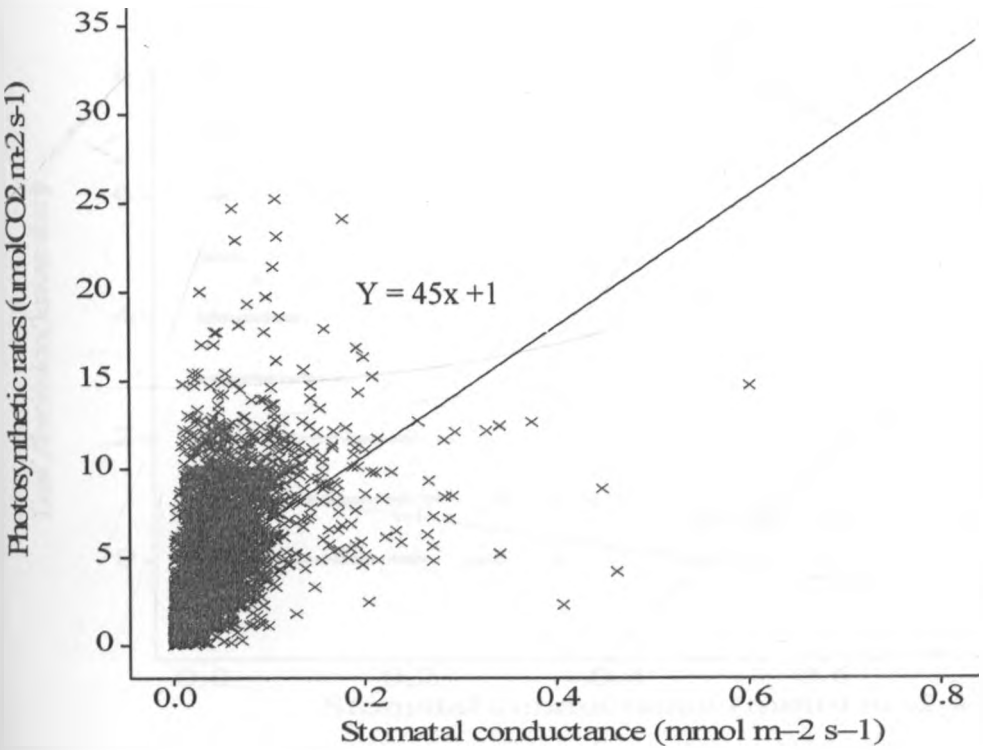


Figure 1: *Significant (P≤ 0.01) positive correlation between rates of photosynthesis (P_N) and Stomatal conductance (Gs)*

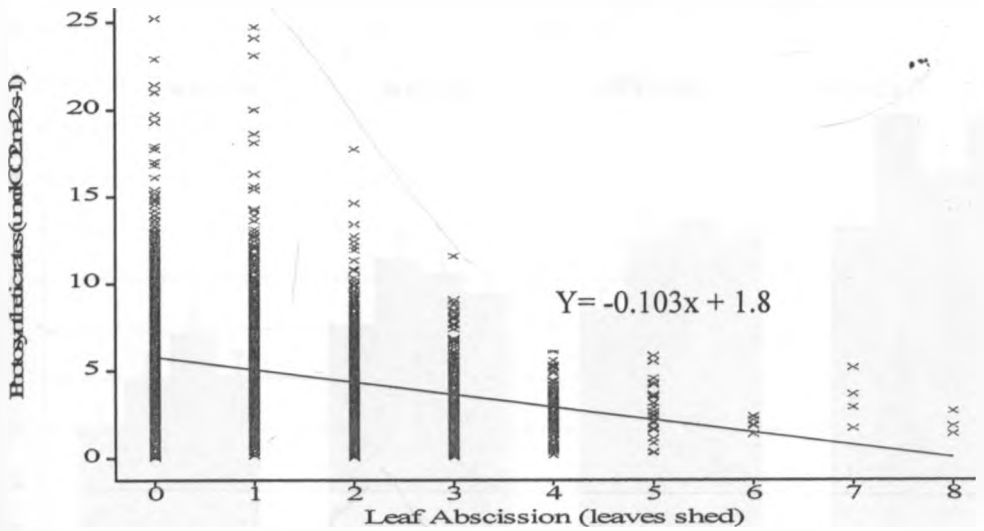


Figure 2: *Significant (P≤ 0.01) negative correlation between Leaf Abscission (LA) and Photosynthetic Rates (P_N)*

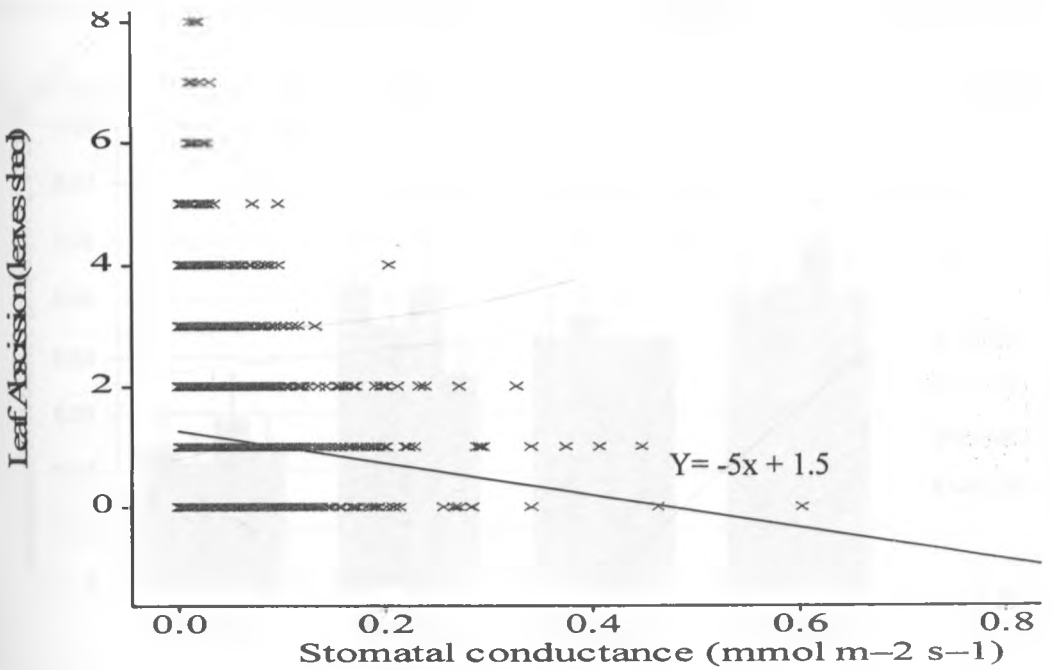


Figure 3: *Significant (P≤ 0.01) negative correlation between Leaf Abscission (LA) and Stomatal conductance (Gs)*

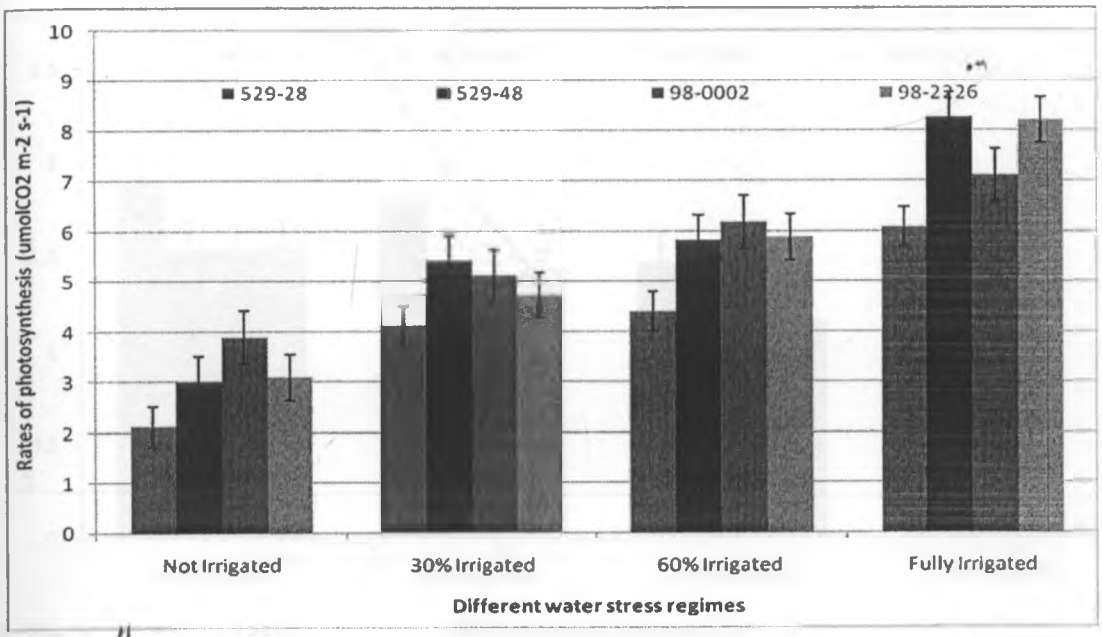


Figure 10: *Photosynthetic rates versus levels of drought stress treatments of transgenic genotypes (529-28 and 529-48) and non transgenic genotypes (98-0002 and 98-2226)*

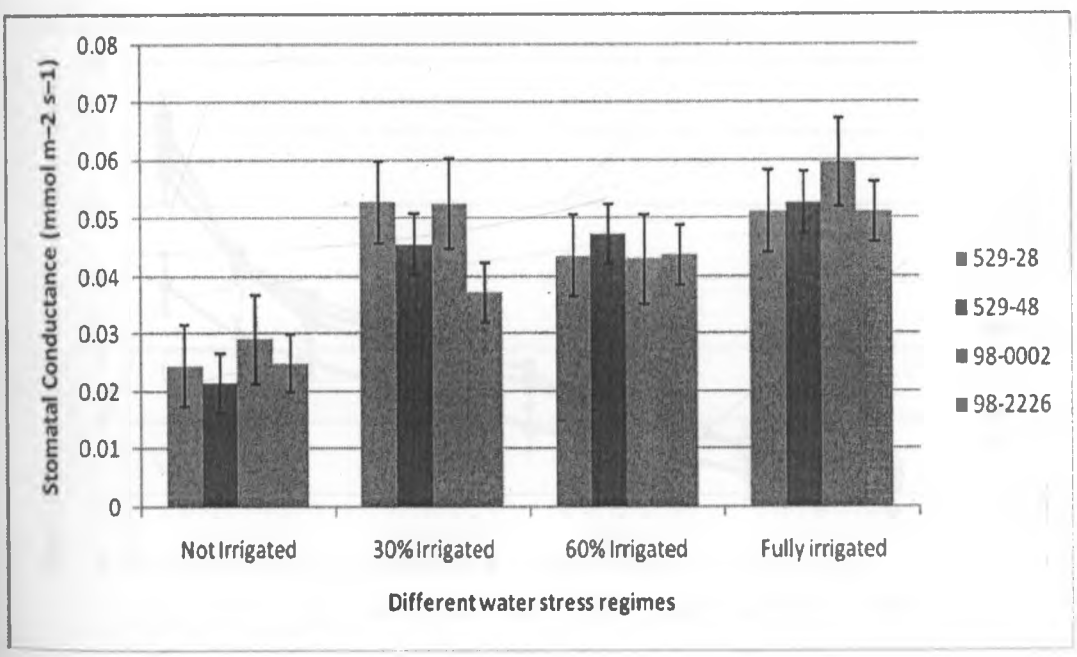


Figure 5: *Stomatal Conductance versus levels of drought stress treatments of transgenic genotypes (529-28 and 529-48) and non transgenic genotypes (98-0002 and 98-2226)*

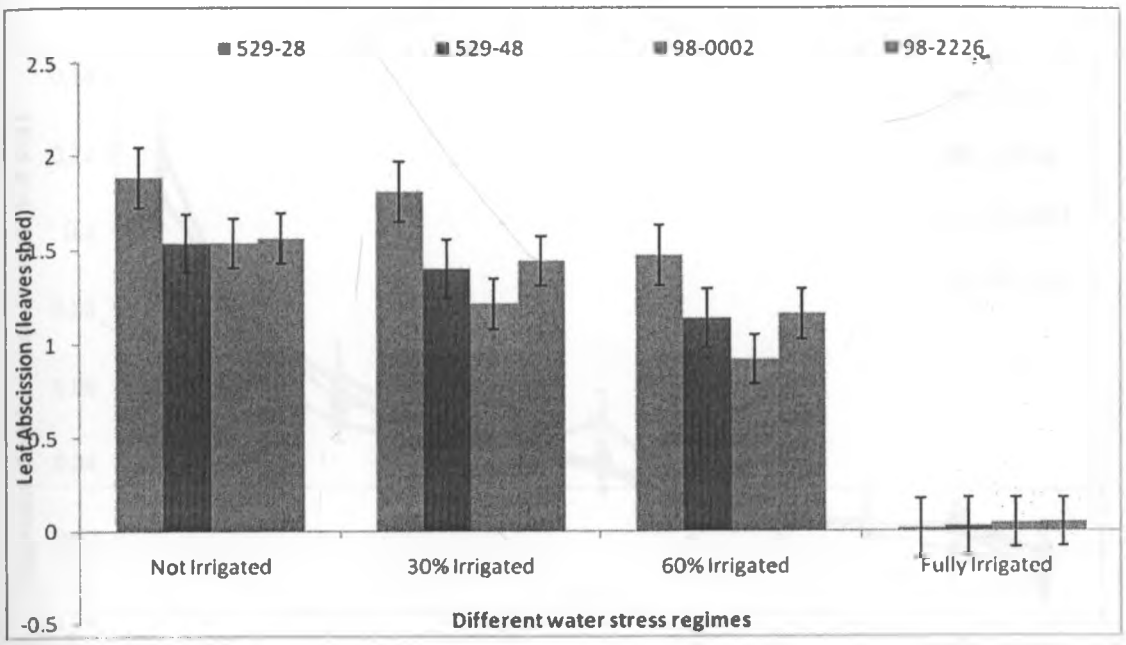


Figure 6: *Leaf Abscission versus levels of drought stress treatments of transgenic genotypes (529-28 and 529-48) and non transgenic genotypes (98-0002 and 98-2226)*

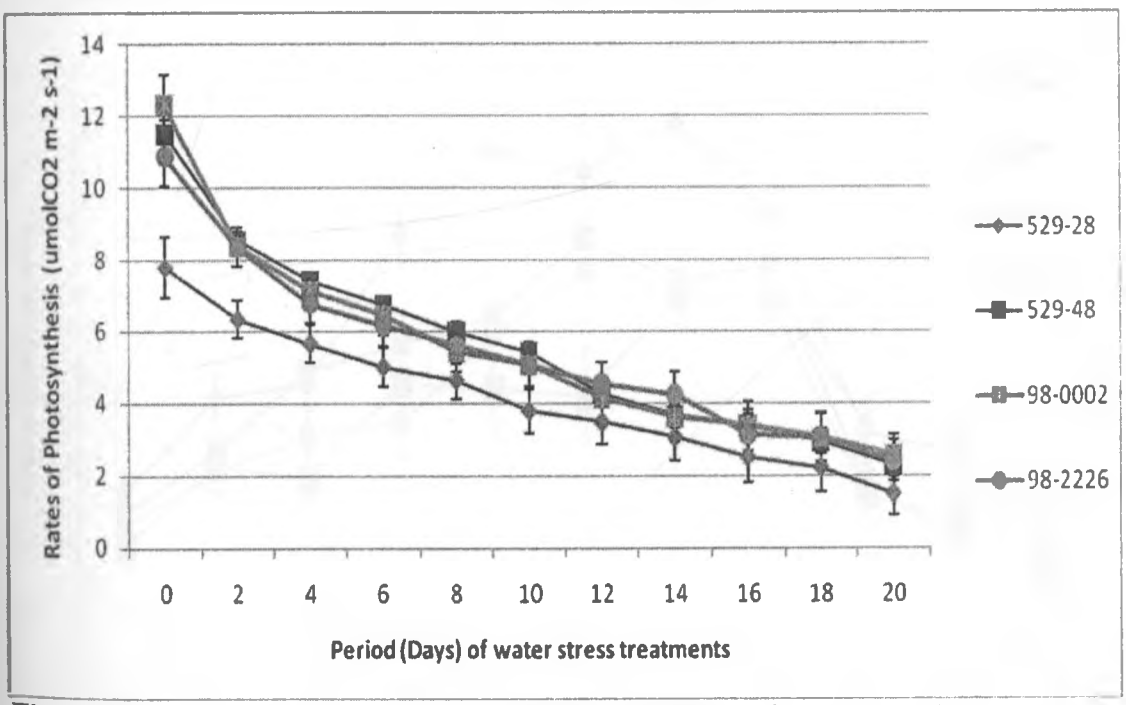


Figure 7: *Photosynthetic rates response with time (Days of water stress) in transgenic genotypes (529-28 and 529-48) and non transgenic genotypes (98-0002 and 98-2226)*

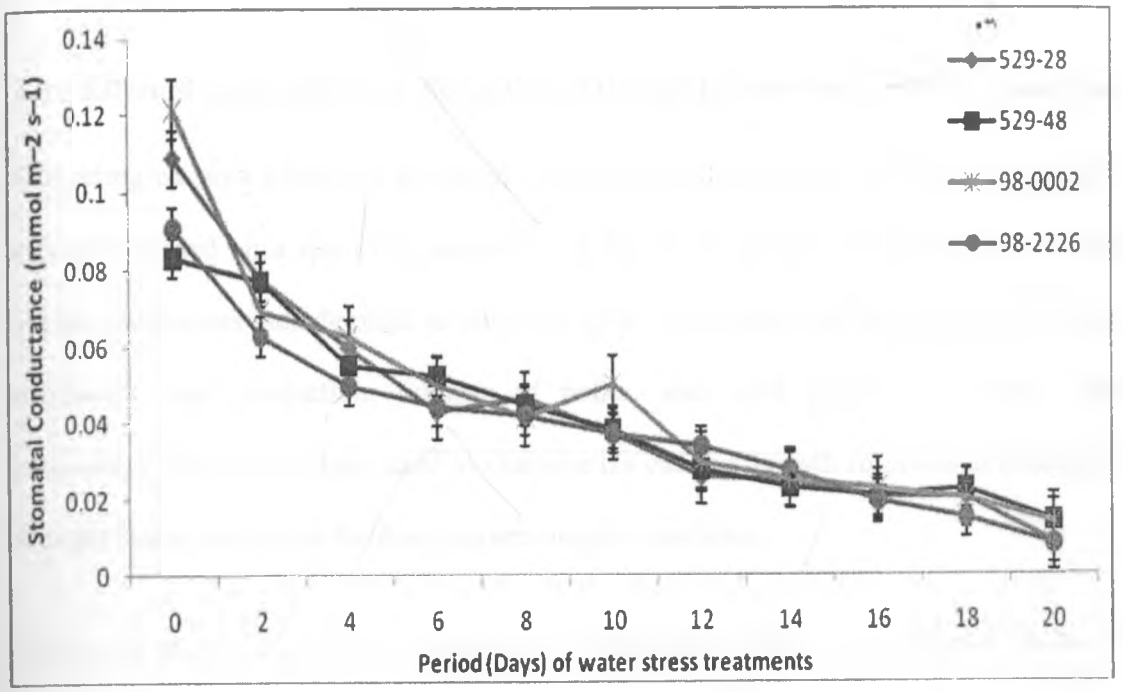


Figure 8: *Stomatal Conductance response with time (Days of water stress) transgenic genotypes (529-28 and 529-48) and non transgenic genotypes (98-0002 and 98-2226)*

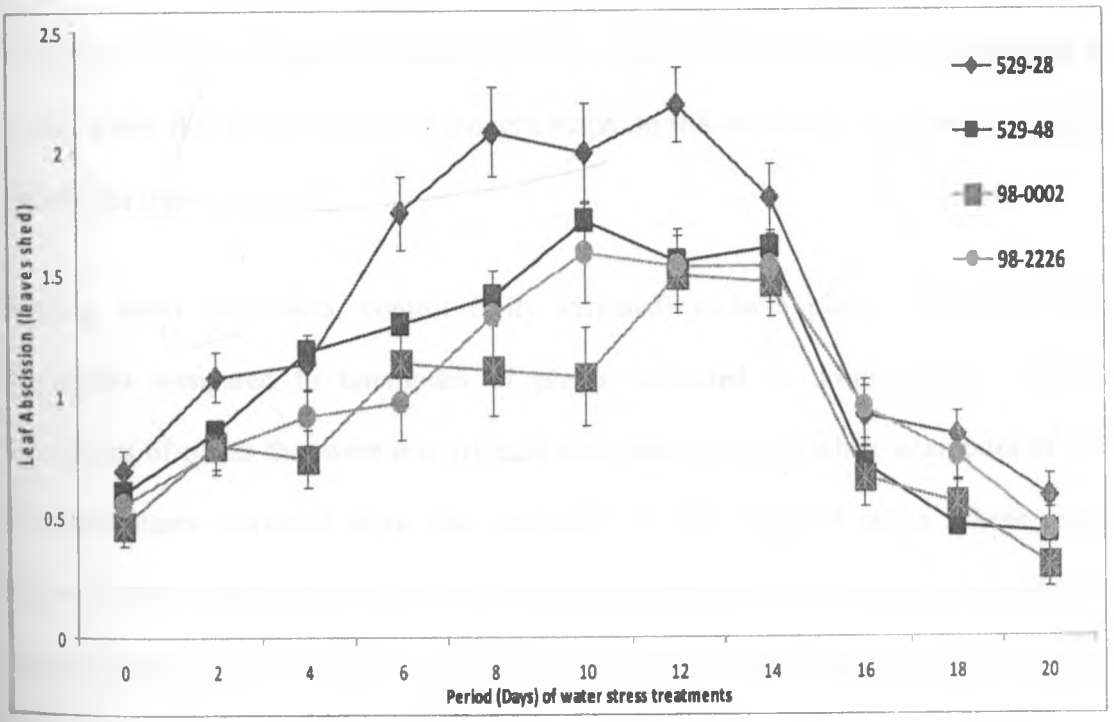


Figure 9: *Leaf Abscission response with time (Days of water stress) in transgenic genotypes (529-28 and 529-48) and non transgenic genotypes (98-0002 and 98-2226)*

3.6 Discussion

3.6.1 Effect of water deficit on elongation of the last internodes in cassava genotypes

Subjecting cassava genotypes to drought stress and analyzing for differences in growth increment based on a specified parameter (s) has been carried out before. Some of the growth parameters include plant height, stem girth, root length, number of roots, leaf area expansion, leaf production, number of nodes, and seed production among other parameters. These have been used to characterize cassava growth response to drought or drought stress under both field and screen-house conditions.

Measuring growth or elongation of the last internodes in cassava genotypes subjected to drought stress to characterize drought tolerance in a greenhouse or in a field has not been done before. Elongation of cassava internodes has been characterized only as a 'striking' symptom of super-elongation disease caused by *Sphaceloma manihoticola* (Zeigler et al., 1980). From this study, effects of drought stress on the increment or growth of cassava internodes were observed.

Among water treatments, control (fully irrigated) cassava plants expressed longer internodes compared to internodes of plants subjected to drought stress regimes. Internodes of plants that were not irrigated elongated the least, while internodes of 60% irrigated plants increased more than internode of 30% irrigated plants. These results showed that internode growth in cassava plants depend on water supply or availability implying that internode elongation is positively correlated with amount of water supplied. Genotypic differences in internode growth were observed in specific water regimes.

Among fully irrigated plants, internode of genotypes 98-2226 and 98-0002 increased longer than (but relatively same length) in TME-3, 91-02322 and 95-0306. The inter-nodal growth reduced from TME-3, 91-02322 and 95-0306 respectively. Transgenic line 529-48 grew the most, followed by wild type TMS 60444 and line 529-28 grew the least.

Line 529-48 elongated more than non transgenic genotypes while elongation between wild type TMS 60444 and genotypes 98-0002 and 98-2226 were relatively the same. Internode of line 529-28 grew longer than of genotype 95-0306. Within the fully irrigated plants, the differences in internode growth among the genotypes could be attributed to individual genotype ability to sustain high photosynthetic rates under well watered conditions. Rates of photosynthesis in cassava are genotype dependent (Alves, 2002; Lenis et al., 2006; El-Sharkawy, 2003, 2007).

For example, it was observed that genotypes (such as 98-2226, 98-0002 and line 529-48) that expressed higher photosynthetic rates probably generated more photoassimilates and rapidly translocated to the stems for improved internode growth, compared to variety 95-0306 and line 529-28 that showed least internode elongation as well as low photosynthetic rates.

Genotypic variation of internode elongation under 0% irrigation was also noted in this study. Genotype TME-3 elongated the most, followed by genotypes 98-2226 and 91-02322 respectively (in a descending order), genotype 98-0002 and 95-0306 elongated to relatively similar levels, (but they grew the least).

Transgenic line 529-48 grew more than wild type TMS 60444 and line 529-28. Internode elongations for lines 529-28 and wild type TMS 60444 elongated to similar levels. Elongation between lines 529-28, TMS 60444, genotypes 98-0002, and 95-0306 were the same. Similar observations were made between genotypes 98-2226, TME-3, and line 529-48. The variation in internode growth under 0% irrigation was not consistent compared to internodal genotypic growth variation observed in other drought stress regimes in this study.

This is can be attributed to the fact that under this treatment, soil moisture loss was too rapid and most of the genotypes had wilted, collapsed or ceased growing by day 12 of the stress treatment. Similarly El-Sharkawy (2007) reported that the expansion of cassava leaves (another parameter used to measure cassava growth) was nearly halted by water stress. Despite this, genotypes TME-3 and 95-0306 elongated the most and the least under this drought stress respectively.

Consistent genotypic differences were observed under 30 and 60% drought stress treatments. Genotype 98-2226 grew the most, while genotype 95-0306 grew the least among non transgenic genotypes. Internodes of genotypes TME-3, 98-0002, and 91-02322 grew to relatively similar levels. Transgenic line 529-48 grew the most, followed by wild type TMS 60444, while internode of line 529-28 lengthened the least. Internode growth was more in line 529-48 compared non transgenic genotypes.

These results were consistent in both 30 and 60% water stress. Internode increment of wild type TMS 60444 was relatively similar to genotypes 91-02322, 98-0002 and TME-3, less than genotype 98-2226, and more than genotype 95-0306 respectively under 30% irrigation. Internode of all non transgenic genotypes elongated more than line 529-28 with the exception of genotype 95-0306 (grew less).

Under 60% water stress, internodes of non transgenic genotypes grew less than internode of wild type TMS 60444, with the exception of genotype 98-2226 (no differences) and again internodes non transgenic genotypes elongated more than internode of line 529-28, with the exception of genotype 95-0306 (no differences). The significant differences in internode elongation (from this study) indicated that sustained cassava growth under drought depended on the severity of drought stress as well as individual cassava genotype. El-Sharkawy (2003) also reported that cassava's internode length and mass depended on the age of the plant, genotype and environmental factors such as drought.

Similar significant impacts of soil moisture stress on cassava's vegetative growth have also been cited. For example, Aina et al. (2007) reported a significant reduction in plant height and stem girth among drought stressed cassava plants (both in a screen-house as well as in the field). The well watered plants sustained their growth. The reduction of plant height and stem girth under moisture stress as reported by Aina et al. (2007), correlated directly with internode growth reduction as observed in 0, 30 and 60% drought stress compared to control plants in this study.

Cassava's stem and hence total height is known to increase via internodal growth. Other related growth parameters found to be genotype and drought stress dependent include root length, shoot/canopy growth (stems, petioles and leaves), (Okogbenin et al., 2003; El-Sharkawy, 2007).

A decline in cassava's shoot growth was attributed to moisture stress (Okogbenin et al., 2003; El-Sharkawy, 2007). Shoot development in cassava are susceptible to soil water content variations (Connor and Cock, 1981) as cassava plants previously exposed to drought showed less shoot dry matter compared to well watered controls (Pardales and Esquibel, 1996). The shoot (stems and leaves) growth reduction under moisture stress, therefore inferred the internode elongation growth too was also retarded (as shown in this study). The last internodes are presumed to be part of the shoot (i.e. top most section of the cassava stem that is growing).

In this study, it was found that genotypes that expressed low leaf abscission (high leaf retention) such as 98-0002, 98-2228 and line 529-48, also expressed higher photosynthetic rates during water stress, while low leaf retaining genotypes such as 95-0306 and 52-28, expressed low rates of photosynthesis. The presumably high and low amount of photosynthates or photoassimilates (depending on leaf retention and photosynthesis) produced by these varieties or genotypes, were probably translocated and utilized in sustaining the arrested growth i.e. internode elongation. This explains the genotypic growth variations.

The partitioning of photoassimilates in cassava and their translocation for the growth of shoots (petioles, nodes, stems and leaves) depends on growth conditions such as temperature, photoperiod, water regime and cultivar (Cock et al., 1979; Tan and Cock, 1979; Pellet and El-Sharkawy, 1997; Alves, 2002; El-Sharkawy and Cadavid, 2002).

Another factor that might have played a role in variation of the genotypes in terms of their internode elongation is variation in plant height. Aina et al., (2007) reported reduction in total plant height due to moisture stress and still the reduction was genotype dependent. In this study, the taller genotypes were 98-2226, 98-0002 and line 529-48 and the shorter genotypes included 91-02322, 95-0306 and line 529-28 (data not provided). Since the genotypes and treatments were randomly assigned, the effect of shading (of taller varieties onto shorter varieties) was or was not expected to influence internode growth between the two groups.

Additionally, the taller genotypes expressed higher leaf retention (larger leaf area index) than shorter genotypes across the drought stress treatments and therefore the effect of shading on internode elongation was expected. However, internodes of taller genotypes elongated more than shorter genotypes. These results were in contrast with the earlier reports by Janick (2003) that associated shading effect with higher internode elongation. Cassava does not tolerate shading since it leads to stem and internode elongation leaving no carbohydrate for root growth.

Increasing plant population beyond the optimum number may cause higher internode elongation due to decrease in light penetration inside the canopy and increase plant height (Janick, 2003). Plant populations, at which cassava cultivars are grown, widely vary between 10, 000 and 20, 000 (El-Sharkawy, 2003; Janick, 2003). However, plant population cannot be attributed to the differences in internode growth between taller and shorter genotypes (as shown in this study), since the population sample that was used in this experiment, was far below the optimum population size that Janick (2003) indicated would have an effect on elongation of the internodes through canopy and light blockage. The shade effect has also been measured and found to reduce elongation of cassava fibrous roots (Aresta and Fukai, 1984; Alves, 2002).

3.6.2 Effect of water deficit on leaf abscission (LA) or leaf retention in cassava genotypes

Itani et al., (1999) studied mechanisms of leaf maintenance in cassava under water deficits and Lenis et al., (2006) reported that leaf retention has become a rather frequent trait in evaluating cassava populations under drought conditions. Cassava may possess a high ability to maintain its leaves (Cock et al., 1979). Various levels of drought stress regimes (0, 30, 60% and control) applied in this study significantly induced cassava genotypic differences in levels of leaf retention (assessed through leaf abscission). Leaf abscission method has been used to evaluate leaf retention among cassava genotypes under drought (Rosas et al., 1976; El-Sharkawy et al., 1992).

Okogbenin et al., (2003) measured cumulative leaf scars (shed) by counting number of leaves dropped, while El-Sharkawy and Cock (1987) and El-Sharkawy et al., (1992) evaluated leaf retention in cassava by counting the total number of leaves falling per hectare under drought stress.

In this study, variation in leaf abscission was observed between drought stress treatments. For example, when ranked in a descending order (from high to low LA means, hence level of leaf retention), non irrigated (0%) plants lost more leaves, followed by 30, 60% and control plants respectively. Control plants showed least LA. These findings suggest that leaf abscission is dependent of severity of drought stress regime. This has been confirmed by earlier reports which showed that cassava leaf life (from emergence to abscission) depended on water deficit (Cock et al., 1979; Irikura et al., 1979; Alves, 2002).

Generally at the onset of dry season, cassava plants reduce canopy by shedding leaves (Okogbenin et al., 2003) therefore limiting transpiration (El-Sharkawy and Cock, 1987). Cassava is known to adapt to conditions of soil water shortage through various mechanisms such as shedding leaves (Aina et al., 2007). When LA response within specific drought stress treatment (s) was assessed or screened, genotypic variation in leaf retention was observed. Among control genotypes, LA were relatively similar i.e. control plants maintained relatively similar number of leaves. Since the level of leaf retention was screened via number of leaves shed or abscised (Rosas et al., 1976; El-Sharkawy et al., 1992; Okogbenin et al., 2003), as a result of drought stress treatment in this study, the level of leaf retention in fully irrigated genotypes could not be discerned.

Leaf abscission was not expected in control plants in this study. Although not significantly expressed, the LA in control plants could be attributed to ageing/senescence. The older, lower cassava leaves often senesce and fall (Rosas et al., 1976; El-Sharkawy and Cock, 1987; Yao et al., 1988; Lenis et al., 2006). Leaf retention in control plants should have been, but was not, quantified using a simple visual score as described by Cock, (1984) or Lenis et al., (2006). When total irrigation was withheld (0% drought stress), genotypes 91-02322, 98-0002 and 98-2226 dropped relatively similar (but low) total number of leaves, compared to genotype 95-0306.

Despite this, observed trends showed 91-02322, 98-2226 and 98-0002 expressing high to low LA means (in a descending order respectively). Genotype 95-0306 expressed the highest LA. Leaf abscission in transgenic line 529-28, wild type TMS 60444, and non-transgenic genotype 95-0306 as well as TME-3 was relatively the same. A similar response was also observed between transgenic line 529-48 and non-transgenic genotypes 91-02322, 98-0002 and 98-2226.

Most genotypes (except 95-0306) showed similar LA hence difficulty in assigning leaf retention level. Although a comprehensive explanation could not be provided, a possible reason could be that withholding total irrigation might have caused a rapid soil moisture loss from the potted plants. In addition, it was observed that most of cassava genotypes had discolored, wilted and 'buckled' by day 12 of withholding total water supply. Ike and Thurtell (1981) also observed that only under severe drought stress do cassava's leaves discolor, wilt and abscise rapidly from the basal leaves.

Variations in leaf retention among cassava genotypes under both 30 and 60% drought stress were evidently discernible. For example, genotypes 98-0002 and 95-0306 consistently expressed low and high LA (respectively) than other genotypes under the two drought stress regimes. Although, genotypes TME-3, 98-2226 and 91-02322 consistently expressed relatively similar level of LA (under the two treatments), observed LA trends showed a slight shift in leaf retention when specific treatment was analyzed.

For example, under 30% irrigation, genotype TME-3 lost more leaves, followed by 98-22226, and 91-02322 in a descending order respectively, while under 60% irrigation, genotype TME-3 lost more leaves followed by 91-02322, while 98-2226 expressed least LA. The interchanging variation in leaf retention between genotypes 91-02322 and 98-2226 when submitted to the two different drought stress regimes could not be explained. Genotype 98-0002 expressed steadily lower LA than transgenic lines 529-28, 529-48 and wild type TMS 60444 under both 30 and 60% stress regimes, while line 529-48 retained more leaves than non transgenic genotypes TME-3 and 95-0306.

Line 529-48 expressed either similar or low LA than other non transgenic genotypes such as 91-02322, 98-2226, 95-0306 and TME-3 under 30 and 60% water stress. Genotypes TME-3, 98-2226, 98-0002 and 91-02322 retained more leaves than line 529-28 and wild type TMS 60444 under the two treatments. The variation in leaf retention ability of cassava genotypes as shown in the study, substantiate earlier research which indicated that leaf retention in cassava under drought stress is genotype dependent (El-Sharkawy and Cock, 1987; El-Sharkawy et al., 1992; Alves and Setter, 2000; Alves, 2002; Okogbenin et al., 2003; El-Sharkawy, 2005, 2006; Lenis et al., 2006).

In this study, the observed differences in leaf retention had been attributed to levels of water deficits as earlier supported by Cock et al., (1979), Irikura et al., (1979) and Alves, (2002). Calatayud et al., (2000) had also reported that a 45-day limited water supply in cassava resulted in a pronounced reduction in shoot growth and high leaf fall. However, Lenis et al., (2006) observed and cautioned that differences in leaf retention appear to be an inherent physiological characteristic of the individual genotypes and not a differential response to stress. The variations can also be attributed to differences in traits such as plant height, leaf area index and hence shading.

In this study, taller genotypes included 98-2226, TME-3, 529-48, and 98-0002, while shorter genotypes were 95-0306, 529-28, and 91-02322 (data not provided). Generally, taller and shorter genotypes expressed low and higher leaf abscission respectively. The differences in heights of the two groups could have played a role in limiting access to light through shading and hence affecting leaf fall or abscission. Shading of leaves limits leaf life and accelerates leaf fall (Okogbenin et al., 2003).

In evaluating leaf fall in cassava under drought, Rosas et al., (1976) noted that leaves on the outer sides of the plots, which received light, abscised much later than those on the inner and shaded sides of the stems, thus suggesting mutual importance of shade in controlling leaf abscission. The border effects are therefore likely to be important in evaluating leaf retention (Lenis et al., 2006). However, Alves, (2002) stated that the shortening of leaf life is only possible under severe shading.

Levels of shade up to about 75% have very little effect on leaf life, but under 95-100% shade, cassava leaves abscise within 10 days (Cock et al., 1979). Lenis et al., (2006) also correlated plant heights and number of branching with leaf retention. Cassava leaves ceases to grow when moisture availability is low (Itani et al., 1999). However, because leaf shedding occurs among older leaves, allowing xerophytic leaves to emerge (El-Sharkawy and Cock, 1987), a certain percentage of the leaf area can be maintained even under severe drought conditions.

This was observed in genotype 98-0002 under 0% irrigation in this study after most genotypes had collapsed by day 12 of 0% water stress. Among transgenic genotypes, line 529-48 consistently expressed lower leaf abscission means than line 529-28 and wild type TMS 60444, while LA mean of line 529-28 and TMS 60444 were similar. These observations were consistent across the three (0, 30 and 60%) drought stress regimes.

Despite line 529-28 and wild type TMS 60444 expressing relatively similar LA across the three treatments, observed trends indicated a slight shift depending on a specific water regime. For example, under both 30 and 60% water stress, TMS 60444 lost more leaves than 529-28, while when total water supply was withheld, line 529-28 showed high LA than TMS 60444. This shift in LA, between 529-28 and TMS 60444, depending on the drought stress level as observed in this study could not be explained.

Transformation of line 529-28 and 529-48 was based on expression of the *isopentenyl transferase* (*ipt*) gene which encodes a key enzyme for cytokinin biosynthesis from *Agrobacterium tumefaciens* under control of the senescence-induced *SAG12* promoter from *Arabidopsis thaliana*, hence a delay in leaf senescence (Gan and Amasino, 1995; Zhang and Gruissem, 2004; 2005). Delayed senescence or prolongation of leaf life (under water stress) had already been achieved in transgenic tobacco carrying the *ipt* gene (Gan and Amasino, 1995) and in cassava under drought stress; this should also lead to delayed leaf senescence via an auto-regulatory senescence inhibition system (Zhang and Gruissem, 2004).

Leaf abscission as expressed by line 529-28 (under 30 and 60% water stress) and line 529-48 (under 0, 30 and 60% water stress) against wild type TMS 60444 in this study, concurred with an earlier experiment done by Zhang and Gruissem (2005) who had reported that under drought stress, line 529-28, line 529-48 and wild type TMS 60444 abscised 10, 20 and 50% of their leaves respectively. However, when total irrigation was withheld (0%), LA of line 529-28 against wild type TMS 60444, and line 529-48 versus line 529-28, contrasted with results reported by Zhang and Gruissem (2005).

The low LA of 98-0002, moderate LA of 91-02322 and 98-2226 and the high LA of 95-0306, should further be assessed and their leaf retention correlated with physiological traits such as leaf water potential, leaf conductance, stomatal properties, leaf growth hormone imbalances, biomass production, and storage root yield among other traits under drought stress in field conditions.

The long advocated strategy of breeding for longer leaf life and better leaf retention (El-Sharkawy and Cock, 1987; Cock and El-Sharkawy, 1988; El-Sharkawy, 1993, 2005, 2006; Lenis et al., 2006), especially among the leaf retaining genotypes 98-0002, 98-2226 and 91-02322 identified in this study (under greenhouse conditions), should be carried out under field conditions. A potential benefit for greater leaf retention in cassava genotypes or clones under both drought stress and irrigated conditions has been highlighted.

Lenis et al., (2006) positively correlated improved longevity of leaves with increased productivity as cassava clones with the leaf retention trait produced more total fresh biomass and yielded 33% more root dry matter than clones without the trait. Other advantages that correlated with extended leaf longevity or leaf retention include greater optimal harvestable index (Cock and El-Sharkawy, 1988) and sustained high potential rates of photosynthesis (Cock et al., 1979; Cock, 1984).

High drought tolerance, root productivity (El-Sharkawy et al., 1992; Osiru et al., 1994) and high root quality (Fregene and Puonti-Kaerlas, 2002) have also been associated with leaf retention. Prolonging the leaf life of individual leaves of cassava genotypes (through genetic engineering as observed in line 529-28 and 529-48) or through conventional breeding could aid production of cultivars with improved root yield and quality and permit more frequent harvesting of the leaves, while maintaining a satisfactory photosynthetic area to ensure storage root production (Fregene and Puonti-Kaerlas, 2002).

In situations where the market value of the cassava leaves (in the areas where they are consumed), becomes higher than that of storage roots (Lutaladio and Ezumah, 1981), household economies could be greatly improved (Fregene and Puonti-Kaerlas, 2002) thus reducing extreme hunger and poverty. In addition the leaves from cassava contain valuable high quality protein and could also provide a reliable low cost source of vitamins, minerals and protein (Eggum, 1970; Balagopalan et al., 1988).

3.6.3 Effect of drought stress on rates of photosynthesis (P_N; umolCO_2 m^{-2} s^{-1}) of cassava genotypes

Studying cassava under water deficit episodes or conditions and determining the genotypic variation in their rates of CO_2 uptake or rates of photosynthesis (P_N) has been previously done (Ike, 1982; El-Sharkawy and Cock, 1990; El-Sharkawy et al., 1992; Pellet and El-Sharkawy, 1994; De Tafur et al., 1997; Itani et al., 1999; El-Sharkawy, 2003, 2007; El-Sharkawy et al., 2008).

From this study, it can be concluded that P_N varied with water availability. For example a high rate of CO_2 uptake was observed in control (fully irrigated) cassava plants, with subsequent reduction in P_N of 60, 30%, and none (0%) irrigated plants respectively. Non-irrigated plants exhibited the least P_N. These results corroborated other findings. For example, while studying photosynthesis in drought-adapted cassava, Calatayud et al. (2000) observed high P_N in well irrigated plants. Water limitation however altered this P_N. El-Sharkawy (2003) also reported reduced rates of photosynthesis in attached cassava leaves under prolonged drought. The findings also substantiated earlier studies which stated P_N as one of the most affected processes under water deficit largely due sensitivity of cassava stomata to lowered water status (Setter and Fregene, 2007).

A primary response to drought stress in cassava is stomatal closure, which decreases photosynthetic CO_2 assimilation (Ike, 1982; El-Sharkawy, 1990; El-Sharkawy and Cock, 1990; De Tafur et al., 1997; Calatayud et al., 2000; Alves, 2002; El-Sharkawy, 2004, 2006; Aina et al., 2007). Despite reports that maximum well watered cassava photosynthetic rates under greenhouse conditions varying from 13 to 24 umolCO_2 m^{-2} s^{-1} (Mahon et al., 1977; Edwards et al., 1990; Alves, 2002), no particular control (fully irrigated) genotype expressed P_N within this expected range in this study.

The high P_N noted in control plants could be attributed to the ideal treatment and greenhouse conditions under which the experiment was set up. The conditions included a constant water supply (fully irrigated), a relatively high RH of 60/50% (day/night), favorable temperature (26°C), 14 hours of light and 84.7% air ventilation. High P_N in cassava has been observed under high humidity, wet soil, high leaf temperature, and high solar radiation (El-Sharkawy et al., 1992, El-Sharkawy, 1993; El-Sharkawy, 2003). The optimum or corresponding temperature for cassava photosynthesis ranges between 25°C and 35°C (El-Sharkawy and Cock, 1990; El-Sharkawy et al., 1992).

Generally genotypes 98-0002 and 98-2226 expressed relatively similar (but high) P_N than other genotypes, while P_N of genotype 95-0306 was the least. Line 529-48 expressed high P_N among transgenic genotypes and the P_N of line 529-28 and wild type TMS 60444 were relatively similar. Transgenic line 529-48 exhibited more P_N than non transgenic genotypes, while P_N of line 529-28 and wild type TMS 60444 varied with P_N of most non transgenic genotypes.

When P_N within specific treatments were analyzed, transgenic and non transgenic cassava genotypes subjected to full irrigation expressed varying rates of photosynthesis. Among non transgenic genotypes, genotype 98-2226 expressed higher rates of CO_2 uptake, followed by genotype TME-3. Rates of CO_2 uptake in genotypes 98-0002 and 91-02322 were relatively the same, while genotype 95-0306 exhibited lower rates of CO_2 uptake. Transgenic line 529-48 expressed higher P_N than both wild type TMS 60444 and transgenic line 529-28. Although the P_N between line 529-28 and wild type TMS 60444 were relatively the same, the P_N value of wild type TMS 60444 was relatively high.

Line 529-48 and genotype 98-2226 expressed relatively similar but higher P_N than all genotypes. With the exception of genotype 95-0306 (that expressed low P_N), all other non transgenic genotypes expressed higher P_N than line 529-28 and wild type TMS 60444. These observations indicated that even under well watered conditions, P_N in cassava is genotype dependent (El-Sharkawy, 2003; 2007). The P_N of cassava plants subjected to 0% irrigation were the lowest in this study. This can be attributed to a number of factors which included wilting and collapse of most of the plants by day 12 of this treatment.

Ike (1982) reported that P_N in cassava were considerably reduced before cassava leaves were wilted as a result of reduced leaf conductance under water deficits. Despite this, genotypic P_N variations were still observed under this treatment, perhaps because the drooped or folded leaves of the stressed cassava genotypes or leaves, though reduced transpirational water loss, were able to sustain or maintain a reasonable yet varying photosynthetic rates (El-Sharkawy, 2003).

For example although most of non-transgenic genotypes such as 98-2226, 91-02322, TME-3, and 95-0306 expressed relatively similar P_N, genotype 98-0002 was able to exhibit high rate of CO_2 uptake and transgenic line 529-48 also expressed higher P_N than its wild type TMS 60444 and line 529-28. Genotype 98-0002 exhibited high P_N than transgenic lines 529-28, 529-48 and their wild type TMS 60444, while non-transgenic genotypes expressed higher P_N than line 529-28.

Another factor that contributed to the observed genotypic P_N variation under 0% irrigation was variation in levels of abscission. Genotype 98-0002 and line 529-48 that expressed high P_N also expressed high levels of leaf retention (see discussion on leaf abscission). Leaf retention has been positively correlated with photosynthetic rates in cassava under drought stress (El-Sharkawy et al., 1992; Osiru et al., 1994; Lenis et al., 2006). Consistent genotypic variation in P_N was observed under 30 and 60% water deficit regimes among transgenic genotypes. For example, line 529-28 and wild type TMS 60444 consistently expressed relatively similar and lower rates of CO_2 uptake than line 529-48. Line 529-48 maintained a higher P_N under the same treatments. Similar observations were made under 0% irrigation.

Transgenes 529-28 and 529-48 had been transformed by introducing an expression construct consisting of the cytokinin synthesis gene, *ipt*, under the control of senescence associated gene promoter *SAG12* to address the problem of excessive leaf abscission in response to drought stress (Zhang and Gruissem, 2004). Tobacco plants had also been successfully transformed with the *ipt* gene to prolong photosynthetically active leaf life under drought conditions, by arresting leaf senescence (Gan and Amasino, 1995).

During the development of the transgenic lines (529-28 and 529-48), Zhang and Gruissem (2005) reported a repressed decrease in chlorophyll (improved retention of green leaf color), total protein and Rubisco contents. The high rates of CO_2 uptake of line 529-48 (than wild type TMS 60444) can perhaps be attributed to the improved chlorophyll retention (improved leaf retention) and Rubisco contents.

While Rubisco is known to have less affinity to CO_2, particularly at temperatures higher than 25°C, its repressed decrease or degradation achieved in line 529-48 under drought stress (Zhang and Gruissem, 2005), might have contributed to its high P_N. The relatively low P_N of 529-28 was in contrast to the expected results.

Zhang and Gruissem (2005) had also reported an increased expression of *ipt* hence low leaf senescence in line 529-28 under drought stress i.e. when compared, line 529-28, 529-48 and wild type TMS 60444 lost 10, 20 and 50% of leaves respectively. It was therefore expected that the previously reported high leaf retention of line 529-28 could translate to a higher P_N than both 529-48 and wild type TMS 60444. This is because leaf retention had previously been positively correlated with P_N and yield in cassava cultivars under drought stress (Fregene and Puonti-Kaerlas, 2002; Lenis et al., 2006).

The relatively low (and similar to wild type TMS 60444) rates of CO_2 uptake of line 529-28 as expressed in this study, contrasted with the expected results. However, it is noted that Zhang and Gruissem (2004; 2005) never measured the P_N during assessment of the transgenes (529-28 and 529-48) and wild type TMS 60444 under water stress.

Although the P_N values of some non-transgenic cassava genotypes not significantly differing from each other under both 30 and 60% drought stress treatments, the general trend showed genotype 98-0002 consistently expressing high P_N, followed by P_N of 98-2226, TME-3, 91-02322, and 95-0306 in a descending order respectively. Genotypes 95-0306 expressed lower P_N. All non-transgenic genotypes showed higher rates of CO_2 uptake than transgenic line 529-28 and wild type TMS 60444, while line 529-48 expressed relatively similar and high rates of CO_2 with genotypes 98-0002 and 98-2226. The observed trends in P_N variation can be attributed to various factors among them differences in levels of leaf retention.

Genotype 98-0002 expressed high leaf retention ability, while genotypes 98-2226, 91-02322 and TME-3 expressed moderately leaf retention. Genotypes 95-0306 expressed the highest leaf abscission. Level of leaf retention directly correlated with P_N (Lenis et al., 2006). Similar variations in P_N among cassava genotypes under drought stress have been reported. Cassava genotypes with high P_N have been identified in seasonally dry and semi-arid hot climates (El-Sharkawy et al., 1990; De Tafur et al., 1997; El-Sharkawy, 2006, 2008).

Other factors that might have contributed to variation in P_N among cassava genotypes in this study, include differences in total number and distribution of stomata per leaf area (Alves, 2002), total biomass (Ramanujam, 1990; El-Sharkawy, 2004), leaf area index, leaf longevity (Iglesias et al., 1995; El-Sharkawy, 1993, 2006), leaf orientation (droop or fold), leaf conductance, leaf water potential (Ike, 1982; Cock and El-Sharkawy, 1988; El-Sharkawy and Cock, 1990; El-Sharkawy, 2003, 2006, 2007) among other factors.

Stomata are the routes by which CO_2 enters the leaf and any stress induced (such as water deficit) decreases in stomatal aperture, can limit the rate of CO_2 diffusion into the leaf and hence affect rate of photosynthesis (Alves, 2002). Cassava's high stomatal sensitivity to atmospheric and edaphic water deficits have been confirmed (El-Sharkawy and Cock, 1984; El-Sharkawy et al., 1985; Cock et al., 1985; El-Sharkawy, 1993; El-Sharkawy, 2006). Itani et al., (1999) observed that even under severe water stressed cassava plants, the stomata opened sometimes slightly without resumption of photosynthesis.

With the exception of leaf retention determined under 0, 30 and 60% irrigation, most of these factors were however not measured in this study. Since clear genotypic differences in rates of CO_2 uptake under 30 and 60% drought stress regimes was discerned, it is thus recommended to utilize either of the treatments in further screening or analysis of P_N variation among cassava genotypes under greenhouse conditions. El-Sharkawy et al. (2008) recommended that in addition to the choice of cassava genotypes, there was also the need to choose relevant environmental stresses (drought stress included) so that genetic variation in photosynthetic efficiency can express and be assessed.

Selection and breeding for high photosynthetic rates in cassava cultivars has been advocated for (Lenis et al., 2006; El-Sharkawy, 2003, 2007). Therefore, the highly photosynthesizing varieties (98-0002 and 98-2226) as identified in this study should be selected and be bred for improved performance. However they should be evaluated under water stressed field conditions. The yield performance and other drought tolerant properties of transgenic line 529-28 and 529-48 are currently being assessed under water stressed field conditions in China (Zhang Peng, Personal communication, 2008).

3.6.4 Effect of drought stress on stomatal conductance (Gs; mmol m^{-2} s^{-1}) of cassava genotypes

Reviews and reports on cassava's stomatal conductance (Gs) under drought stress have been documented (Ike, 1982; De Tafur et al., 1997; Itani et al., 1999; El-Sharkawy, 2003; 2007; El-Sharkawy et al., 2008). From this study, high and low stomatal conductance was observed in control (fully irrigated) and non irrigated plants respectively, while plants subjected to 30 and 60% water stress, exhibited relatively similar Gs. These Gs variations can be directly attributed to water availability. Earlier studies on Gs in cassava under drought buttress these findings.

For example, when irrigation was withheld or soil water was depleted, transpiration rates decreased, indicating a decline in stomatal conductance (Alves and Setter, 2000). Itani et al., (1999) previously observed high stomatal conductance from leaves of control plants. When water is available, cassava maintains a high stomatal conductance, and can keep internal CO_2 concentration high; but when water becomes limiting, the plant closes the stomata in response to even small decreases in soil water potential (El-Sharkawy and Cock, 1984; Alves, 2002; Aina et al., 2007).

Stomatal conductance in cassava decreases during episodes of drought stress (De Tafur et al., 1997; Alves, 1998; Alves and Setter, 2000; Calatayud et al., 2000). Stomatal closure reduces CO_2 uptake and transpiration (hence reduced Gs) in cassava genotypes (El-Sharkawy and Cock, 1984; El-Sharkawy et al., 1984; El-Sharkawy, 1990; Itani et al., 1999; El-Sharkawy, 2007).

The resulting decline in transpiration lessens the decrease in leaf water potential and soil water depletion thereby protecting cassava leaf tissues from desiccation (Ike, 1982; Palta, 1984; El-Sharkawy and Cock, 1984; Cock et al., 1985). Generally, lines 529-28, 529-48 and wild type TMS 60444 expressed relatively similar Gs. Genotypes 98-0002, 95-0306, and TME-3 also expressed relatively similar (but higher) Gs, while Gs of genotypes 98-2226 and 91-02322 were also the same (but lower).

The Gs of transgenic lines 529-28, 529-48 and wild type TMS 60444 was more than Gs of genotypes 98-2226 and 91-02322 and relatively similar to the Gs of genotypes 98-0002, TME- and 95-0306. Similar Gs differences in cassava genotypes have also been observed in related drought stress studies (El-Sharkawy, 1993; De Tafur et al., 1997; Alves, 2002; El-Sharkawy, 2007). When specific drought stress regime was analyzed, genotypic variation in Gs was also observed. Among control plants for example, genotypes 98-0002, 95-0306 and TME-3 conducted relatively similar (but high) amount of water vapor compared to genotypes 91-02322 and 98-2226 which exhibited lower and relatively similar Gs.

Transgenic lines 529-28, 529-48 and their wild type TMS 60444 exhibited relatively similar Gs. In addition, Gs means of these transgenes and wild type were relatively similar to Gs of genotypes TME-3, 95-0306, 98-2226, higher than the Gs of genotype 91-02322 and lower than Gs of genotype 98-0002 respectively. Under 0% irrigation, genotypes TME-3, 98-2226, 98-0002, and 91-02322 conducted relatively similar (but lower) amount of water vapor compared to genotype 95-0306.

Wild type TMS 60444 expressed higher Gs compared to transgenic lines (529-28 and 529-48) and relatively similar (but high) Gs compared to genotype 95-0306. The Gs of 529-28 and 529-48 were similar. Transgenic line 529-48 also exhibited similar Gs as of genotypes 98-2226. In analysis of 30% water stress, Gs of genotypes 98-0002 and 95-0306 were high (but similar) than Gs of genotypes TME-3, 98-2226 and 91-02322, which were also similar, (but low). Although Gs of transgenes and wild type were same, observed trends showed Gs of line 529-28, 529-48 and TMS 60444 descending respectively.

Line 529-28 and wild type TMS 60444 expressed relatively similar Gs as of genotypes 95-0306 and 98-0002. When 60% water regime was analyzed, transgenes and wild type expressed relatively similar Gs. With the exception of genotype 91-02322, that showed lower Gs, all non-transgenic genotypes expressed relatively similar Gs. Transgenic lines expressed same Gs as genotypes 95-0306, 98-2226 and 98-0002.

Although no distinct cassava genotype could clearly be designated or selected in terms of high or low Gs under drought stress in this study, the finding nevertheless enhanced or conformed to earlier reports that stomatal conductance in cassava, is genotype dependent. For example, Gs of genotype 91-02322 did not significantly vary from Gs of other non-transgenic genotypes, but observed trends indicated its Gs value as relatively low across the three drought stress regimes. Similarly, the trends also showed that genotypes 98-0002 and 95-0306 conducted more water vapor. Related studies have confirmed such Gs variation in other cassava genotypes or cultivars (Porto, 1983; Calatayud et al., 2000; Okogbenin et al., 2003; El-Sharkawy, 2007).

From the findings in this study, it can be hypothesized that Gs of cassava under drought stress is influenced by a number of inherent genotypic and environmental factors and that these factors affect effective Gs measurement and hence use in the assessment of drought tolerant traits of cassava.

The factors include vapor pressure difference or deficit, leaf temperature, air velocity, leaf water potential, leaf water conductance, transpiration rates, stomatal size, distribution, opening or closure, and leaf area expansion. Cassava stomata close in large leaf-air to vapor pressure differences, its leaf conductance decreases rapidly with increasing vapor pressure deficit or varies widely with time of the day and transpiration rates decreases over the same range of vapor pressure differences without change in bulk leaf water potential, and the rates of water loss measured as a function of leaf temperature (Ike, 1982; El-Sharkawy, 1990; El-Sharkawy and Cock, 1990; Alves, 2002; Alves and Setter, 2004b).

High transpiration rates have been associated with high stomatal conductance in cassava (Itani et al., 1999). Leaf area expansion or growth in cassava decreases upon imposition of water deficit (Connor et al., 1981; Palta, 1984; El-Sharkawy and Cock, 1987; Baker et al., 1989). This limits expansion and development of transpirational surface area during water deficit (Alves, 2002; Alves and Setter, 2004b) and thus affecting stomatal conductance. In addition, reviews have shown that under greenhouse conditions, the stomata may close or open depending on the cultivar or wind velocity (Dixon and Grace, 1984; El-Sharkawy, 1990).

Cassava possess a tight stomatal control over leaf gas exchange that reduces water losses when the plants are subjected to soil water deficits as well as to high atmospheric evaporative demands (El-Sharkawy, 2007). Transpiration is substantially reduced (in response to mild drought) as do other species that act to retain water during drought episodes (El Sharkawy et al., 1984; Tardieu and Simonneau, 1998; Alves and Setter, 2000). Most of these factors affecting stomatal conductance of cassava genotypes under water deficits were not determined in this study.

However, since transgenes (529-28 and 529-48) had been transformed for enhanced leaf retention (through reduced chlorophyll degradation; Zhang and Gruissem, 2005) under drought stress, it was expected that they could either conduct more water vapor, due to enhanced stomata life (i.e. sustained stomatal opening before closure), or lower Gs as a result of increased stomatal sensitivity to drought stress and decreased leaf water potential. The results were however in contrast as both transgenic lines expressed relatively similar Gs as their wild type TMS 60444.

The findings could also not be fully explained as Gs response to drought stress was not determined during screening of the transgenes (Zhang and Gruissem, 2004, 2005). Evidently, stomatal conductance cannot be effectively used or entirely relied on to characterize staygreen or drought tolerance in cassava submitted to drought stress under greenhouse conditions. No significant differences in Gs were also observed in most cassava cultivars under either rain-fed or well watered (El-Sharkawy, 2003).

Despite this, cassava varieties that conducted relatively low amount of vapor across drought stress treatments (i.e. 91-02322, TME-3, and 98-2226) as identified in this study should warrant for further studies especially under field conditions. Genotypes expressing high water use efficiency (i.e. high ratio of leaf CO_2 uptake per amount of water conducted or transpired) under drought stress conditions should be bred for the arid and semi-arid conditions under which substantial expansion cultivation of cassava has recently occurred.

3.6.5 Correlations between photosynthetic rates, stomatal conductance and leaf abscission

The positive correlation between photosynthetic rates and stomatal conductance, the negative correlation between photosynthetic rates and leaf abscission as well as the negative correlation between leaf abscission and stomatal conductance as observed in this study have also been previously reported (Calatayud et al., 2000; El-Sharkawy, 2003, 2007). Obviously, low leaf abscission (or high leaf retention) under drought stress implies that the retained leaves maintains both their stomatal conductance and photosynthetic activities. In this study, the genotypic variations in these correlations were also observed.

For example, among transgenic genotypes, line 529-48 generally dropped fewer leaves and expressed high photosynthetic rates when compared to line 529-28 and wild type TMS 60444. Stomatal conductance between 529-28, 529-48 and TMS 60444 were relatively similar. In non-transgenic plants, genotype 98-0002 abscised fewer leaves, conducted more amount of water vapor and exhibited higher photosynthetic rates compared to other non transgenic genotypes.

Genotype 95-0306 expressed high leaf abscission, high stomatal conductance and low photosynthetic rates. Similarly genotype 98-2226 expressed relatively low leaf abscission, conducted low amount of water vapor, and high photosynthetic rates. The contrast in the positive correlation between stomatal conductance and photosynthetic rates as observed in the two genotypes (95-0306 and 98-2226) in this study could not be explained.

Cassava genotypic variations in correlation of the above parameters as reported in this study have also been noted. For example, Cock et al., (1979) and Cock, (1984) reported varietal differences in leaf longevity or retention and also indicated that the retained leaves maintain high potential rates of photosynthesis until abscission.

In addition, Calatayud et al., (2000) reported that although limited water supply resulted in a pronounced reduction in shoot growth and high leaf fall, the water status of remaining attached leaves was not affected, thus sustaining a relatively stable photosynthesis. A strong correlation between photosynthetic rates and relative humidity has been reported (El-Sharkawy, 2003; 2007). For example, a decrease in stomatal conductance strongly limited rates of photosynthesis at lower humid conditions (Grantz, 1990; Dai et al., 1992; Ohsumi et al., 2008).

3.7 General Discussion, Conclusions and Recommendations

3.7.1 General discussion

This study investigated and revealed that generally drought stress treatment under greenhouse conditions generated significant variations in rates of CO_2 uptake (photosynthesis), amount of water vapor conducted (stomatal conductance), leaf abscission (leaf retention) and elongation of last internodes (internodal growth) among cassava genotypes.

With regard to specific drought stress regime, results in this study showed that control (fully irrigated) plants expressed significantly higher values of the above measured parameters compared to performance of plants submitted to 0, 30 and 60% water stress. Cassava plants submitted to 0% irrigation expressed the least values of photosynthesis, stomatal conductance, leaf retention and internodal elongation, while the values of the same parameters in plants under 30 and 60% irrigation were also significantly different.

Cassava plants expresses high and reduced stomatal conductance, rates of photosynthesis, leaf retention as well as high shoot growth under unstressed and stressed conditions respectively (El-Sharkawy and Cock, 1987; El-Sharkawy et al., 1992; El-Sharkawy, 2003, 2006, 2007, 2008; Lenis et al., 2006). The genotypic differences in terms of photosynthetic rates, stomatal conductance, leaf abscission, and internodal growth, were clearly observed or differentiated in control, 30, and 60% drought stress treatments. Under these three treatments, high performing genotypes were 98-0002, 98-2226 and line 529-48, the low responsive genotypes were 95-0306, line 529-28 and TMS 60444, while genotypes 91-02322 and TME-3 were considered moderately responsive.

When total irrigation was withheld, most of these cassava genotypes expressed relatively similar values of the measured parameters. Therefore no clear distinction could be concluded as to the high, low, or moderate performing genotype under 0% irrigation.

Cassava genotypes expressed significantly varied values of photosynthetic rates, leaf abscission, and internodal growth clearly showing the most and less likely to be adapted to drought stress. However, no significant genotypic variation was recorded in stomatal conductance. This observation has also been reported by (El-Sharkawy, 2003). This makes stomatal conductance a less suitable parameter to be used in the analysis of drought tolerance among cassava genotypes.

Based on correlation matrices of the studied parameters, drought stress ultimately affects leaf retention, rates of photosynthesis, and stomatal conductance. Similar correlations were observed in these parameters before (Calatayud et al., 2000; El-Sharkawy, 2003, 2007). The response of these parameters are intertwined i.e. a reduction in the number of leaves (due to leaf fall), causes a decline in photosynthetic rates, and stomatal conductance and vice versa. These correlations or responses can be attributed to the high sensitivity of cassava stomata to both atmospheric and edaphic drought stress (El-Sharkawy, 2003; 2007; El-Sharkawy et al., 2008).

A general decline in photosynthetic rates and stomatal conductance with extended period of drought stress in all cassava genotypes were observed. Similar declining curves in photosynthetic rates and stomatal conductance have also been reported (El-Sharkawy et al., 1992; Itani et al., 1999; El-Sharkawy, 2003; 2007).

Leaf abscission versus days of drought stress curve was bell shaped. The leaf abscission response curve as shown in this study, have also been confirmed by El-Sharkawy and Cock (1987), but contrasted by the cumulative curves of leaf fall or shoot growth as reported by El-Sharkawy and colleagues (1992).

3.7.2 Conclusion

The study revealed that the application of 30, 60, and 100% (control) drought stress regimes, were successful in distinguishing cassava genotypic variations in terms of their photosynthetic rates, leaf abscission, and internode growth. Non irrigated drought stress regime or totally withholding irrigation produced no significant genotypic differences. Therefore, the three (30, 60 and 100%) drought stress treatments are suitable for application in the analysis of staygreen trait in cassava. Stomatal conductance is not a favorable parameter to characterize a staygreen phenotype.

The general cassava genotypic performance was ranked in terms of their variations in leaf abscission, rates of photosynthesis, stomatal conductance and internode growth responses to drought stress treatment. For example leaf abscissions in non-transgenic genotypes were ranked as follows (i.e. from low to high leaf abscission): 98-0002, 98-2226, 91-02322, TME-3 and 95-0306. Similarly, among transgenic genotypes, leaf abscission was ranked such that line 529-48, 529-28 and wild type TMS 60444 showed low to high abscission respectively.

Non-transgenic genotypes 98-0002, 98-2226, 91-02322, TME-3 and 95-0306 expressed their rates of photosynthesis in descending trend respectively, while photosynthetic rates in transgenic lines 529-48, wild type TMS 604444, and line 529-28 also reduced respectively. The ranking of stomatal conductance was based on observed relative trends as no significant variations were observed among the genotypes.

Stomatal conductance (from low to high trends) in non-transgenic genotypes 95-03036, 98-0002, TME-3, 98-2226 and 91-02322, while in transgenic plants, wild type TMS 60444, line 528-28 and 529-48 conducted high to low amount of water vapor respectively. High to low internode growth or elongation was expressed by non transgenic genotypes 98-2226, TME-3, 98-0002, 91-02322, and 95-006 respectively. Transgenic line 529-48 expressed higher internode increment, followed by wild type TMS 60444, while internode of transgenic line 529-28 increased the least.

Based on these rankings, tentative levels of drought stress tolerance or staygreen trait under greenhouse conditions can be assigned. For example genotypes 98-0002 and 98-2226 were regarded as highly staygreen, genotypes 91-02322 and TME-3 considered moderately staygreen and low level of staygreen assigned to genotype 95-0306. Transgenic line 529-48 was tentatively regarded as highly staygreen than line 529-28 and wild type TMS 60444. Wild type TMS 60444 was considered drought stress susceptible.

3.7.3 Recommendations

Based on the general discussion and conclusions, the following recommendations can be made:

(1) The three (30%, 60% and well watered) levels of drought stress treatments are recommended for application in the analysis of staygreen or drought tolerance in cassava genotypes under greenhouse conditions. Application of 0% irrigation or totally withholding water supply is not recommended for use as most plants wilted and 'collapsed' in the course of the experiment (as observed in this study).

(2) Photosynthetic rates, leaf abscission and internode growth are among other parameters that can be suitably measured and used to characterize drought tolerance or levels of staygreen among cassava genotypes under greenhouse and field conditions.

(3) Although stomatal conductance has been considered not a suitable parameter to measure and use to distinguish levels of drought tolerance in this study, it can be recommended that other associated factors that affect its response to drought stress should also be determined before a holistic conclusion can be made. Some of these factors include: leaf water potential, leaf conductance, vapor pressure deficits and rates of transpiration.

(4) Field trial on the tentatively high staygreen genotypes 98-0002, 98-2226 and line 529-48, moderate staygreen genotypes 91-02322, TME-3 and line 529-28 and drought susceptible genotypes 95-0306 and TMS 60444 should be made to assess their yielding abilities under field-droughted conditions. The genotypes also warrant further investigations as source materials for breeding cultivars for drought prone areas.

(5) In addition to photosynthetic rates, stomatal conductance, leaf abscission and internode growth, it can be recommended that other physiological and morphological characteristics of cassava that are affected by drought and therefore can also be used to characterize staygreen trait, should be also be analyzed. This is because drought tolerance is a complex trait that cannot be defined by a few factors. Recommended factors for further analysis under drought stress includes leaf water conductance, leaf water potential, stomatal activity, leaf area index, expansion, and production, root lengths and sink strength.

(6) It is recommended that effect of prolonged or shortened drought stress period be further experimented on, especially for the analysis of stomatal conductance and photosynthetic rates. El-Sharkawy et al., (1992) studied and recorded significant response of stomatal conductance and photosynthetic rates with 105 days of water stress, while Itani et al., (1999) reported reducing rates of stomatal conductance between 6.00 and 18.00 (12 hours) of a day. These showed wide variations and high sensitivity of stomata to water stress. This will also enhance an understanding on the maximum drought period beyond which cassava can sustain growth and produce reasonable yield.

(7) The results on analysis of staygreen trait in transgenic and non-transgenic cassava genotypes in this study are greenhouse based. It is however recommended that the analysis should be replicated under field conditions.

3.7.4 References (Chapter three)

Aina, O.O., Dixon, A.G.O., and Airline, E.A., 2007: Effect of soil moisture stress on growth and yield of cassava in Nigeria. Pakistan Journal of Biological Sciences, Vol. 10 (18), pp. 3085-3090.

Alves, A.A.C., and Setter, T.L., 2004a: Abscisic acid accumulation and osmotic adjustment in cassava under water deficit. Environmental and Experimental Botany, Vol. 51, pp. 259-271.

Alves, A.A.C., and Setter, T.L., 2004b: Response of Cassava Leaf Area Expansion to Water Deficit: Cell Proliferation, Cell Expansion and Delayed Development. Annals of Botany, Vol. 94, pp. 605–613.

Alves, A.A.C., 2002: Cassava botany and physiology. In: Hillocks, R.J., Thresh, J.M. and Bellotti, A.C. (Eds.). Cassava: Biology, production and utilization, CABI Publishing (2002), pp. 67-89.

Alves, A.A.C., and Setter, T.L., 2000: Response of Cassava to Water Deficit: Leaf Area Growth and Abscisic Acid. Crop Science, Vol. 40, pp. 131–137.

Alves, A.A.C., 1998: Physiological and developmental changes in cassava (*Manihot esculenta* Crantz) under water deficit. PhD thesis, Cornell University, Ithaca, New York, 160 pp.

Aresta, R.B., and Fukai, S., 1984: Effects of solar radiation on growth of cassava (*Manihot esculenta* Crantz). II. Fibrous root length. Field crops research, Vol.9, pp. 361-371.

Baker, G.R., Fukai, S., and Wilson, G.L., 1989: The response of cassava to water deficits at various stages of growth in the sub-tropics. Australian Journal of Agricultural Research, Vol.40, pp. 517-528.

Baker, G.R., Fukai, S., and Wilson, G.L., 1989: The response of cassava to water deficits at various stages of growth in the sub-tropics. Australian Journal of Agricultural Research, Vol.40, pp. 517-528.

Balagopalan, C., Padmaja, G., Nanda, S.K., and Moorthy, S.N., 1988: Cassava in food, feed, and industry. CRC Press, Inc., Boca Raton, Florida.

Calatayud, P.A, Llovera, E., Bois, J.F, and Lamaze, T., 2000: Photosynthesis in drought-adapted cassava. Photosynthetica, Vol.38, pp.97-104.

Cock, J.H., and El-Sharkawy, M.A., 1988: Physiological characteristics for cassava selection. Experimental agriculture, Vol.24 (4), pp. 443-448.

Cock, J.H., Porto, M.C.M., and El-Sharkawy, M.A., 1985: Water use efficiency of cassava. III. Influence of air humidity and drought stress on gas exchange of field grown cassava. Crop sciences, Vol. 25, pp. 265-272.

Cock, J.H., 1984: Cassava. In: P.R. Goldsworthy and N.M. Fisher (Eds.). The Physiology of Tropical Field Crops, Wiley, New York, pp. 529-549.

Cock, J.H., Franklin, D., Sandoval, G., and Juri, P., 1979: The ideal cassava plant for maximum yield. Crop Sciences, Vol.19, pp. 271 - 279.

Connor, D.J., and Cock, J.H., 1981: Response of cassava to water shortage. II. Canopy Dynamics. Field Crops Research, Vol.4, pp. 285-296.

Connor, D.J., Cock, J.H., and Parra, G.E., 1981: Response of cassava to water shortage. (Growth and Yield). Field Crops Research, Vol. 4, pp. 181-200.

Dai, Z., Edwards, G.E., and Ku, M.S.B., 1992: Control of photosynthesis in and stomatal conductance in *Ricinus communis* L. (castor bean) by leaf to air pressure deficit. Plant physiology, Vol. 99, pp. 1426-1434.

De Tafur, S.M., El-Sharkawy, M.A., and Calle, F., 1997: Photosynthesis and yield performance of cassava in seasonally dry and semi-arid environments. Photosynthetica, Vol. 33, pp. 249-257.

Dixon, M., and Grace, J., 1984: Effect of wind on the transpiration of young trees. Annals of Botany, Vol. 83, pp. 811-819.

Edwards, G.E., Sheta, E., Moore, B.D., Dai, Z., Franceschi, V.R., Cheng, S.H., Lin, C.H., and Ku, M.S.B., 1990: Photosynthetic characteristics of cassava (*Manihot esculenta* Crantz), a C_3 species with chlorenchymatous bundle sheath cells. Plant and Cell physiology, Vol. 31, pp. 1199-1206.

Eggum, O.L., 1970: The protein quality of cassava leaves. British Journal of Nutrition, Vol. 24, pp. 761-769.

El-Sharkawy, M.A., Lopez, Y., and Bernal, L.M., 2008: Genotypic variations in activities of *phosphoenolpyruvate carboxylase* and correlations with leaf photosynthetic characteristics and crop productivity of cassava grown in low-land seasonally-dry tropics. Photosynthetica, Vol. 46 (2), pp. 238-247.

El-Sharkawy, M.A., 2007: Physiological characteristics of cassava tolerance to prolonged drought in the tropics: Implications for breeding cultivars adapted to seasonally dry and semiarid environments. Plant Physiology, Vol. 19 pp. 257-286.

El-Sharkawy M.A, 2006: International research on cassava photosynthesis, productivity, eco-physiology, and responses to environmental stresses in the tropics. Photosynthetica, Vol. 44 (4) pp. 481-512.

El-Sharkawy, M.A., 2005: How can calibrated research-based models be improved or use as a tool in identifying genes controlling crop tolerance to environmental stresses in the era of genomics from an experimentalists's perspective. Photosytnthetica, Vol. 43, pp. 161-176.

El-Sharkawy, M.A., 2004: Cassava biology and physiology. Plant Molecular Biology, Vol. 56 (4), pp. 481-501.

El-Sharkawy M.A, 2003: Cassava biology and physiology. Plant Molecular Biology, Vol.53, pp.621-641.

El-Sharkawy, M.A., and Cadavid, L.F., 2002: Response of cassava to prolonged drought stress imposed at different stages of growth. Experimental Agriculture, Vol. 38, pp. 333-350.

El-Sharkawy, M.A., Cadavid, L.F., De Tafur, S.M., and Caicedo, J.A., 1998: Genotypic differences in productivity and nutrient uptake and use efficiency of cassava as influenced by prolonged water stress. Acta Agron. Univ. National-Palmira-Colombia, Vol. 48, pp. 9-22.

El-Sharkawy, M.A, 1993: Drought tolerant cassava for Africa, Asia and Latin America. Biosciences, Vol. 43, pp. 441-451.

El-Sharkawy, M.A., Hernandez, A.D.P., and Hershey, C., 1992: Yield stability of cassava during prolonged mid-season water stress. Experimental Agriculture, Vol. 28, pp. 165-174.

El-Sharkawy, M.A. and Cock, J.H. 1990: Photosynthesis of cassava *(Manihot Esculenta* Crantz*)*. Experimental Agriculture, Vol. 26, pp. 325-340.

El-Sharkawy, M.A., 1990: Effect of humidity and wind on leaf conductance of field grown cassava. Brazilian Journal of physiology and vegetation, Vol. 2 (2), pp. 17-22.

El-Sharkawy, M.A. and Cock, J.H. 1987: Response of cassava to water stress. Plants and Soils, Vol. 100, pp. 345-360.

El-Sharkawy, M.A., Cock, J.H., and Hernandez, A.D.P., 1985: Stomatal response to air humidity and its relation to stomatal density in a wide range of warm climate species. Photosynthetic research, Vol. 7, pp. 137-149.

El-Sharkawy M.A and Cock J.H, 1984: Water use efficiency of cassava. Effects of air humidity and drought stress on stomatal conductance and gas exchange. Crop Sciences, Vol.24, pp. 497-502.

El-Sharkawy, M.A., Cock, J.H., Held, K.A., 1984: Water use efficiency of cassava (*Manihot esculenta*) and differing sensitivity of stomata to air humidity in cassava and other warm climate species. Crop Sciences, Vol. 24, pp. 503–507.

Fregene, M., and Puonti-Kaerlas, J., 2002: Cassava biotechnology. In Cassava: Biology, Production and Utilization (Eds Hillocks, R.J., Thresh, J.M., and Bellotti, A. C), pp. 179-208.

Gan, S., and Amasino, R.M., 1995: Inhibition of leaf senescence by auto-regulated production of cytokinin. Science, Vol. 270, pp. 1986-1988.

Grantz, D.A., 1990: Plant response to humidity. Plant Cell Environment, Vol. 13, pp. 667-679.

Iglesias, C., Bonierbale, M., El-Sharkawy, M., Lozano, L., Bellotti, A., and Wheatley, C., 1995: Focusing basic research for cassava varietal improvement. In: Howeler RH (Eds), Cassava Breeding, Agronomy Research and Technology Transfer in Asia, pp.40-60.

Iglesias, C., Bonierbale, M., El-Sharkawy, M., Lozano, L., Bellotti, A., and Wheatley, C., 1995: Focusing basic research for cassava varietal improvement. In: Howeler RH (Eds), Cassava Breeding, Agronomy Research and Technology Transfer in Asia, pp.40-60.

Itani, J., Oda, T., and Numao, T., 1999: Studies on mechanisms of dehydration postponement in cassava leaves under short-term soil water deficits. Plant Production Science, Vol. 2, pp.184-189.

Ike, I.F., 1982: Effect of water deficits on transpiration, photosynthesis and leaf conductance in cassava. Physiologia Plantarum, Vol. 55, pp. 411-414.

Ike, F., and Thurtell, G.W., 1981: Response of indoor-grown cassava water deficit and recovery of leaf water potential and stomatal activity after water stress. Journal of experimental botany, Vol. 32 (5), pp. 1029-1034.

Irikura, Y., Cock, J.H., and Kawano, K., 1979: The physiological basis of genotype-temperature interactions in cassava. Field Crop Research, Vol. 2, pp. 227-239.

Janick, J., 2003: Horticultural reviews, Google books, page 371.

Lenis, I.J., Calle, F., Jaramillo, G., Perez, J.C., Ceballos, H., and Cock, J.H., 2006: Leaf retention and cassava productivity. Field Crops Research, Vol. 95, pp. 126–134.

Lutaladio, N.B., and Ezumah, H.C., 1981: Cassava leaf harvesting in Zaire (Democratic republic of Congo). In: Terry, E., Oduro, K., and Caveness, F., (Eds) Tropical root crops: Research strategies for the 1980s. IITA, Ibadan, Nigeria, pp. 134-136.

Mahon, J.D., Lowe, S.B., and Hunt, L.A., 1977: Variation in the rate of photosynthetic CO_2 uptake in cassava cultivars and related species of *Manihot*. Photosynthetica, Vol. 11, pp. 131-138

Oguntunde, G.P., 2005: Whole-plant water use and canopy conductance of cassava under limited available soil water and varying evaporative demand. Plant and soil, Vol. 278, pp.371-383.

Ohsumi, A., Hamasaki, A., Nakagawa, H., Homma, K., Horie, T., and Shiraiwa, T., 2008: Response of leaf photosynthesis to vapor pressure difference in rice (*Oryza sativa* L) varieties in relation to stomatal and leaf internal conductance. Plant Production Sciences, Vol. 11 (2), pp. 184-191.

Okogbenin, E., Ekanayake, I.J and Porto, C.M., 2003: Genotypic variability in adaptation responses of selected clones of cassava to drought stress in the Sudan savanna zone of Nigeria. Agronomy and Crop Science, Vol. 189, pp. 376-389.

Osiru, D.S.O., Hahn, S.K., and Osonubi, O., 1994: Mechanisms of drought tolerance in cassava. Journal of crop sciences, Vol. 2, pp. 233-246.

Palta, J. A., 1984: Influence of water deficits on gas-exchange and the leaf area development of cassava (Manihot esculenta Crantz) cultivars, Journal of Experimental Botany, Vol. 35, pp. 1441–1449.

Pardales, J.R., and Esquibel, C.B., 1996: Effect of drought during the establishment period on the root system development of cassava. Journal of Crop Sciences, Vol. 65, pp.93-97.

Payne, R., Murray, D., Harding, S., Baird, D., Soutar, D., and Lane, P., 2007: Genstat® for Windows (3rd Edition). VSN International, Oxford, UK. 342 pp. ISBN-1-904375-08-1

Payne, R., Murray, D., Harding, S., Baird, D., Soutar, D., and Lane, P., 2007: Genstat® for Windows (3rd Edition). VSN International, Oxford, UK. 342 pp. ISBN-1-904375-08-1

Pellet, D., and EL-Sharkawy, M.A., 1997: Cassava varietal response to fertilization: Growth dynamics and implications for cropping sustainability. Experimental Agriculture, Vol.33, pp. 53-365.

Pellet, D., and El-Sharkawy, M.A., 1994: Sink-source relations in cassava: Effects of reciprocal grafting on yield and leaf photosynthesis. Experimental Agriculture, Vol. 30, pp. 359-367.

Porto, M.C.M. 1983: Physiological mechanisms of drought tolerance in cassava (*Manihot esculenta* Crantz). PhD thesis, University of Arizona, USA.

Ramanujam, T., 1990: Effect of moisture stress on photosynthesis and productivity of cassava. Photosynthetica, Vol. 24, pp. 217-224

Rosas, C., J. H. Cock, and G. Sandoval, 1976: Leaf fall in cassava Experimental agriculture, Vol. 12, pp. 395-400.

Setter, T.L., and Fregene, M.A., 2007: Recent advances in molecular breeding of cassava for improved drought stress tolerance. In: M.A. Jenks et al. (eds.), Advances in molecular breeding toward drought and salt tolerant crops, pp. 701–711.

Tan, S.I., and Cock, J.H., 1979: Branching habit as a yield determinant in cassava. Field crops research, Vol. 2, pp. 281-289.

Tardieu, F., and Simonneau, T., 1998: Variability among species of stomatal control under fluctuating soil water status and evaporative demand: modeling isohydric and an-isohydric behaviors. Experimental Botany, Vol. 49, pp. 419-432.

Zeigler, R.S., Powell, L.E., and Thurston, H.D., 1980: Gibberellin A4 production by *Sphaceloma manihoticola,* causal agent of cassava super elongation disease. Plant Physiology and Biochemistry, Vol. 70, (7), pp. 589-593.

Yao, N.R., Goue, B., Zeller, B., and Monteny, B., 1988: Effect of drought on leaf development and dry matter production of the cassava plant. Tropical Agriculture, Vol.65, pp.84-88.

Zhang, P., and Gruissem, W., 2005: Leaf senescence-inducible expression of isopentenyl transferase in cassava rendering it resistant to drought stress. Deutscher Tropentag, Abstract, p.1

Zhang, P., and Gruissem, W., 2004: Leaf Senescence-Inducible Expression of Isopentenyl Transferase in Cassava Rendering it Resistant to Drought Stress. In Sixth International Scientific Meeting of the Cassava Biotechnology Network. CIAT/CBN, Cali, Colombia (2004), pp. 99.

3.7.5 List of Appendices

Appendix 1: Analysis of variance (ANOVA) of internode growth or elongation of cassava genotypes subjected to drought stress treatment under greenhouse conditions.

Source of variation	d.f (m.v)	s.s	m.s	v.r	Cov.ef.	F pr.
Genotype	7	11.12816	1.58974	20.51	0.94	<.001
Treat	3	76.47105	25.49035	328.84	0.96	<.001
Genotype. Treat	21	3.32245	0.15821	2.04	1.00	0.003
Covariate	1	0.02772	0.02772	0.36		0.550
Residual	3134 (1)	242.93693	0.07752		1.00	
Total	3166 (1)	346.32310				

Appendix 2: Analysis of variance (ANOVA) of total number of leaves abscised (LA) in cassava genotypes subjected to drought stress treatment under greenhouse conditions.

Source of variation	d.f	s.s	m.s	v.r	Cov.ef.	F pr.
Genotype	7	81.947	11.707	10.75	0.94	<.001
Treat	3	1322.950	440.983	440.88	0.98	<.001
Genotype. Treat	21	30.898	1.471	1.35	0.99	0.131
Covariate	1	6.595	6.595	6.06		0.014
Residual	3135	3414.556	1.089		1.00	
Total	3167	4850.864				

Appendix 3: Analysis of variance (ANOVA) of rates of photosynthesis of cassava genotypes subjected to drought stress treatments under greenhouse conditions.

ource of variation	d.f	s.s	m.s	v.r	F pr.
enotype	7	988.387	141.198	14.38	<.001
reat	3	7031.198	2343.733	238.77	<.001
enotype. Treat	21	333.278	15.870	1.62	0.037
esidual	3136	30782.799	9.816		
otal	3167	39135.662			

Appendix 4: Analysis of variance (ANOVA) of Stomatal conductance of cassava genotypes subjected to drought stress treatment under greenhouse conditions

ource of variation	d.f	s.s	m.s	v.r	F pr.
enotype	7	0.032331	0.004619	2.29	0.025
reat	3	0.258898	0.086299	42.87	<.001
enotype. Treat	21	0.035137	0.001673	0.83	0.683
esidual	3136	6.312851	0.002013		
otal	3167	6.639217			

CHAPTER FOUR

Optimization of an efficient protocol (s) to purify RNA from cassava leaves

Abstract

RNA integrity, quality and quantity are critical for plant molecular studies such as microarray-based gene expression profiling. Extracting high quality RNA from cassava (leaves) and other recalcitrant plant tissues is difficult due to the presence of polysaccharides, polyphenolics and other secondary metabolites that often co-precipitate with the final RNA extract.

Therefore to optimize a CTAB-based method to suit RNA extraction from cassava leaves, modifications were introduced to a version of CTAB protocol as described by Gasic et al., (2004). Rates of EB per 1g ground tissue were increased, Tissue-EB and Chl: Iaa (24:1) mixture was incubated at a lower (water-bath) temperature. Centrifugation steps were carried out at 4°C, high concentration of PVP-K-30 (soluble) and NaCl were used, sodium acetate pH was lowered, Ethyl alcohol concentration was raised and a two-step high molarity LiCl precipitation was applied.

The modified CTAB protocol was then compared with four other different RNA extraction methods (obtained from literature). The modified CTAB method produced RNA of high concentration (more than 1 microgram), high quality (A_{260}:A_{280} and A_{260}:A_{230} ratios more than 2.0) and high integrity (distinct and visible 28S and 18S rRNA bands) from young and old cassava leaves, compared to RNA (from the same leaf tissues) generated by the other four methods.

Therefore the objective of the study was achieved as shown by the modifications on the CTAB-based protocol that allowed purification of total RNA of high quality, integrity and in sufficient concentrations.

4.1 Abbreviations

EB: extraction buffer; CTAB: cetyl-trimethyl-ammonium-bromide; β-ME: Beta-mercaptoethanol; Chl: Iaa: chloroform-isoamylalcohol; PVP: polyvinylpyrrolidone; NaCl: sodium chloride; LiCl: lithium chloride; HCl: hydrochloric acid; EDTA: ethylene-diamine-tetra-acetic acid; SDS: sodium dodecyl sulfate; DEPC: diethyl-pyrocarbonate; RNA: ribonucleic acid; rRNA: ribosomal RNA; DNA: deoxyribonucleic acid; cDNA: complementary DNA; RT-PCR: real-time polymerase chain reaction;; RT: room temperature; °C; degree celcius; rpm: revolution per minute; H_2O: water; ml: millitres; µl: micro-litre; M: molarity; mM: millimoles; ng; nanogram; µg: microgram.

4.2 Introduction

RNA extraction is an essential step for most molecular techniques, including cDNA library construction, northern blot analysis, gene isolation with RT-PCR, hybridization and gene expression profiling (Gehrig et al., 2000; Azevedo et al., 2003; Carra et al., 2007; Li et al., 2006; Luoime et al., 2008; Gambino et al., 2008). Extracting a high-quality RNA and consequently preserving the transcriptome are essential for obtaining accurate information about the level of gene expression (Rosic and Hoegh-Guldberg, 2009).

A successful RNA isolation procedure is often assessed by the quantity, quality and integrity of the purified RNA (Suzuki et al., 2003). RNA quality is determined by means of spectrophotometric ratios, A_{260}:A_{280} and A_{260}:A_{230}, that correlate with level of protein, polysaccharides and polyphenol contamination respectively (Iandolino et al., 2004). High quality RNA should have an A_{260}:A_{280} ratio between 1.9–2.1 and an A_{260}:A_{230} ratio of 1.8–2.3 (Sambrook et al., 1989). The verification of RNA integrity is regularly performed using Agarose gel electrophoresis and the appearance of 18S and 28S ribosomal RNA (rRNA) bands (Sambrook et al. 1989; Fleige and Pfaffl, 2006).

Several RNA extraction methods have been successfully applied to various plant species (Logemann et al., 1987; Hughes and Galau, 1988). However, isolating suitable RNA remains problematic especially from recalcitrant plant species or tissues with high levels of proteins, phenolic compounds and/or polysaccharides (Baker et al., 1990; Schneiderrbauer et al., 1991; Gehrig et al., 2000). RNA yields from such plant species (based on method used) are often of poor quality and too low for further downstream application (Gehrig et al., 2000; Alemzadeh et al., 2005; Fleige and Pfaffl, 2006; Rosic and Hoegh-Guldberg, 2009).

The cellular components that inhibit high quality and quantity RNA isolation includes endogenous RNases, polysaccharides, polyphenols, proteins, lipids and other secondary metabolites (Azevedo et al 2003; Alemzadeh et al., 2005; Gambino et al., 2008; Vasanthaiah et al., 2008). Phenolic compounds readily oxidize to form covalently linked quinones and easily bind proteins and nucleic acids resulting in high molecular weight complexes (Azevedo et al., 2003; Loomis, 1974).

Polysaccharides tend to co-purify and co-precipitate with the RNA in the presence of alcohols or low ionic strength buffers (Levi et al., 1992; Lopez-Gomez and Gomez-Lim, 1992; Richards et al., 1994; Gehrig et al., 2000; Malnoy et al., 2001; Carra et al., 2007). Polysaccharide contamination hinder re-suspension of the precipitated RNA, interfere with absorbance-based RNA quantification, and may inhibit enzymatic manipulations, poly(A)$^+$-RNA isolation as well as electrophoretic migration (Wilkins and Smart, 1996).

Endogenous ribonucleases reduce the integrity of the RNA, particularly when their amount increases, such as during senescence, wounding, or pathogen attack (Logemann et al., 1987; López-Gómes and Gómes-Lim, 1992; Green, 1994). Homogenization triggers inevitably the mixture of RNA and endogenous RNases (Wan and Wilkins, 1994). Polysaccharides elimination by selective ethanol precipitation of the RNA in the presence of a high salt (Fang et al., 1992; Chang et al., 1993) has been successfully attempted.

Phenolic compounds have been rendered ineffective by the use of antioxidants, high ionic-strength extraction buffers in the alkaline pH range, or in reagents such as diethyldithiocarbamic acid and polyvinylpyrrolidone (Loomis, 1974; Kirk and Kirk, 1985; Hughes and Galau, 1988). Inclusion of spermidine, a polyamine, to extraction buffers may also deter co-isolation of nucleic acids, polysaccharides and phenolics (Chang et al., 1993), while chaotropic agents such as *guanidium isothiocyanate* (Chirgwin et al., 1979) have been widely employed to inhibit RNase activity (Valenzuela-Avendaño et al. 2005).

A strong protein denaturant or proteinase K (Hall et al., 1978) is used to degrade endogenous enzymes such as RNases during homogenization hence preventing RNA degradation. It is however cautionary to note that, if not carefully isolated and discarded, these contaminants not only affect the yield and quality of the final RNA extract (Wang et al., 2007), but they also interfere with downstream applications (Alemzadeh et al., 2005; Salzmann et al., 1999) such as cDNA synthesis or hybridization (Rubio-Pina and Vàzquez-Flota, 2008).

The above mentioned contaminants can occur at various concentrations depending on the plant species and organs that are considered for nucleic acid extraction. Although extraction protocols can be modified to suit particular plant species or tissues (Gruffat, 1998), in order to obtain RNA sample with a quality that is sufficient for downstream applications, the optimization process is often tedious, time consuming and may often require a lot of starting material (Luoime et al., 2008).

Geuna et al., (1998) reported that extracting RNA from plant tissues that are characterized by highly variable composition may prove especially difficult. Cassava is no exception since it is rich in polysaccharides, phenolic compounds, and other secondary metabolites. In addition, an experiment that involves isolation of RNA from stressed tissues can increase ribonucleases activity that degrades RNA (Logemann et al., 1987; Green, 1994). The high amounts of secondary compounds in cassava plants are expected to pose a serious challenge in isolating RNA especially from the leaves.

It is therefore important to study, experiment and optimize various RNA isolation methods and recommend the most suitable procedure for purifying total RNA of high quality, concentration and integrity from cassava leaves that can be effectively and efficiently be applied for downstream cDNA synthesis, labeling and hybridization on cassava-leafy spurge cDNA microarray for differential gene expression analysis upon drought stress treatment.

4.3 Materials and Methods

Establishment of the model cassava genotype TMS 60444 (for RNA extraction) used in this study was carried out in a greenhouse (Lindau-Eschikon, Zürich, Switzerland) and optimization of the various RNA isolation methods was carried out at the plant biotechnology laboratory (ETH-Zentrum, Zurich, Switzerland). The RNA was purified from young (top most fully expanded) and old (at mid-stem) leaves of 4 month-old cassava model genotype TMS 60444.

The protocols tested and optimized included: Trizol Reagent based protocol (Invitrogen, Cat #. 15596-018; as developed by Chomczynski and Sacchi (1987), Total nucleic acid extraction + DNase treatment (a modification of Soni and Murray (1994) method), Reilly et al., (2001) method, RNeasy Plant Mini Kit (Qiagen, Cat. # 74903), and CTAB-based protocol adopted from original as described by Gasic et al., (2004). The quantity and quality of the purified RNA was determined by measuring the concentrations (ng/µl) of the total RNA extracted, the A_{260}:A_{280} ratio (to indicate protein contamination) and A_{260}:A_{230} ratio (for polysaccharide and phenolic compound contaminations) using a NanoDrop® ND-1000. The extractions were replicated five times.

4.3.1 TRI®$_{ZOL}$ Reagent Method

TRI®$_{ZOL}$ Reagent protocol was an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). The extraction reagent in the EB is a monophasic solution or mixture of phenol and *guanidine isothiocyanate*. Using an autoclaved and cooled mortar and a pestle, cassava leaves were homogenized in liquid Nitrogen to a fine powder.

The powdered tissues were then transferred to a 2 ml micro-centrifuge tube containing Trizol reagent (Invitrogen, Cat #. 15596-018) at rates of 1 ml Trizol per 50 to 100 mg powdered tissue. 0.2 ml chloroform was added to the Trizol and powdered tissue mixture. The entire mixture was vortexed thoroughly for 30 seconds, incubated for 5 minutes at room temperature (RT), and centrifuged at 13 000 rpm for 10 minutes The chloroform separated the solution into an aqueous phase (exclusively containing the RNA) and an organic phase.

The aqueous phase was transferred to fresh 2 ml eppendorf tubes and 0.5 ml Isopropyl alcohol (Isopropanol) was added and incubated for 30 minutes on ice to precipitate the RNA. The mixture was then centrifuged at 13 000 rpm, 4°C, for 10 minutes. The resulting supernatant was discarded and the precipitated pellet was washed with 0.5 ml 75% ethanol. Centrifugation at 13 000 rpm, 4°C, for 10 minutes was performed to recover the pellet. The ethanol was discarded and the pellet air dried. The dry pellet was then re-suspended in 188µl sterile distilled H_2O (RNase-free).

DNase treatment was carried out by adding 10µl 2X DNase Buffer (Promega, Cat. #. M198A), 1µl RNase inhibitor/RNase out (Invitrogen, Cat. #. 10777-019) and 1µl RNase-free-DNase (Promega, Cat. #. M6101) to the re-suspended pellet. The mixture was then incubated for 30 minutes at 37°C. One volume phenol: chloroform (1:1) was added to the sample in order to remove the DNase. The mixture was vortexed for about 5 seconds and centrifuged at 13 000 rpm, RT, for 5 minutes. The resultant supernatant was transferred to fresh eppendorf tubes and 1 volume chloroform added. The mixture was then vortexed for 5 seconds and centrifugation step repeated as above.

The new supernatant was transferred to fresh eppendorf tubes, 1 volume LiCl (4 M) added and the mixture incubated overnight at 4°C to precipitate the RNA. After centrifugation at 13 000 rpm, 4°C, for 10min, the resulting supernatant was discarded and precipitated RNA pellet was washed with 0.2 ml 75% ethanol. Centrifugation at 13 000 rpm, 4°C, for 10 minutes was performed to recover the RNA pellet. The ethanol was discarded, the pellet air-dried, and afterwards re-dissolved in 40µl RNase-free sterile distilled H_2O.

A sample of the RNA (1.2 µl) was then pipetted and used to assess the RNA purity and concentration by measuring the spectrophotometric absorbance (in water) at wavelengths 230, 260 and 280 nm and hence A_{260}:A_{280} and A_{260}:A_{230} ratios using spectrophotometer (NanoDrop®ND-1000, Technologies Inc.). The remainder of the samples was stored at -80°C.

4.3.2 Modified Total Nucleic Acid extraction + DNase treatment method

This method was a modified version of isolating intact DNA and RNA from plant tissues developed by Soni and Murray (1994). Extraction Buffer (EB) was constituted from 50mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0), 2% SDS, 100mM LiCl, proteinase K (10 µg/ ml) and sterile distilled H_2O. Proteinase K was added to the EB just before use. Using a scalpel, cassava leaves were cut, frozen in liquid nitrogen, and ground to fine powder with a pre-chilled mortar and pestle. 500mg of the powder was transferred to a 2.2 ml eppendorf tube containing 1.2 ml EB.

The tubes were vortexed for 5 seconds, swirled and inverted for thorough mixing. The mixture was then centrifuged at 16 100 rpm, 4°C, for 5 minutes and the resultant supernatant transferred to fresh eppendorf tubes. One volume phenol was added to the supernatant, the mixture briefly vortexed and then centrifuged 13 200 rpm, 4°C, for 6 minutes. The supernatant was transferred to fresh eppendorf tubes containing 0.5 ml Phenol: Chloroform (1:1). This mixture was briefly vortexed and centrifuged as described above. The resultant supernatant was transferred to fresh eppendorf tubes containing 0.5 ml Phenol: Chloroform: Isoamyl alcohol (25:24:1).

The mixture was vortexed for 5 seconds and centrifuged as described above. Precipitation of the total nucleic acid pellet was achieved by adding one fourth (¼) volume ammonium acetate (10 M) and 4 volumes 99% ethanol (from -20°C) to the supernatant in fresh eppendorf tubes and the mixture incubated for 5min at RT. The mixture was then centrifuged at 13 200 rpm, 4°C, for 10 minutes.

The resultant supernatant was decanted and the precipitated pellet washed with 1 ml 70% ethanol. The pellet was recovered by centrifugation at 13 200 rpm, 4°C, for 5 minutes. The ethanol was carefully drained off and the pellet vacuum dried in a SpeedVac. for 3 minutes. The total nucleic acid pellet was re-suspended in 130µl sterile distilled H_2O, followed by centrifugation at 13 200 rpm, 4°C, for 15 minutes. The dissolved nucleic acids were carefully pipetted out into fresh eppendorf tubes and the remnant pellets and other contaminants were discarded.

RNA was obtained by carrying out DNase treatment on the dissolved nucleic acids. This was achieved by adding 10µl 2X DNase Buffer (Promega, Cat. #. M198A), 1µl RNase inhibitor/RNase out (Invitrogen, Cat. #. 10777-019) and 1µl RNase-free-DNase (Promega, Cat. #. M6101) to the nucleic acids in fresh eppendorf tube. The mixture was incubated for 30 minutes at 37°C. One volume phenol: chloroform (1:1) was added to the mixture, the mixture briefly vortexed and then centrifuged at 13 000 rpm, 4°C, for 10 minutes.

The resultant supernatant was transferred to fresh eppendorf tubes. The RNA pellet was precipitated by adding 1 volume LiCl (4 M) followed by incubating the mixture at 4°C for 60 minutes. The RNA pellet was recovered by centrifuging the mixture at 13 000 rpm, 4°C, for 10 minutes. The resultant supernatant was carefully discarded and the RNA pellet was washed with 200µl 75% ethanol. After centrifugation at 13 000 rpm, 4°C, for 10min, the ethanol was carefully decanted and the RNA pellet vacuum dried in a SpeedVac, for 3 minutes. The RNA pellet was then re-dissolved in 40µl sterile distilled H_2O.

A sample of the RNA (1.2 µl) was then pipetted and used to assess the RNA purity and concentration by measuring the spectrophotometric absorbance of the sample (in water) at wavelengths 230, 260 and 280 nm and hence A_{260}:A_{280} and A_{260}:A_{230} ratios using spectrophotometer (NanoDrop®ND-1000, Technologies Inc.). The remainder of the samples was stored at -80°C.

4.3.3 RNA Extraction Method as described by Reilly et al., (2001).

The EB was composed of 100mM Tris-Hcl (pH 7.5), 20mM EDTA (pH 8.0), 100mM NaCl, 1% Sodium lauryl-sarcosine and Diethyl-pyrocarbonate-treated (DEPC) H_2O. Sodium Lauryl-sarcosine denatures cellular and nucleoprotein complexes and thus releasing the RNA. Cassava leaves were homogenized to a fine powder using a pre-chilled pestle and mortar. Aliquots of Liquid N_2 were continuously added during grinding to prevent thawing and consequent degradation by RNase in the sample.

Five hundred (500) mg of the homogenized tissue were half filled into 2 ml eppendorf tubes using a pre-chilled spatula, 1 ml of EB was added and the mixture vortexed vigorously. An equal volume of phenol was added and the mixture centrifuged at 12,000 rpm, 4°C, for 5 minutes. The aqueous phase was transferred to fresh eppendorf tubes, an equal volume of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) added, vortexed and centrifuged as described above. The upper phase was transferred to fresh eppendorf tubes, an equal volume of chloroform added the mixture vortexed and centrifuged as described above.

The upper phase was transferred to fresh eppendorf tubes. The RNA was precipitated by adding 1 volume of pre-chilled isopropanol followed by incubation at -70°C for 15 minutes. The RNA pellet was recovered by centrifuging the mixture at 12 000 rpm, 4°C, for 5 minutes. The resultant supernatant was decanted off and the RNA pellet re-suspended in 500µl DEPC treated H_2O. The isopropanol precipitation was repeated (as described above) and the new pellet was again dissolved in 500µl DEPC treated H_2O. One-third ($^1/_3$) volume LiCl (8 M) was added and the RNA allowed to precipitate overnight at 4°C.

The mixture was then centrifuged as described above and the new RNA pellet re-suspended in 300µl DEPC treated H_2O. One-tenth (0.1) volume sodium acetate (3 M; pH 4.8) and 2 volumes 70% ethanol were added. The mixture was incubated at -70°C for 30 minutes. The mixture was centrifuged at 12,000 rpm, 4°C, for 10 minutes, and the resultant supernatant discarded. The RNA pellet was washed with 0.2 ml 70% ethanol. After centrifugation (as above), the ethanol was carefully decanted off and the RNA pellet dried in a SpeedVac, for 3 minutes. The RNA pellet dissolved in 100µl DEPC treated H_2O.

A sample of the RNA (1.2 µl) was then pipetted and used to assess the RNA purity and concentration by measuring the spectrophotometric absorbance of the sample (in water) at wavelengths 230, 260 and 280 nm and hence A_{260}:A_{280} and A_{260}:A_{230} ratios using spectrophotometer (NanoDrop®ND-1000, Technologies Inc.). The remainder of the samples was stored at -80°C.

4.3.4 RNeasy Plant Mini Kit Protocol

RNA was isolated from cassava leaves following instructions from the manufacturer of the kit (Qiagen, Cat. #. 74903). The following buffers (supplied with the kit), were used for total RNA extraction from cassava leaves: Lysis buffers; RLT and RLC containing *guanidine thiocyanate* and guanidine hydrochloride respectively, washing buffer RPE (mixed with ethanol before use) as well as buffer RW1; a mixture of small amount of guanidine thiocyanate and ethanol. Buffer RLT was prepared by adding 10µl β-mercapto-ethanol (β-ME) per 1 ml of the buffer and 4 volumes of 100% ethanol was added to Buffer RPE to obtain a working solution before being used in the extraction process.

A maximum of 100mg pre-homogenized cassava leaf powder was weighed into RNase-free, Liquid Nitrogen-chilled 2 ml micro-centrifuge tubes. 450µl Buffer RLT was added and the mixture vortexed vigorously. The lysate was then pipetted onto QIAshredder spin column (Qiagen, Cat. #.74903; lilac in color) placed in a 2 ml collection tube and centrifuged at 16 100 rpm, 4°C, for 2 minutes. Supernatant of the flow-through fraction was carefully transferred to a new micro-centrifuge tube without disturbing the cell-debris pellet in the collection tube.

Ethanol (0.5 volumes; 99%) was added to the cleared lysate, and mixed immediately by pipetting. The sample was then applied to an RNeasy mini column (Qiagen, Cat. # 74903; pink in color) placed in a 2 ml collection tube. The tube was closed gently and centrifuged at 10 000 rpm, RT, 30 seconds. The flow through was discarded and 700µl Buffer RW1 was added to the RNeasy column. The tube was then closed gently and centrifuged as described above (to wash the spin column membrane).

The flow-through and collection tube were discarded and the RNeasy column transferred into a fresh 2 ml collection tube. The RNeasy column was then washed by pipetting 500µl Buffer RPE into it, followed by centrifugation as described above. The flow through was discarded and the collection tube re-used to repeat Buffer RPE washing as described above. The collection tube with the flow through were discarded and the RNeasy silica gel membrane dried by placing the RNeasy column into a fresh 2 ml collection tube and centrifuging at 16 100 rpm, RT, for 1 minute. This was to eliminate any chance of possible Buffer RPE carry over hence contamination.

The RNeasy column was then transferred to a fresh 1.5 ml collection tube and 40µl RNase-free H_2O was pipetted directly onto the RNeasy silica gel membrane. The tube was closed gently and the RNA eluted by centrifuging the column at 10 000 rpm, RT, for 1 minute. A sample of the RNA (1.2 µl) was then pipetted and used to assess the RNA purity and concentration by measuring the spectrophotometric absorbance of the sample (in water) at wavelengths 230, 260 and 280 nm and hence A_{260}:A_{280} and A_{260}:A_{230} ratios using spectrophotometer (NanoDrop®ND-1000, Technologies Inc.).

4.3.5 Modified CTAB-Based Method

This method was adopted from a modified procedure of extracting RNA from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction by Gasic et al., (2004). The EB was composed of CTAB (2%), PVP K-30 (2%), Tris-HCl (100 mM; pH 8.0), EDTA (25 mM), NaCl (2 M), spermidine (0.5g/l; free acid-HRS), β-mercaptoethanol (2%), and Sterile RNase-free H_2O.

Other reagents included Chloroform: Isoamyl-alcohol (24:1), Lithium Chloride (LiCl; 10 M), Sodium acetate (3 M; pH 5.2), and Ethyl alcohol (100%).

Cassava leaves were homogenized in liquid Nitrogen to a fine powder. Liquid N_2 was constantly added to the tissue during grinding to prevent thawing. The frozen powdered tissues were then quickly transferred to a pre-chilled 50 ml falcon tube containing EB at a rate of 20 ml of the extraction solution per 1g tissue. The mixture was vortexed for 10 seconds and then incubated on ice for 5 minutes. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added. The sample was then heated on a water bath at 50°C for 15 minutes and then centrifuged at 5 000 rpm, RT, for 10 minutes.

The resulting supernatant was transferred to fresh 50 ml falcon tube, where an equal volume of Chloroform: Isoamyl alcohol (24:1) was again added, mixed, vortexed and centrifuged as described above. The resultant supernatant was transferred to fresh 50 ml tube, 0.25 volumes LiCl (10 M) added and the mixture incubated on ice overnight at 4°C.

The sample was then centrifuged at 5000 rpm, RT, for 20 minutes the resultant supernatant decanted and the precipitated pellet was dried by inverting the tubes for 10 minutes on a kimwipe. The pellet was then re-suspended in 250 µl sterile RNase-free H_2O and transferred into a 2 ml micro-centrifuge tube where 250 µl LiCl (10 M) was added. The mixture was flicked to mix and the RNA precipitated by incubating on ice for 2 ½ hours.

The sample was then centrifuged at 13 000 rpm, 4°C, for 10 minutes. The resultant supernatant was decanted; the RNA pellet re-suspended in 250 µl sterile RNase-free H_2O and 25 µl sodium acetate (3 M; pH 5.2) and 1 ml 100% ethanol were added. The mixture was then incubated at -20°C for 60 minutes and then centrifuged as described above.

The resultant supernatant was decanted and the RNA pellet vacuum dried in a SpeedVac for 3 minutes. The dried RNA pellet was then re-suspended in 100 µl sterile RNase-free H_2O. A sample of the RNA (1.2 µl) was then pipetted and used to assess the RNA purity and concentration by measuring the spectrophotometric absorbance of the sample (in water) at wavelengths 230, 260 and 280 nm and hence A_{260}:A_{280} and A_{260}:A_{230} ratios using spectrophotometer (NanoDrop®ND-1000, Technologies Inc.). The remainder of the samples was stored at -80°C.

4.3.6 Data analysis

Total RNA concentration, A_{260}:A_{280} and A_{260}:A_{230} ratios values were subjected to analysis of variance (ANOVA) using the PROC ANOVA procedure of Genstat Discovery Edition 3 (Lawes Agricultural Trust Rothamsted Experimental Station, UK). The means of the above three parameters (between protocols and leaf tissues) were compared using the Fisher's protected Least Significant Differences (LSD) test at 5% probability level (Payne et al., 2007).

4.4 Results

4.4.1 Concentration (ng/µl) of RNA obtained from young and old leaf tissues of cassava genotype TMS 60444 from five different protocols

There was significant ($P \leq 0.05$) variation in the amount of RNA yielded between protocols and within leaf tissues, while the protocol x leaf tissue interaction produced no significant ($P > 0.05$) difference (Appendix 5; Table 6). CTAB based method produced significantly the highest ($P \leq 0.05$) amount of RNA when compared with the RNA yielded by the other four methods. RNeasy method produced the least amount of RNA (Table 6). Although RNA from Total Nucleic Acid + DNase method did not significantly ($P > 0.05$) differ from those of Trizol and Reilly et al., (2001) procedures, the RNA yield however significantly varied ($P \leq 0.05$) with the RNA concentrations from RNeasy method.

The RNA generated using Trizol, Reilly et al., (2001) and RNeasy methods were not significantly ($P > 0.05$) different (Table 6). The amount of RNA produced also significantly varied within leaf tissues. Generally, RNA concentrations from younger leaves were significantly higher ($P \leq 0.05$) than RNA from older leaves across all the protocols with the exception of RNeasy method where the amount of RNA from both young and old leaves were not significantly ($P > 0.05$) different (Table 6).

When RNA from specifically younger leaves were observed, CTAB based protocol generated significantly higher ($P \leq 0.05$) amounts of RNA compared to the other four protocols, while RNeasy method produced the least. Young leaves' RNA from Total Nucleic Acid + DNase method also varied significantly ($P \leq 0.05$) from those of Trizol, Reilly et al., (2001) and RNeasy methods (Table 6).

The RNA (young leaves) from Trizol and Reilly et al., (2001) procedures did not significantly differ ($P>0.05$) from each other, although Trizol method produced significantly varied ($P\leq0.05$) amounts of RNA from RNeasy method. Reilly et al., (2001) and RNeasy methods did not produce significantly different ($P>0.05$) amount of RNA from young leaves (Table 6). Analysis of total RNA from older leaves showed that CTAB based protocol produced significantly ($P\leq0.05$) more RNA from these leaves than the other four protocols.

Despite RNA yield (old leaves) from Trizol method not significantly ($P>0.05$) varying with RNA yield obtained from Total Nucleic Acid + DNase and Reilly et al., (2001) procedures, it did however significantly vary ($P\leq0.05$) with RNA concentrations from RNeasy method (Table 6). The RNA generated from similar leaf tissues (old) using Total Nucleic Acid + DNase, Reilly et al., (2001) and RNeasy methods showed no significant ($P>0.05$) differences (Table 6).

Table 6: Mean concentrations (ng/µl) at LSD P≤0.05 of total RNA extracted using five different protocols from young and old leaves of cassava genotype TMS 60444.

Leaf Tissues	**Protocols**					
	CTAB	Reilly	RNeasy Kit	Total+DNase	Trizol	**Tissue (mean)**
Young Leaves	1793g (t)	284ij (v)	161j (x)	617h (y)	420i (r)	**655e**
Old Leaves	1078k (u)	100mp (w)	66np (x)	212mp (z)	248m (s)	**340.8f**
Protocol (mean)	**1436a**	**192bd**	**114cd**	**414b**	**334bd**	

LSD (P≤0.05) Protocol = 253.3 LSD (P≤0.05) Leaf Tissue = 160.2

Means of total RNA concentration followed by the same letter in specific protocol column and leaf tissue row in table 6 (above) are not significantly different at 5% significant level. For each leaf tissue 5 samples were acted during three independent experiments were measured.

4.4.2 Purity (A_{260}:A_{280} ratio) of RNA obtained from young and old leaf tissues of cassava genotype TMS 60444 from five different protocols

The RNA (A_{260}:A_{280}) ratio was significantly varied (P≤0.05) between protocols and not significantly varied (P>0.05) within the leaf tissues as well as the protocol x leaf tissue interaction (Appendix 6; Table 7). The ratio obtained using CTAB method was significantly higher (P≤0.05) than the ratios generated from the other four protocols, while Reilly et al., (2001) method generated the least A_{260}:A_{280} ratio. Total Nucleic Acid + DNase method A_{260}:A_{280} ratio was significantly different (P≤0.05) from the A_{260}:A_{280} ratio values of Reilly et al., (2001), RNeasy and Trizol methods (Table 7).

Although the A_{260}:A_{280} of Trizol and RNeasy protocols did not significantly differ ($P>0.05$) from each other, their values were significantly different ($P\leq0.05$) from the A_{260}:A_{280} ratio obtained from Reilly et al., (2001) method. The A_{260}:A_{280} ratios between young and old leaves were not significantly different ($P>0.05$) from each other in CTAB, Total Nucleic Acid + DNase, Trizol and Reilly et al., (2001) methods. The only exception was observed in RNeasy protocol where the A_{260}:A_{280} ratio was significantly higher ($P\leq0.05$) in young leaves than old leaves (Table 7).

Specific leaf tissue A_{260}:A_{280} ratio variation was also significant. CTAB method produced significantly the highest ($P\leq0.05$) A_{260}:A_{280} ratio in young leaves compared to other methods, while Reilly et al., (2001) method generated the least A_{260}:A_{280} ratio in young leaves. The young leaf A_{260}:A_{280} ratio observed from Trizol and RNeasy methods did not significantly vary ($P>0.05$) from each other.

However, the ratio A_{260}:A_{280} of the two methods varied significantly ($P\leq0.05$) with those from Total Nucleic Acid + DNase and Reilly et al., (2001) procedures (Table 7). Similar observations (as described for young leaves) were also made on analyzing the A_{260}:A_{280} ratio from older leaves. CTAB produced significantly the highest A_{260}:A_{280} ratio (from old leaves) than other protocols, while Reilly et al., (2001) produced the least A_{260}:A_{280} ratio.

Although old leaves' A_{260}:A_{280} ratios obtained from Trizol and RNeasy methods did not significantly differ ($P\leq0.05$) from each other, the ratios varied significantly ($P\leq0.05$) with those obtained from Total Nucleic Acid + DNase and Reilly et al., (2001) protocols (Table 7).

Table 7: Mean Purity (A_{260}:A_{280} ratio; mean; Lsd P≤0.05) of total RNA from young and old leaves of cassava genotype TMS 60444 extracted using five different protocols

Leaf Tissues	**Protocols**					
	CTAB	Reilly	RNeasy	Total+DNase	Trizol	**Tissue (mean)**
Young Leaves	2.19e (t)	1.18h (u)	1.56g (v)	1.93f (x)	1.52g (y)	**1.68d**
Old Leaves	2.16i (t)	1.18m (u)	1.41k (w)	1.83j (x)	1.42k (y)	**1.60d**
Protocol (mean)	**2.18a**	**1.18d**	**1.49c**	**1.88b**	**1.47c**	

LSD (P≤0.05) Protocol = 0.17; LSD (P≤0.05) Leaf Tissue = 0.11

Means of A_{260}:A_{280} ratios followed by the same letter in specific protocol column and leaf tissue row table 7 (above) are not significantly different at 5% significant level. For each leaf tissue 5 samples were extracted during three independent experiments were measured.

4.4.3 Purity (A_{260}:A_{230} ratio) of RNA obtained from young and old leaf tissues of cassava genotype TMS 60444 from five different protocols

Significant (P≤0.05) differences in the A_{260}:A_{230} ratio between protocols were observed, but both leaf tissue and protocol x leaf tissue interaction yielded no significant (P>0.05) variations (Appendix 7; Table 8). The RNA (A_{260}:A_{230}) ratio from CTAB method was significantly higher (P≤0.05) than the ratios obtained from the other four protocols (Table 8). The least A_{260}:A_{230} value was observed in Reilly et al., (2001) method.

Although the A_{260}:A_{230} ratio obtained from both Total Nucleic Acid + DNase and Trizol methods were not significantly different ($P>0.05$), their values varied significantly ($P\leq0.05$) from those of Reilly et al., (2001) and RNeasy kit methods. The A_{260}:A_{230} values from Reilly et al., (2001) and RNeasy kit methods were also not significantly ($P>0.05$) different (Table 8).

When comparing the A_{260}:A_{230} ratio between young and old leaves across the protocols, a highly significant ($P\leq0.05$) ratio was observed in young leaves than old leaves under Reilly et al., (2001) and RNeasy methods, while the ratios did not significantly vary ($P>0.05$) under CTAB, Total Nucleic Acid + DNase and Trizol protocols. Despite this, a general trend was observed where higher A_{260}:A_{230} ratios were generated in young leaves compared to old leaves across most of the protocols with the exception of Trizol method where the A_{260}:A_{230} did not significantly ($P>0.05$) differ (Table 8). There was significant variation ($P\leq0.05$), when the A_{260}:A_{230} ratios were specifically analyzed in young leaves across the protocols.

When ranked, CTAB based protocol produced significantly higher ($P\leq0.05$) ratio, followed by Total Nucleic Acid + DNase, Trizol, RNeasy and Reilly et al., (2001) methods in that order. Reilly et al., (2001) protocol produced the least significant ($P\leq0.05$) A_{260}:A_{230} ratio (Table 8). When the A_{260}:A_{230} of older leaves were specifically analyzed across the protocols, the ratio observed in CTAB based method was significantly higher ($P\leq0.05$) compared to the rest of the protocols. Although Total Nucleic Acid + DNase and Trizol method ratios were not significantly different ($P>0.05$), their values varied significantly ($P\leq0.05$) with those from RNeasy and Reilly et al.,

(2001) methods. No significant differences (P>0.05) were observed in the A_{260}:A_{230} ratios of RNeasy and Reilly et al., (2001) methods (Table 8).

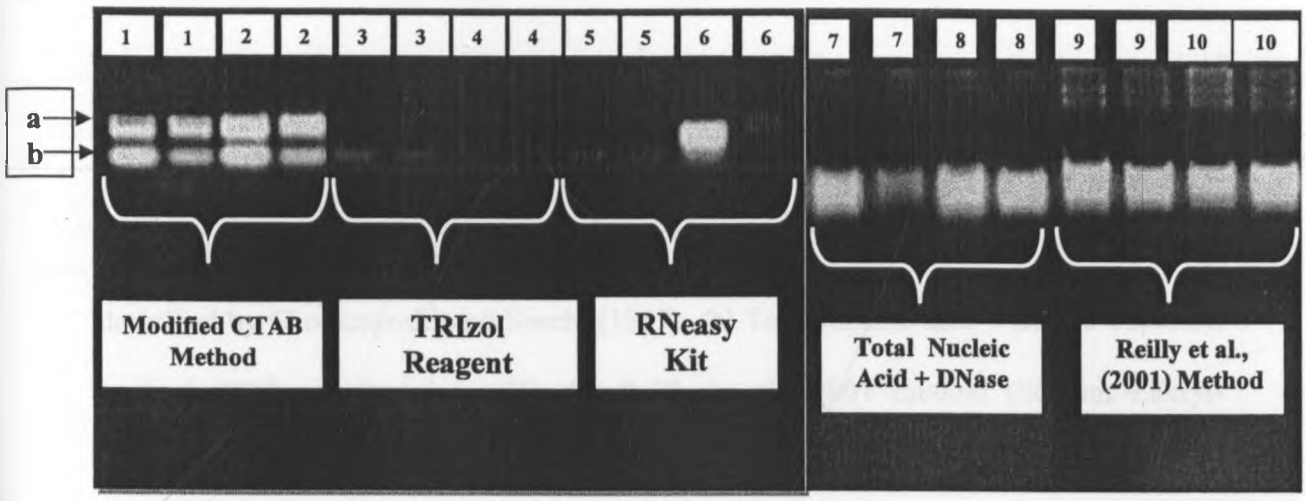
Table 8: Mean Purity (A_{260}:A_{230} ratio; mean; Lsd P≤0.05) of total RNA from young and old leaves of cassava genotype TMS 60444 extracted using five different protocols

Leaf Tissues	**Protocols**					
	CTAB	Reilly	RNeasy	Total+DNase	Trizol	**Tissue (mean)**
Young Leaves	2.21e (t)	0.68i (u)	0.93h (w)	1.38f (y)	1.23g (z)	**1.29d**
Old Leaves	2.20j (t)	0.40m (v)	0.44m (x)	1.26k (y)	1.24k (z)	**1.11d**
Protocol (mean)	**2.21a**	**0.54c**	**0.69c**	**1.32b**	**1.25b**	

LSD (P≤0.05) Protocol = 0.23; LSD (P≤0.05) Leaf Tissue = 0.14

Means of A_{260}:A_{230} ratios followed by the same letter in specific protocol column and leaf tissue row table 8 (above) are not significantly different at 5% significant level. For each leaf tissue 5 samples were extracted during three independent experiments were measured.

Figure 10: Total RNA extracted from young cassava leaf tissues (samples 2, 4, 6, 8 & 10) and old cassava leaf tissues (samples 1, 3, 5, 7, & 9) using the modified-CTAB, TRIzol Reagent, RNeasy Plant Kit, Total Nucleic Acid + DNase Treatment and Reilly et al., (2001) extraction methods.



The RNA was separated by 2% Agarose gel electrophoresis (stained with ethidium bromide). Bands (a) and (b) corresponding to 28S rRNA and 18S rRNA respectively were more distinctly visible in lanes 1 and 2 of the modified CTAB method. This indicated that the modified CTAB method was effective in extracting high quality non-degraded RNA from the polysaccharide and polyphenol rich cassava leaves.

4.4.4 Discussion

Transcriptomic analysis of cassava genome necessitates initial optimization of protocols for isolating total RNA so that gene expression profiling using cDNA based microarrays can be performed. To achieve this, the efficiency of five different protocols was compared for RNA purification. All the 5 tested protocols permitted extraction of total RNA of different quantities, qualities and integrity from both young and old cassava leaf tissues.

The first four methods: (a) Trizol Reagent (phenol and guanidine isothiocyanate) as described by Chomczynski and Sacchi (1987), (b) Total nucleic acid + DNase treatment method (SDS and Proteinase K), (c) Reilly et al., 2001 method (Sodium Lauryl-sarcosine) and (d) RNeasy plant minikit (guanidine thiocyanate and guanidine hydrochloride) produced total RNA from both young and old cassava leaves that were relatively low in yield (RNA less than 500ng; Table 6). The only exception was RNA of more than 500ng obtained from young leaves by Total nucleic acid + DNase method (Table 6).

The RNA samples from the above four protocols were also significantly contaminated with polysaccharides, phenolic compounds and proteins as shown by the low A_{260}:A_{280} and A_{260}:A_{230} ratios i.e. all below 1.8 (Table 7 and Table 8). Although RNA extract (young leaf) of Total nucleic acid + DNase method was protein-free RNA, i.e. A_{260}:A_{280} ratio more than 1.9 (Table 7), the sample was still significantly contaminated with phenolic compounds i.e. A_{260}:A_{230} ratio below 1.8 (Table 8).

In comparison, the modified CTAB-based method was the most efficient protocol for extracting RNA from both young and old cassava leaf tissues as indicated by the high RNA yield i.e. more than 1µg (Table 6) as well as high A_{260}:A_{280} ratio i.e. more than 1.9 (Table 7) and high A_{260}:A_{230} ratios i.e. more than 1.8 (Table 8). These results showed that the modified CTAB-RNA sample was not only sufficient in concentration, but was also free from polysaccharides, phenolic compounds and protein contaminations.

Using a closely related (but not a replica) modified CTAB based method, Gasic et al., (2004) obtained similarly high quantity and quality RNA from various apple tissues rich in polyphenols and polysaccharides. The successful extraction of RNA from cassava leaves using the modified CTAB-based method can be attributed to a number of changes introduced on the original procedure as described by Gasic et al., (2004). These modifications included using 20 ml EB for every 1g tissue (instead of 5 ml per 1 g tissue). This improved the efficiency of separating organic and aqueous phase after centrifugation and minimized loss of starting material. The Tissue-EB and Chl: Iaa mixture was incubated in a pre-warmed (50°C) water-bath for 15 minutes (instead of 15 minutes at 60°C).

The lowered incubation temperature reduced the chances of RNA fragmentation. Finally a two-step precipitation (overnight and $2^1/_2$ hrs) with LiCl (2.5 M), instead of a single LiCl (2.5 M) precipitation, was introduced into the protocol. This further eliminated polysaccharides and thus improved and increased the purity and yield of final RNA (Rubio-Piña and Vázquez-Flota, 2008; Carra et al., 2007; Iandolino et al., 2004).

In addition, the reagents used to constitute the EB of modified CTAB method also contributed to extraction of high quantity and quality RNA from both cassava leaf tissues. The CTAB is a detergent that preserves the integrity of nuclear and organelle membranes yielding total RNA with lower concentrations of un-spliced heteronuclear transcripts, as well as increases the RNA-to-DNA ratio (Mejjad et al., 1994; Dellaporta et al., 1983).

The (PVP) K-30 (soluble) in the EB improved sequestration and elimination of phenolic compounds (Gambino et al., 2008; Woodhead et al., 1997; Salzman et al., 1999) and inclusion of low spermidine concentration stimulated an unexplained increase of RNA synthesis (Swanson and Gibbs, 1980; Chang et al., 1993).

The high molarity of NaCl (5 M) and the strong reductant β-ME in the EB increased the solubility of polysaccharides, reducing their co-precipitation with RNA in later steps of the protocol and denatured ribonucleases and other contaminating proteins that are released during tissue disruption and homogenization (Iandolino et al., 2004; Fang et al., 1992; Lodhi et al., 1994).

The high molarity LiCl not only differentially precipitated RNA from admixture with DNA, but it also increased RNA yield and favored precipitation of larger transcripts over smaller ones (Carra et al., 2007). The centrifugation steps were carried out at 4°C because the low temperature reduced RNA degradation and decreased the rate of chemical reactions between nucleic acid and phenolic compounds hence improving elimination of phenolic compounds (Gambino et al., 2008).

RNA purification protocols with CTAB in the EB has also been adapted to extract RNA from polysaccharides and polyphenol-rich plant tissues including *Arabidopsis siliques*, sweet potatoes and grape berries as well as other woody plants (Gambino et al., 2008; Carra et al., 2007; Meisel et al., 2005; Hu et al., 2002; Malnoy et al., 2001; Jaakola et al., 2001; Zeng and Yang, 2002; Iandolino et al., 2004).

In the two studies, involving isolation and characterization of cassava catalase expressed during post-harvest physiological deterioration and towards identifying the full set of genes expressed during cassava post-harvest physiological deterioration (Reilly et al., 2001) sodium Lauryl-sarcosine in the EB was used to extract total RNA from storage roots of cassava cultivar CM2177-2 or leaves for northern hybridizations.

However, when the EB containing sodium lauryl-sarcosine was tested and used to extract RNA from young and old leaf tissues of cassava genotype TMS 60444, the final RNA extract was of low quantity and purity (Tables 4.3.1, 4.3.2 and 4.3.3). This suggests that RNA extraction in cassava is possibly genotype or leaf-tissue-age depended. The RNeasy plant mini kit EB containing *guanidinium isothiocyanate, guanidinium hydrochloride* and Tris-Hcl NaCl, was used (following the manufacturer's instructions (Qiagen, Cat. #. 74903) in the extraction of RNA. The method produced RNA of low yields and of poor-quality from both young and old cassava leaf tissues.

Although Mackenzie et al. (1997) successfully extracted RNA from grapevines with the RNeasy kit, they used a different extraction buffer (Gambino et al., 2008). Nassuth et al. (2000) used the same protocol and obtained similar results but reported problems in extraction from old grapevine tissues.

RNeasy protocol has also been used to extract RNA from apple, citrus, olive, pear, and plum (Bertolini et al., 2001; Ragozzino et al., 2004) for virus detection by RT-PCR, but little information was provided on the quality and quantity of the isolated RNA (Gambino et al., 2008).

Purification of RNA using Trizol reagent according to manufacturer's (Invitrogen, Cat #. 15596-018) instructions is based upon a guanidinium thiocyanate-phenol-chloroform extraction method originally described by Chomczynski and Sacchi (1987). The addition of acidic guanidinium thiocyanate is widely employed to inhibit RNase activity (Chomczynski and Sacchi, 1987; Valenzuela-Avendaño et al. 2005). Total nucleic acid + DNase method constituted SDS, Proteinase K, phenol: chloroform: Isoamyl alcohol extraction and isopropanol precipitation procedure as describe by Soni and Murray (1994). The addition and incubation with Proteinase K decreased RNase activity during the extraction process (Azevedo et al., 2003).

The main modification on this protocol was that after the first phenol: chloroform: Isoamyl alcohol (25:24:1) centrifugation and precipitation with Ammonium acetate (10 M; + 99% Ethyl alcohol) step, the re-dissolved total nucleic acid was treated with DNase buffer (Promega, Cat. #. M198A), RNase inhibitor (Invitrogen, Cat. #. 10777-019) and RQ1 RNase-Free DNase 1 (Promega, Cat. # M6101). Treatment of the RNA sample with RQ1 RNase-Free DNase was to degrade contaminating genomic DNA. The RNA samples (young and old cassava leaf tissues) from the two protocols (Trizol reagent method and the modified Total nucleic acid + DNase method) were of low quantity and poor quality.

For example, the samples contained significantly high levels of polysaccharides, polyphenolic compounds, and protein contaminants as determined by $A_{260:230}$ and $A_{260:280}$, ratios (Logemann et al., 1987; Manning 1990).

4.4.5 Conclusion and recommendation

The increasing popularity of microarray technique for gene expression profiling has created the need to isolate high quality total RNA. With the aim of optimizing and recommending the most suitable method for extracting RNA from cassava leaves and hence establish a cassava-leafy spurge cDNA microarray procedure (in the future) to characterize cassava transcriptome upon drought stress treatment, five RNA extraction protocols were tested on the 4-month old young and old leaves of model cassava genotype TMS 60444 grown under greenhouse conditions.

Based on the basic knowledge that successful optimization of microarray procedure and thus gene expression profiling analysis after hybridization onto cassava-leafy spurge cDNA microarray chip, limits microarray experiments to RNA samples with A_{260}:A_{280} and $A_{260:A230}$ ratios between than 1.9-2.1, it is recommended thus to employ the optimized CTAB-based method (modified from the original by Gasic et al., (2004), to extract RNA of sufficient quantity and quality from cassava leaves.

The recommended optimized CTAB method is efficient, simple, and reproducible procedure. The purified RNA is of high quality and could be a suitable template for cDNA synthesis, cDNA labeling and hybridization of the labeled cDNA onto a cassava-leafy spurge cDNA microarray chip for identification of up and down regulated genes upon drought stress.

4.4.6 References (Chapter four)

Azevedo, H., Lino-Neto, T., and Tavares, R.M., 2003: An improved Method for high-quality RNA Isolation from needles of Adult Maritime Pine Trees, Plant molecular biology reporter 21, pp. 333–338.

Alemzadeh, A., Fujie, M., Usami, S., and Yamada, T., 2005: Isolation of High-Quality RNA from High-Phenolic tissues of Eelgrass (*Zostera marina* L.) by keeping temperature low. Plant Molecular biology reporter 23, pp. 421a-421h.

Baker, S.S., Rugh, C.L., and Kamalay, J.C., 1990: RNA and DNA isolation from recalcitrant plant tissues. Biotechniques, Vol. 9, pp. 268-272.

Bertolini, E., Olmos, A., Martínez, M.C., Gorris, M.T., and Cambra, M., 2001: Single-step multiplex RT-PCR for simultaneous and colourimetric detection of six RNA viruses in olive trees. Journal of Virology and Methods, Vol.96, pp. 33-41.

Carra, A., Gambino, G., and Schubert, A., 2007: Acetyltrimethylammonium bromide-based method to extract low-molecular-weight RNA from polysaccharide-rich plant tissues. Analytical Biochemistry, Vol. 360, pp. 318–320.

Chang, S., Puryear, J., and Cairney, J., 1993: A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter, Vol.11, pp. 113-116.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J., 1979: Isolation of biologically active ribonucleic acid from sources enriched in Ribonuclease. Biochemistry, Vol. 18, pp. 5294-5299.

Chomczynski, P., and Sacchi, N., 1987: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol chloroform extraction. Analytical Biochemistry, Vol. 162, (1), pp. 156-159.

Dellaporta, S, L., Wood, J., and Hicks, J.B., 1983: A plant DNA mini preparation: version II. Plant Molecular Biology Reporter, Vol.1, pp.19-21.

Fang, G., Hammar, S., and Grumet, R., 1992: A quick and inexpensive method for removing Polysaccharides from plant genomic DNA. Biotechniques, Vol. 13, pp. 52-56.

Fleige, S., Pfaffl, M.W., 2006: RNA integrity and the effect on the real-time qRT-PCR performance. Molecular Aspects of Medicine, Vol. 27, pp.126–139.

Gambino, G., Irene, P., and Ivana, G., 2008: A Rapid and Effective Method for RNA Extraction from Different Tissues of Grapevine and Other Woody Plants. Phytochemical Analysis, DOI: 10.1002.pca, pp. 1-6.

Gasic, K., Hernandez, A., and Korban, S.S., 2004: RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Molecular Biology, Reporter, 22, pp. 437a–437g.

Gehrig, H., Winter, K., Cushman, J., Borland, A., and Taybi, T., 2000: An Improved RNA Isolation Method for Succulent Plant Species Rich in Polyphenols and Polysaccharides. Plant Molecular Biology Reporter, Vol. 18, pp. 369-376.

Geuna, F., Hartings, H., and Scienza, A., 1998: A new method for rapid extraction of high quality RNA from recalcitrant tissues of grapevine. Plant Molecular Biology Reporter, Vol. 16, (1), pp. 61-67.

Green, P.J., 1994: The ribonucleases of higher plants. Annual Review of Plant Physiology, Vol. 45, pp. 421-445.

Gruffat, D., 1998: Isolation of RNA from mammalian cells: application to large mRNA. In: Siebert PD and Larrick JW (Eds), Gene cloning and analysis by RT-PCR. Bio-techniques, Vol. 23, pp. 35-55.

Hall, T.C., Ma, Y., Buchbinder, B.U., Pyne, J.W., Sun, S.M., and Bliss, F.A., 1978: Proceedings from National Academy of Sciences, USA, 75, pp. 3196-3200.

Hu, C.G., Honda, C., Kita, M., Zhang, Z., Sudan, T., and Moriguchi, T., 2002: A simple protocol for RNA isolation from fruit trees containing high levels of polysaccharides and polyphenol compounds. Plant molecular biology Reporter, Vol. 20, pp. 69a–69g.

Hughes, D.W., and Galau, G., 1988: Preparation of RNA from cotton leaves and pollen. Plant molecular biology, reporter, 6, pp. 253-257.

Iandolino, A.B., Goes da-Silva, F., Lim, H., Choi, H., Williams, L.E., and Cook, D.R., 2004: High-quality RNA, cDNA, and derived EST libraries from grapevine (*Vitis vinifera* L.). Plant Molecular Biology, Reporter, 22, pp. 269-278.

Jaakola, L., Pirttila, A.M., Halonnen, M., and Hohtola, A., 2001: Isolation of high quality RNA from Bilberry (*Vaccinum myrtillus* L.) fruit. Molecular biotechnology, Vol. 19, pp. 201-203.

Kirk, M. M., and Kirk, D. L., 1985: Translational regulation of protein synthesis, in response to light, at a critical stage of Volvox development. Cell biology, Vol. 41, pp. 419-428.

Levi, A., Galau, G.A., and Westzstein, H.Y., 1992: A rapid procedure for the isolation of RNA from high-phenolic containing tissues of pecan. Horticultural Science, 1992, Vol. 27, (12) pp. 1316-1318.

Li, B., Bochu, W., Kun, T., Yilong, L., Jian, W., and Jinmin, W., 2006: A simple and convenient approach for isolating RNA from highly viscous plant tissue rich in polysaccharides. Colloids and Surfaces B: Bio-interfaces, Vol. 49, pp. 101–105.

Lodhi, M.A., Ye G-N, Weeden, N.F, and Reisch, B.I., 1994: A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant molecular biology Reporter, Vol.12, pp. 6-13.

Logemann, J., Schell, J., and Willmitzer, L., 1987: Improved method for the isolation of RNA from plant tissues. Annals Biochemistry, Vol. 163, pp. 16-20.

Loomis, W.D., 1974: Overcoming problems of phenolics and quinines in the isolation of plant enzymes and organelles. *Methods in Enzymology,* Vol.31, pp. 528-545.

López-Gómes, R., and Gómes-Lim, M.A., 1992: A method for extraction of intact RNA from fruits rich in polysaccharides using ripe mango mesocarp. Horticultural Science, Vol. 27, pp. 440-442.

Louime, C., Vasanthaiah, H., Jittayasothorn, Y., Lu, J., Basha, S., Thipyapong, P., and Boonkerd, N., 2008: A simple and efficient protocol for high quality RNA extraction and cloning of chalcone synthase partial cds from Muscadine grape cultivars (*Vitis rotundifolia* Michx.). European Journal of Scientific Research, Vol.22 (2), pp.232-240.

Mackenzie, D.J., Mclean, M.A., Mukerji, S., and Grenn, M., 1997: Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. Plant Dis. Vol. 81, pp.222–226.

Malnoy, M., Reynoird, J.P., Mourgues, F., Chevreau, E., and Simoneau, P., 2001: A method for isolating total RNA from pear leaves. Plant Molecular biology Reporter, Vol. 19, (1), pp. 69a-69f.

Meisel, L., Fonseca, B., González, S., Baeza-Yates, R., Cambiazo, V., Campos, R., Gonzalez, M., Orellana, A., Retamales, J., and Silva, H., 2005: A rapid and efficient

method for purifying high quality total RNA from peaches (*Prunus persica*) for functional genomics analyses. Biological Resources, Vol. 38, pp. 83–88.

Manning, K., 1990: Isolation of nucleic acids from plants by differential solvent precipitation. Annals of Biochemistry, Vol. 195, pp. 45-50

Mejjad, M., Vedel, F., and Ducreux, G., 1994: Improvement of DNA preparation and of PCR cycling in RAPD analysis of marine microalgae. Plant Molecular Biology Reporter, Vol.12, pp. 6-13.

Nassuth, A., Pollari, E., Helmeczy, K., Stewart, S., and Kofalvi, S.A., 2000: Improved RNA extraction and one-tube RT-PCR assay for simultaneous detection of control plant RNA plus several viruses in plant extracts. Journal of Virology and Methods, Vol. 90, pp. 37–49.

Payne, R., Murray, D., Harding, S., Baird, D., Soutar, D., and Lane, P., 2007: Genstat® for Windows (3rd Edition). VSN International, Oxford, UK. 342 pp. ISBN-1-904375-08-1

Ragozzino, E., Faggioli, F., and Barba, M., 2004: Development of a one tube one step RT-PCR protocol for the detection of seven viroids in four genera: *Apscaviroid*, *Hostuviroid*, *Pelamoviroid* and *Pospiviroid*. Journal of Virology and Methods, Vol. 121, pp. 25–29.

Reilly, K., Han, Y., Tohme, J., and Beeching, J.R., 2001: Isolation and characterization of a cassava catalase expressed during Post-harvest Physiological Deterioration. Biochemica et Biophysica Acta, issue.1518, pp. 317-323.

Richards, E., Reinhardt, M., and Rogers, S., 1994: Preparation of genomic DNA from plant tissue. In: F. M. Ausubel., R. Brent., R. E. Kingston., D. D. Moore., J.G. Seidman., J.A. Smith. and K. Struhl (Eds): Current protocols in Molecular Biology. J Wiley and Sons, New York. pp 231-23

Rosic, N.N., and Hoegh-Guldberg, O., 2009: A method for extracting a high-quality RNA from *Symbiodinium sp.* Journal of Applied Phycology, DOI 10.1007/s10811-009-9433-x, pp. 1 – 8.

Rubio-Pina, J. A., and Vàzquez-Flota, F. A., 2008: Isolation of functional total RNA from *Argemone mexicana* tissues. Electronic Journal of Biotechnology, Vol.11, (4), pp. 1-5

Salzman, R.A., Fujita, T., Salzman, Z., Hasegawa, P.M., and Bressan R.A., 1999: An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Molecular Biology Reporter, 1999, vol. 17, (1), pp. 11-17.

Sambrook, J., Fritsch, E., and Maniatis, T., 1989: Molecular cloning: a laboratory manual. Cold Spring Harbor, New York. Cold Spring Harbor Laboratory Press. pp: 7.3-7.4

Schneiderbauer, A., Sandermann, H., and Ernst, D., 1991: Isolation of functional RNA from plant tissues rich in phenolic compounds. Annals of Biochemistry, Vol. 197, pp. 91-95.

Silvah, H., Yoshioka, K., Dooner, H.K., and Klessig, D.F., 1999: Characterization of a new Arabidopsis mutant exhibiting enhanced disease resistance. MPMI, Vol.12, pp.1053-1063.

Soni, R., and Murray, A.H., 1994: Isolation of intact DNA and RNA from plant tissues. Analytical Biochemistry, Vol. 218, pp. 474-476.

Suzuki, Y., Hibino, T., and Kawazu, T., 2003: Extraction of total RNA from leaves of eucalyptus and other woody and herbaceous plants using sodium iso-ascorbate. Biotechniques, Vol. 34 (5), pp. 992–993, 988–990.

Swanson, T.L., and Gibbs, G.E., 1980: Inhibition of lymphocyte growth by spermidine in medium containing fetal bovine serum. Plant in vitro cellular and developmental biology, Vol. 16 (9), pp. 761-766.

Valenzuela-Avendaño, J.P., Estrada-Mota, I.A., Lizama-UC, G., Souza-Perera, R., Valenzuela-Soto, E.M., and Zúñiga-Aguilar, J.J., 2005: Use of a simple method to isolate intact RNA from partially hydrated *Selaginella lepidophylla* plants. Plant molecular biology Reporter, vol. 23, (2), pp.199a-199g.

Vasanthaiah, H., Ramesh, K., and Mehboob, S., 2008: Efficient protocol for isolation of functional RNA from different grape tissue rich in polyphenols and polysaccharides for gene expression studies. Electronic Journal of Biotechnology, Vol.11 (3), pp. 1-8.

Wan, C., and Wilkins, T. A., 1994: A Modified Hot Borate Method Significantly Enhances the Yield of High-Quality RNA from Cotton (*Gossypium hirsutum* L.). Analytical Biochemistry, 223, pp. 7-12.

Wang, X., Tian, W., and Li, Y., 2007: Development of an efficient protocol of RNA isolation from recalcitrant tree tissues. Molecular Biotechnology, Vol. 38, (1), pp. 57-64.

Wilkins, T.A., and Smart, L.W., 1996: Isolation of RNA from plant tissue. In: Krieg PA (Ed), A laboratory guide to RNA, isolation, analysis and synthesis, pp. 21-42.

Woodhead, M., Taylor, M.A., Davies, H.V., Brennan, R.M., and McNicol, R.J., 1997: Isolation of RNA from blackcurrant (*Ribes nigrum* L.) fruit. Molecular biotechnology, Vol. 7 (1), pp. 1-4.

Zeng, Y., and Yang, T., 2002: RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. Plant molecular biology Reporter, Vol.20, pp. 417a-417e.

4.7 List of Appendices

Appendix 5: Analysis of Variance for RNA Yield/Concentrations (ng/µl) from young and old cassava leaves isolated using five different protocols.

Source of Variation	d.f	s.s	m.s	v.r	F.pr
Protocol	4	28402684.	7100671.	36.21	<.001
Tissue	1	3555118.	3555118.	18.13	<.001
Protocol. Tissue	4	1375478.	343869.	1.75	0.143
Residual	110	21567868.	196072.		
Total	119	54901148.			

Appendix 6: Analysis of variance of RNA purity (A260:A280 ratio/protein contamination) from young and old cassava leaves isolated using five different protocols.

Source of Variation	d.f	s.s	m.s	v.r	F.pr
Protocol	4	14.64692	3.66173	40.20	<.001
Tissue	1	0.17176	0.17176	1.89	0.172
Protocol. Tissue	4	0.08931	0.02233	0.25	0.912
Residual	110	10.02008	0.09109		
Total	119	24.92808			

Appendix 7: Analysis of variance of RNA purity (A260:A230 ratio/organic contaminants) from young and old cassava leaves isolated using five different protocols.

Source of Variation	d.f	s.s	m.s	v.r	F.pr
Protocol	4	41.4634	10.3659	66.18	<.001
Tissue	1	0.9135	0.9135	5.83	0.017
Protocol. Tissue	4	1.0141	0.2535	1.62	0.175
Residual	110	17.2304	0.1566		
Total	119	60.6215			