PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR RESPONSES OF TEA (*Camellia sinensis* L. O. Kuntze) CULTIVARS GROWN IN KENYA TO WATER DEFICIT

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EGERTON UNIVERSITY

APRIL, 2014

DECLARATION AND RECOMMENDATION

DECLARATION

I hereby declare that this thesis is my original work and has not been submitted for award in any institution of learning to the best of my knowledge.

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RECOMMENDATION

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DEDICATION

.....to my family for their unwavering support.

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ABSTRACT

Tea is one of the most popular non-alcoholic beverages worlwide, and a leading foreign exchange earner and source of livelihood to over three million people in Kenya. Tea growing areas in Kenya often experience drought periods which cause accumulated soil water deficits. Tea plants respond to water deficit through poorly understood physiological, cellular/biochemical and molecular processes. Development of tea cultivars adapted to water-deficit stress greatly relies on an understanding of mechanisms of plant responses. The present study was therefore designed with the objective of determining the responses of tea plants to water deficit. In the study, 18-months old seedling tea plants from eight drought tolerant and susceptible cultivars were subjected to three levels of treatment consisting of high (34%), moderate (26%) and low (18%) soil moisture content in a rain-out shelter. The experiment was designed in a complete randomized design with three replications. After three months of exposure to treatment, physiological parameters (leaf water status, shoot growth and gas exchange parameters), biochemical parameters (leaf proline and glycinebetaine levels) were determined. The data generated were subjected to two-way analysis of variance using GENSTAT. Molecular responses were analysed on total leaf RNA extracted from tolerant and susceptible tea cultivars under water stressed and unstressed conditions. mRNA was extracted from the total RNA and reverse transcribed to complementary DNA. The sequences/reads generated from the cDNA libraries using a 454 GLX sequencer were analysed *in silico* using bioinformatic tools. The results showed that there was significant reduction ($P<0.05$) in shoot growth, leaf relative water content, shoot water potential and gas exchange parameters with decrease in soil water. Proline was accumulated by droughted tea plants. A total of 232,853 Raw reads generated from the sequencer, assembled into 460 long transcripts (contigs). The contigs showed similarity to proteins in the *Arabidopsis* proteome following annotation by BLAST. Based on Gene ontology analysis, drought response related transcripts including Heat shock proteins, *Hsp70,* antioxidant molecules such as Superoxide dismutase, catalase and peroxidase, signal tranducers, Calmoduline like protein, and Galactinol synthase (*Gols4*) were induced in the water stressed plants. Results from this study therefore confirmed that water stress response in tea is controlled at the genetic level and there is potential to manipulate the genetics of tea to develop drought tolerant cultivars.

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LIST OF ABBREVIATIONS

AAPK	Abscisic acid-activated protein kinase
ABA	Abscisic acid
ATP	Adeneine Triphosphate
APS	Adenosine 5'-Phosphosulfate
BADH	Betaine aldehyde dehydrogenase
BLAST	Basic Local Alignment Search Tools
cDNA	Complementary Deoxyribonucleic acid
CDPK	Calcium dependent Protein Kinase
CIAT	International Centre for tropical Agriculture
CMO	Choline mono-oxygenase
DNA	Deoxyribonucleic acid
DTT	Dithiotreital
DW	Dry weight
emPCR	Emulsion polymerase chain reaction
EST	Expressed Sequence Tags
FLcDNA	Full length complementary Deoxynucleic acid
FW	Fresh weight
GO	Gene Ontology
HSP	Heat shock protein
KEGG	Kyoto Encyclopedia of Genes and Genomes
LRWC	Leaf Relative Water Content
mRNA	Messenger Ribonucleic acid

NCED	9-cisepoxycarotenoid dioxygenase
ncRNA	Non-coding ribonucleic
NR	Non-Redundant
ROS	Reactive oxygen species
Rubp	Ribulose 1,5-bisphosphate
RWC	Relative water content
SOD	Superoxide dismutase
SNP	Single nucleotide polymorphism
SMP	Streptavidin-coated magnetic particle
SMC	Soil Moisture Content
SSH	Suppression substractive hybridization
SWP	Shoot water potential
TBK	Tea Board of Kenya
TRFK	Tea Research Foundation of Kenya
TW	Turgid weight

CHAPTER ONE

INTRODUCTION

1.1 Background information

Tea (*Camellia sinensis* L. (O) Kuntze) is one of the most widely consumed beverages in the world, only second to water. Popularity of this beverage is due to its aroma, pleasant taste and medicinal benefits (Lin *et al.,* 2003). The tea plant from which the beverage is processed is an evergreen bush that grows to 15m high in the wild, and 60 – 100 cm under cultivation to facilitate commercial harvesting either by hand or machines (Vo, 2006). Tea best perfomes in tropical and subtropical areas with adequate rainfall and well drained acidic soils (Graham, 1999). In Kenya, tea was first introduced in 1904 by the British settlers as a cash crop (Matheson, 1950). As of 2002 the crop covered around 157,720ha in the highlands East and West of the Great Rift Valley in Kenya as shown in Figure 1, (Wachira, 2002). These areas receive rainfall of above 1400mm distributed throughout the year, and temperature range from 13-30^{o}C (Willson and Clifford, 1992).

Tea contributes 26% of the total foreign exchange earnings annually and 4% of the Gross Domestic Product in Kenya (Wachira and Ronno, 2004). In 2011, Kenya earned Kshs 109 billion from the sale of 377.9 million kilogram of tea (TBK, 2012). Kenya is a leading exporter of black tea in the world contributing about 22% of the manufactured product (TBK, 2012). The crop offers a direct source of livelihood to about 8% of the total rural population (Mbadi and Owuor, 2008). Tea farming also contributes to environmental conservation through enhanced water infiltration, reduced surface erosion, and mitigation of global warming through carbon sequestration (Cheserek, 2011). Tea is rich in polyphenols and has been associated with numerous pharmacological properties including antidiabetic, antimicrobial (Koech *et al.,* 2013), anticancer, anti-inflammatory, anti-aging (Khan and Mukhtar, 2007) antioxidant (Karori *et al.*, 2007; Kerio *et al.*, 2013), and anti-malarial properties (Sannella *et al.*, 2006). Cultivated tea is diploid with 2n=2x=30 chromosomes (Bezbaruah, 1971; Kondo, 1977) though numerous stable autopolyploids exist (Wachira, 1994). The genome of diploid tea is about 4.0 Gigabases in size (Tanaka and Taniguchi, 2006). Cultivated tea consists of three taxa: *C. sinensis* var. *sinensis* (Chinary type), *C. sinensis* var. *assamica* (assam type) and *C. sinensis* var. *Assamica* ssp *Lasiocalyx* (Cambod type). The three varietal taxa are differentiated by their morphological, biochemical and molecular affinities (Magoma *et al.,* 2003; Wachira and Waugh, 1995; Wachira *et al.,* 2001).

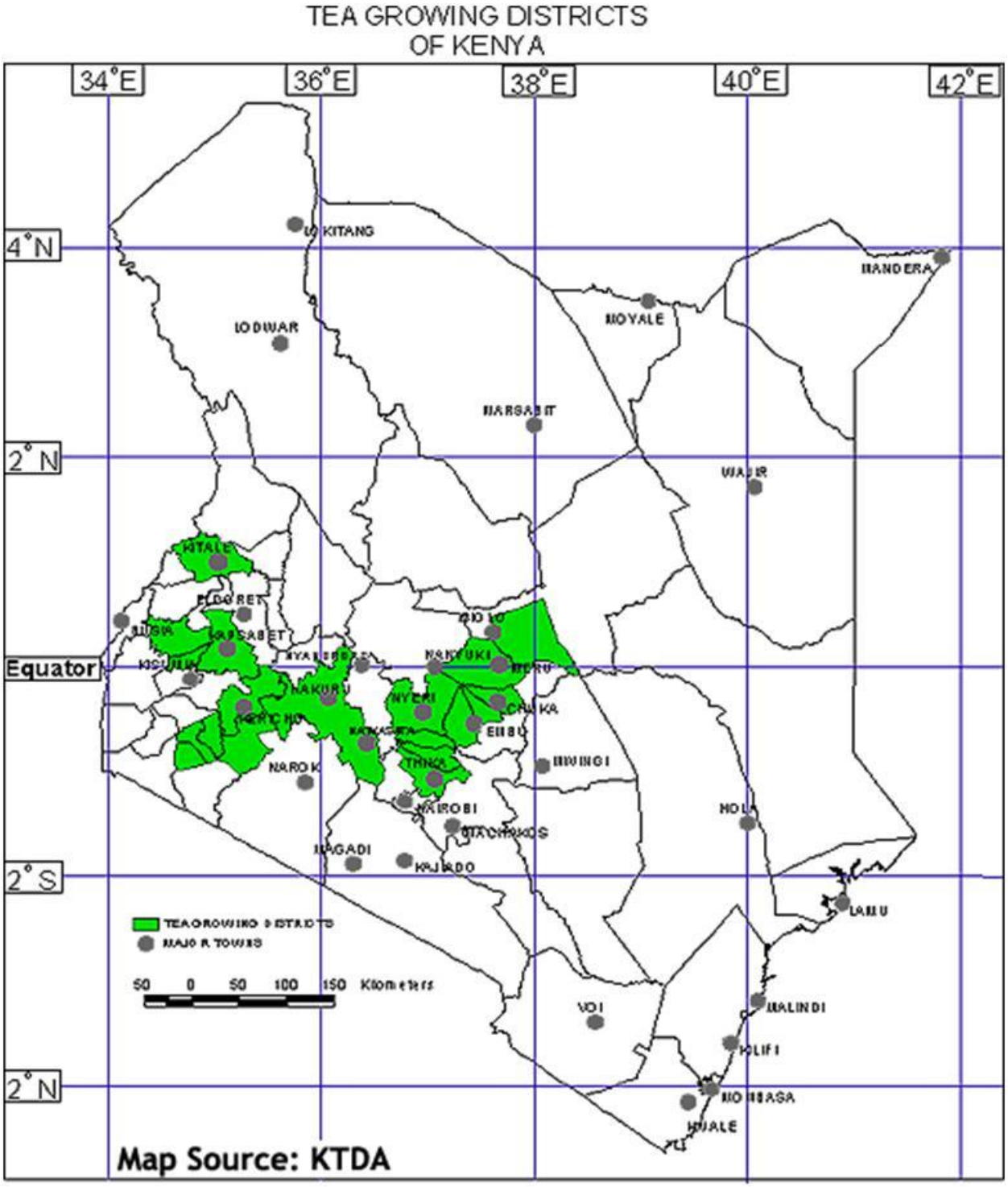


Figure 1; Map of tea growing areas in Kenya.

Tea farming is greatly influenced by environmental conditions, with drought as the most limiting factor affecting crop growth and productivity (Cheruiyot *et al.,* 2010). The global climate change which has been associated with unprecedented weather phenomena has resulted in prolonged drought periods and freak frost incidences (Otieno *et al.*, 2002.). Being a rain-fed crop tea is significatly affected by drought with droughted plants responding by wilting, scorching and eventually death. These responses are attributable to reduced rate of photosynthesis and altered metabolic processes. The drought in turn leads to loss of green leaf production and hence reduced income to farmers (Cheserek, 2011).

Water deficit resulting from drought is characterized by plant water loss exceeding the absorption rate. As a protection strategy, plants employ various physiological, biochemical and molecular response mechanisms to cope with adverse conditions including water stress. During drought, the roots are the primary sites involved in perception of change in soil moisture content (Das *et al.,* 2012). Roots send signals to the leaf tissues of the plant which in turn respond by lowering the leaf water status comprising of relative water content, water potential and gas exchange parameters including net photosynthesis, stomatal conductance and evapotranspiration rate (Bota *et al.,* 2004b). Plant growth rate is also affected by water deficit (Shakeel *et al.,* 2011). At the biochemical level, plants respond by accumulating some organic compounds such as proline, glycinebetaine and non-reducing sugars such as raffinose. These osmolytes are secondary compounds that significantly contribute to plants ability to ameliorate the effects of the stress factor (Hare *et al.,* 1998). The tea plant is rich in flavanoid compounds some of which may serve as osmolytes. Precursor molecules of the flavanoids include malonyl-CoA from carbohydrates metabolism and P-coumaronyl-CoA from the phenylpropanoid pathway (Magoma *et al.,* 2000). The presence of these metabolites therefore may be used as an indication of plant response to stimuli resulting from internal or external stress factors (Magoma *et al.,* 2000). However, biochemical compounds associated with drought are not well elucidated in tea (Cheruiyot *et al.,* 2007). Plants also respond to water deficit through molecular changes that induce tolerance through expression of responsive genes (Shinozaki and Yamaguchi-Shinozaki, 2007). These genes are responsible for the synthesis of several metabolites and proteins, some of which may be responsible for conferring defences to stresses and / or repair of damage caused by drought (Shinozaki and Yamaguchi-Shinozaki, 2007). Various studies are geared towards elucidating the genetic basis of adaptive response to water stress and their utilization in the breeding programmes. Following the availability of genome sequencing data, gene expression profiles have been

used to identify genes involved in adaptive response to drought. The essentials of such studies relate to identification of drought-inducible genes which posses' important functions for drought tolerance (Kin-Won *et al.,* 2011). This is an essential step in understanding the molecular mechanism of stress response and developing plants with enhanced tolerance to water stress (Kin-Won *et al.,* 2011).

With the advancement in technology, improved techniques for determining the genome and or transcriptome profile of an organism have been developed. The Next Generation Sequencing technique is one such approach which comprises of llumina/Solexa, Applied Biosystems SOLiD and Roche/454 pyrosequencing. These techniques are fast, simple, cost effective and provide a more comprehensive and efficient way to measure transcriptome composition, obtain RNA expression profiles and discover new exons and genes (Shi *et al.,* 2011). This method has particularly been used in tea to determine the candidate genes for major metabolic pathways (Shi *et al.,* 2011). In this study, Roche/454 pyrosequencing was used to identify novel genes that are expressed by tea plants upon exposure to water stress. The new insight is essential for development of plants with enhanced tolerance to water stress. Physiological and biochemical responses of the tea plant to water stress were also determined in this study.

1.2 Statement of the problem

Increasing incidences of drought, rising temperatures and freak weather phenomena like frost and hail significantly affect tea cultivation in Kenya. The changing climate scenario is projected to result in some of the traditional tea growing areas in Kenya losing their suitability for tea cultivation. Although tea breeders have endeavoured to develop drought tolerant tea cultivars, these initiatives have been constrained by lack of better understanding of tea plant responses to drought. Indeed, the physiological, biochemical and molecular responses of tea to water stress are poorly understood. The techniques that involve the use of molecular markers that would otherwise have been very powerful in resolving the above challenges have been limited by lack of adequate genetic information on the key genes coding for the drought response traits of interest in tea.

1.3 Objectives

1.3.1 General

To determine the responses of tea (*Camellia sinensis)* to water deficit.

1.3.2 Specific objectives

1. To determine the effects of water deficit in plant water status and gas exchange parameters of tea plants.
2. To determine changes in proline and glycinebetaine profiles in tea due to water deficit.
3. To identify and characterize water stress responsive genes in tea plants.

1.4 Hypotheses

1. Water deficit does not influence water status and gas exchange parameters of tea plants.
2. Water deficit does not influence proline and glycinebetaine profiles in tea plants.
3. Drought responsive genes in tea plants cannot be characterized and identified.

1.5 Justification of the study

Tea is Kenya's main foreign exchange earner. It is also a source of livelihood to over three million Kenyans. However, due to global and local climate changes, drought is increasingly becoming a challenge to tea cultivation. During dry seasons, there is a significant decline in green leaf production by 14-33%, increase in unemployment and reduction in farmer's livelihood. In order to mitigate the adverse effects of drought and adapt to effects associated with climate change, drought tolerant tea cultivars need to be developed.

There has been rapid progress in understanding the tea plant and important insights have been reported on the genomic makeup of the species through molecular marker based techniques. These reports have provided useful information concerning the traits responsible for various plant stresses. However, lack of adequate genomic tools and genomic knowledge on a representation of novel genes and the regulatory networks underlying important traits in tea has contributed to lack of genetic information for modern breeding and tea improvement. Use of genome sequencing technologies coupled with application of bioinformatics to

identify expressed genes is thus a viable option towards identifying genes associated with drought which can then be used in tea improvement initiatives for development of drought tolerant cultivars.

1.6 The scope and limitation

The present study determined the physiological, biochemical and molecular response of different tea cultivars to varying soil moisture content. Leaf proline and glycinebetaine profiles were analysed in the test cultivars in order to determine their use as potential indicators of drought tolerance. Molecular and bioinformatics tools were used to determine the candidate genes responsible for drought response in tea.

CHAPTER TWO

LITERATURE REVIEW

2.1 Tea plant

The tea plant, *Camellia sinensis* (L.) O. Kuntze, is a woody plant belonging to the genus *Camellia* (Chang and Bartholomew, 1984) in the family Theacea, a native to China (Taniguchi *et al.,* 2012). It is a perennial, cross-pollinated plant used as a model in self-incompatibility studies (Wang *et al.,* 2008). It is highly heterogeneous with most of it's morphological, physiological and biochemical descriptors showing continuous variation (Lai *et al.,* 2002). The tea plant has a very large genome of 4.0 Gigabases (Tanaka and Taniguchi, 2006) with 15 basic chromosomes. The tea genome is larger than that of human (3.1 Gb), rice (389 Mb) and *Arabidopsis thaliana* (120 Mb), but not wheat (16 Gb) (Taniguchi *et al.,* 2012). Most *Camellia* species including tea form a stable polyploid series though most cultivated tea accessions are diploid (2x=2n=30). Other relatives of tea like the *Camellia irrawadiensis*, *Camellia caudate* and *Camellia kissi* are also diploid. The constancy in diploid chromosome number suggests a monophyletic origin of all tea species. Most *Camellia* taxa from the Japanese origin deviate from the normal chromosome number; for example *Camellia sasanqua* is hexaploid or tetraploid (2n=90 or 2n=60), *Camellia sinensis var. macrophylla* is triploid (2n=45), while *Camellia rosaeflora* is tetraploid (2n=60). In Kenya, natural polyploids including; tetraploids (2x=4n=60) and triploids (2x=3n=45) have been reported (Wachira and Kiplangat, 1991). Identified polyploids in tea have better rooting ability than the diploids and hence are resistant to environmental stress, though they are poor yielders (Bezbaruah, 1968). Polyploids have also been found to be vigorous, and have higher content of total polyphenols than the diploids (Magoma *et al*., 2000; Wachira, 1994; Wachira and Ngetich, 1999).

Generally, there are three main varieties of tea; *C. sinensis* var. *sinensis* (China type), *C. sinensis* var. *assamica* (assam type) and *C. sinensis* var. *Assamica* ssp *Lasiocalyx* (Cambod type). The three varieties freely interbreed, making the tea plant highly heterogenous and heterozygous (Willson and Clifford, 1992). The varieties are differentiated by foliar, floral, growth features, biochemical and molecular affinities (Magoma *et al*., 2003; Wachira and Waugh, 1995; Wachira *et al*., 2001).

Commercially grown teas are hybrids of Assam (*var assamica*) and china (*var sinensis*) distinct ecotypes. Due to distinctive difference in the ecology of their origins, the two ecotypes and their putative hybrids exhibit considerable variation in their ecophysiology (Carr and Stephen, 1992). The China type is known to be a stronger ecotype than the Assam, and is also more resistant to drought conditions. However, it is considered to be inferior in both quality and yield (Carr and Stephen, 1992). A study to compare tea germplasm from Kenya (mostly Assam varieties), China, Japan and Taiwan (mostly chinery variety) based on the levels of total polyphenols and yield showed that Kenyan cultivars were superior in both attributes (Wachira and Kamunya, 2005).

Other wild species of *Camellia* including*; C sinensis* var. *bulsanensis, C.*var *dehungensis, C. taliensis, C. furfucea, C. japonica* and *C. Gymnogyma* (Ackerman, 1973; Chang and Bartholomew, 1984; Takeda, 1990) have been found to hybridizes well with tea and thus scientists have always been interested in identifying such hybrids due to their suspected involvement in tea domestication (Vo, 2006).

2.2 Economic importance, social and health benefit of tea in Kenya

Tea is the Kenya's main foreign exchange earner, contributing 26% of total foreign exchange earnings annually and 4% of the Gross Domestic Product earnings (Wachira and Ronno, 2004). In 2011 the country earned Kshs 112 billion from the sale of 369.2 million kilogram of tea (TBK, 2012). Tea industry in Kenya is largely based in the rural areas where larger population of Kenyans lives. Over 62% of tea is produced by the smallholder sub-sector offering a direct source of livelihood to about 10% of the total population (over 3 million people) (Mbadi and Owuor, 2008). Its contribution towards poverty eradication and infrastructural development in the rural areas is enormous. It also contributes to environmental conservation through enhanced water infiltration, reduced surface erosion, and mitigation of global warming through carbon sequestration (Cheserek, 2011). Tea is composed of several biochemical molecules which contributes to its medicinal value (Bukowski and Percival, 2008; Jia *et al.,* 2005; Khan and Mukhtar, 2007; Monobe *et al.,* 2008). They include; polyphenols, antioxidants, saponin, polysaccharide, L. Theanine, pigments and tea water extracts. The medicinal properties associated with such biochemical compounds include; antidiabetic, antimicrobial (Koech *et al.,* 2013), anticancer, anti-inflammatory, antiaging (Khan and Mukhtar, 2007), antioxidant nature (Karori *et al.,* 2007; Kerio *et al.,* 2013), and antimalarial properties (Sannella *et al.,* 2006)

2.3 Factors affecting growth and productivity in tea

Plants are often exposed to a plethora of abiotic and biotic stress factors. Abiotic stresses like low temperature, salinity and water stress are the principal causes of crop failure that dip average yields of most crops by more than 50% (Mahajan and Tujeta, 2005). Among the abiotic stress factors, drought is one of the major and ever present threats that affect plant growth and yield. Overall, these stress factors are a menace and prevent the plant from reaching its full genetic potential.

2.4 Drought in tea

Drought, which is the soil and or atmospheric water deficit resulting from extreme temperature, is the most common abiotic stress that plants encounter. It is experienced by the plant when water supply to the roots is less than water loss through transpiration (Shakeel *et al.,* 2011). This leads to adverse effects on the plant through disruption of water potential gradient, loss of turgor, denaturation of proteins and disruption of plant membranes (Kozlowski and Pallardy, 2002). Drought also impairs plant growth and development, hence limiting plant production and performance more than any other environmental factor (Shao *et al.,* 2009). In view of the various climate change models, it has been suggested that the area under drought is expanding (Burke *et al.,* 2006). Global warming is expected to bring about escalation in water deficit by increasing evapotranspiration. This is expected to lead to an increase in frequency and intensity of drought with drought prone areas expected to increase from current 1% to 30% by 2100 (Fischlin *et al.,* 2007). Crop loss due to drought exceeds losses resulting from other factors combined (Chaves *et al.,* 2003).

Tea is vulnerable to water stress. Being a rain-fed crop, it depends on minimally but well distributed rainfall averaging 1200 mm. The productivity of tea lands is therefore largely dependent on soil water. However, most of the world tea-growing areas are prone to drought and hence the tea plant is subjected to water deficit stress (Mondal, 2008). Rise in temperature, increase in ambient CO_2 concentration and drought brought about by global and local climate change can affect production and quality of tea through the influence on carbohydrate assimilation, respiration, evapotranspiration and also gene expression (Levitt, 1980; Xin and Browse, 2000). Studies carried-out in TRFK (Ngetich *et al.,* 2001) showed that droughts impact negatively on tea production and result in a loss of 14-20% in yields annually and about 6-19% plant mortality. Severe drought often leads to scorching of leaf

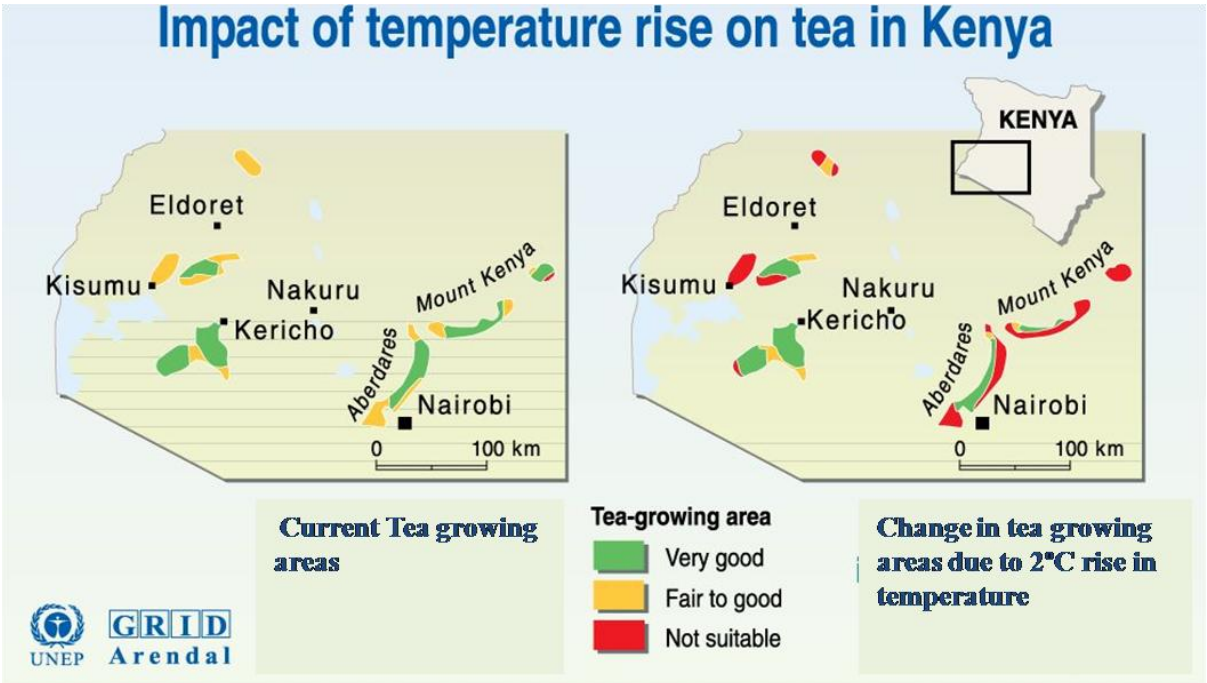
and wilting which may lead to death of crop, resulting in high cost of infilling. Tea plants that are actively growing under optimum water availability maintains the green leaf colour due to the rich chlorophyll content (Fig. 2). Under minimal water availability, the fields look brownish due to leaf scorching and eventual dieing of tea bushes (Fig. 3).

Figure 2.Cultivated tea plants under optimum water availability

Figure 3.Cultivated tea plants under minimal (drought) water availability

Freak weather phenomena are increasingly being experienced in tea growing areas as a consequence of global weather change. Reports indicate that with global weather change, the suitability of some of the tea growing areas is projected to change by 2050 (Managua, 2011). Areas near Nandi will show slight decrease in suitability by 2020 and by 2050 the area is expected to have lost up to 40% suitability (Managua, 2011). This area currently experiences incidences of hail and frost regularly.

Tea producing zones have an optimum altitude of between 1500 and 2100 masl but with global warming, this is expected to increase to altitude of between 2000 and 2300 masl by 2050 (Managua, 2011). By this time, areas with altitudes ranging from 1400 and 2000 masl will suffer the most in terms of decrease in suitability while those at around 2300masl will have had increased suitability for tea farming. Since altitude is correlated with temperature and related variables, temperatures are expected to rise with decreasing altitude and this will have significant impact on tea farming due to decreased soil water availability. Most of the tea growing areas which are fairly suitable for tea farming will loose their suitability as shown in figure 4**.**



Source: Otto Simonett, *Potential impacts of global warming*, GRID-Geneva, case studies on climate change. Geneva, 1989.

Figure 4. Responses to changing climatic condition in suitability of tea growing regions in Kenya.

The rise of temperature mentioned in this report has been experienced in Tea Research Agro-meteorological stations, where there has been an annual rise of 0.02ºC. Analysis of data on

rainfall pattern has also shown that soil water deficit has been increasing by about 3mm annually since 1950s (Cheserek, 2011). The above changes in environmental conditions will therefore continue to exert significant stress to tea plants which must in turn elicit appropriate responses to survive.

2.5 Plant response to drought stress

Plants respond to drought through complex processes that involve adaptive changes or deleterious effects and that are physiological or biochemical due to the changes in water homeostasis (Chaves *et al.*, 2003; Xu *et al.*, 2009). Decrease in cellular water brings about decline in chemical activity and loss of turgor in the plant cell, stomatal closure, repression of cell growth, photosynthesis and activation of respiration. Plants also respond and adapt to water deficit at molecular levels through diverse functions where genes are induced or repressed by stresses (Bartels and Sunkar, 2005; Shinozaki *et al.*, 2003), (Figure 5). The responses act as defense mechanisms to counter the effects of water stress and ensure optimal growth condition.

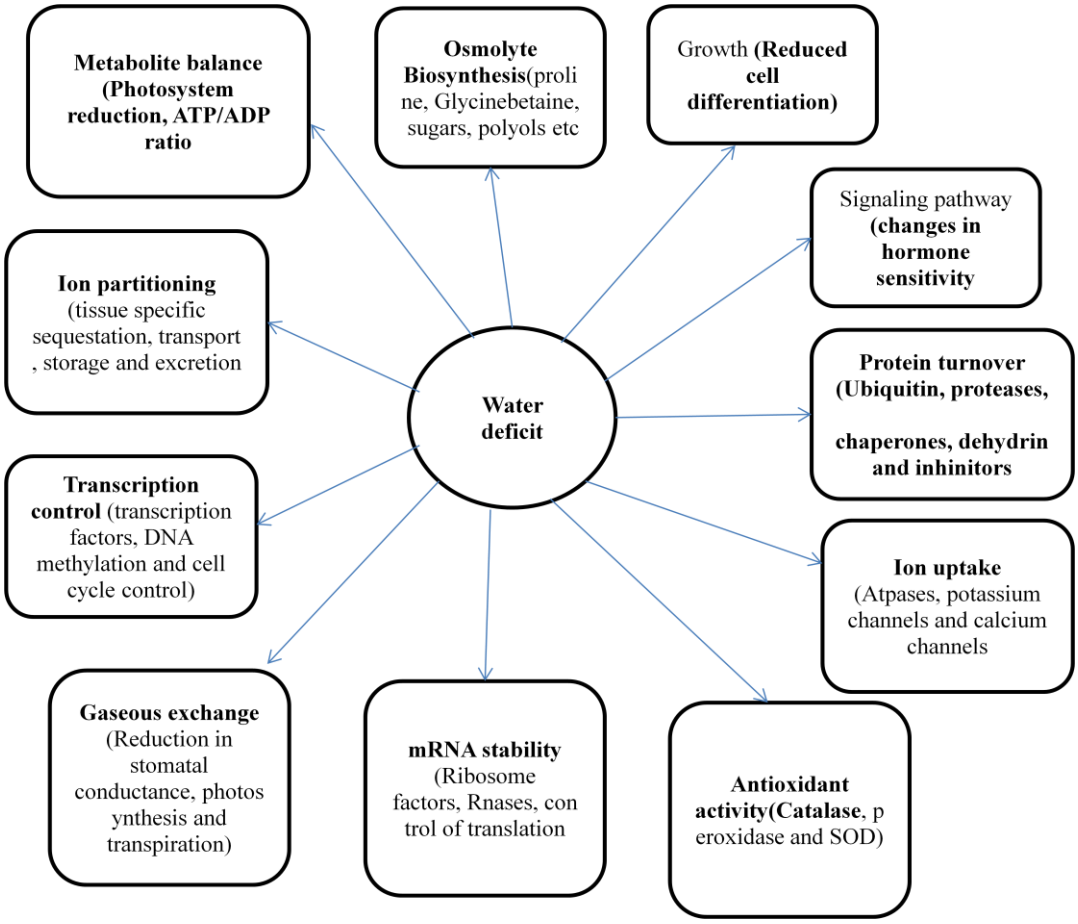


Figure 5; Responses to water deficit in plants (Bohnert *et al.*, 1995) .

Understanding how plants respond to water deficit is essential in the development of stress tolerant varieties (Reddy *et al.,* 2004). The response of plants to water stress depends on plant species, soil condition and the stress exposure period. Generally, these responses occur through complex mechanisms which include the following; escape by completing plant life cycle before severe water deficit, avoidance by enhancing capacity to acquire water by developing root systems (Jackson *et al.,* 2000), plant tolerance mainly through improving osmotic adjustment ability and increasing cell wall elasticity to maintain tissue turgidity, plant resistance by altering metabolic paths for its survival under severe stress for example through increased antioxidant metabolism (Bartoli *et al.,* 1999), through removing some parts of the plant such as the shedding of older leaves (Chaves *et al.,* 2003), plant evolution under long-term drought conditions through genetic mutation and genetic modification (Cheruiyot *et al.,* 2007; Sherrard *et al.,* 2009).

Effects of water deficit in plants occurs either immediately for example by phosphorylation of proteins or can occur over a period of time for example through gene expression (Chakeris, 2009). Plants that can maintain growth during water deficit conditions can be used to identify genes and metabolic pathways that are induced as a result of drought. Such plants are referred to as drought tolerant plants. These plants can be used to study the physiological, biochemical and molecular responses to water stress.

2.5.1 Cellular and Physiological responses to water deficit

The roots are the primary sites involved in perception of water deficit in a plant and are asssumed to trigger tolerance mechanisms (Das *et al.,* 2012). The roots send signal to the leaves causing a reduction in leaf water potential, photosynthesis and stomatal conductance (Bota *et al.,* 2004b). The visible signs of water stress are first evident on the leaves due to wilting and drying. Decrease in water potential leads to reduction in cell growth, root growth and shoot growth. This is due to reduction in stomatal conductance which in turn brings about decline in photosynthesis (Tezara *et al.,* 2002). Photosynthesis is sensitive to water stress and as leaf water content and water potential decreases in C_3 and C_4 plants, stomatal limitation is experienced by the plant. Tea plants exhibit C_3 mechanism of photosynthesis (Janendra *et al.,* 2007). In this mechanism, the carbon dioxide accepting compound is Ribulose bisphosphate (RuBP) (*http://www.differencebetween.com/difference-between-C_3-and-vs-C_4-plants).* The photosynthetic process in tea plants occurs mostly in the leaves although the stems also

contribute to CO_2 assimilation. However, the efficiency of CO_2 fixation by mature stems of a tea plant is low compared to that of leaves (Janendra *et al.,* 2007). Stomatal closure, is the initial event in plants response to drought leading to limitation in carbon uptake in the leaves. These physiological changes are induced in the plant to counteract effects of decline in water potential or leaf turgor. Stomatal closure due to drought, restricts CO_2 entry into the leaf and decrease water loss from the leaves. When water potential is reduced from -1.0 to -2.0Mpa, cells become smaller and leaves less develop resulting in reduced photosynthetic area and slowed ion transport. This may ultimately lead to decrease in yield (Medrano *et al.,* 2002).

Since the cell membrane is the primary target of many plant stresses, osmotic stress induces rapid changes in cell wall conductivity and plasmalemma (Chazen and Neumann, 1994). Cell permeability and regulation during water stress is accomplished by openning and closing of water channels. Water stress affects regular metabolic processes of the cell like carbon-reduction cycle, light reactions, energy charge and proton pumping which may lead to production of toxic molecules like the reactive oxygen species.The morphological effects of water stress in a plant are easily visualized but the cause of this symptoms are not well ellucidated at both biochemical and molecular level (Bruce *et al.,* 2002).

Abscisic acid (ABA) also plays an essential role in physiological response of plants to water stress. ABA is a plant hormone involved in stress response. It's accumulated by many plants when they are exposed to drought. ABA is synthesized *de novo* from xanthophylls basically in response to water deficit where it is known to mediate signals that bring about expression of stress-related genes followed by synthesis of quartenary compounds and compatible osmolytes like glycinebetaine and proline respectively (Ashraf and Foolad, 2007). Three genes involved in ABA biosynthesis have been isolated, they encode abscisic aldehyde oxidase , zeaXanthinepoxidase and 9-cisepoxycarotenoid dioxygenase (NCED) (Satoshi *et al.,* 2001). Studies based on genetic and genomic analyses indicate that over-expression of 9-cisepoxycarotenoid dioxygenase (NCED) improves drought stress tolerance (Mohammad and Lin, 2010). In plants like maize, beans and tomato, expression of NCED genes has been shown to be induced by drought. ABA plays important roles in various physiological processes including induction of seed dormancy and adaptive responses to environmental stresses (Nambara *et al.,* 1998). ABA also regulates many important aspects of plant growth and development including seed maturation and water relations in plants. This therefore means that it's accumulation in plant tissues subjected to water stress acts as a signal for the initiation of acclimation (Shinozaki and Yamaguchi-Shinozaki, 1997). In response to soil

water deficit, ABA is accumulated leading to alteration in ion transport of guard cells which causes closure of stomatal pores, resulting in reduction of plant transpirational water loss. Studies have shown that the level of ABA in water stressed plants stimulates the release of potassium by stomatal guard cells which leads to stomata closure. This involves the action of Abscisic acid-activated protein kinase (AAPK) which is activated by ABA. The same does not apply in conditions of darkness or elevated carbon dioxide level which also trigger stomata closure (Assmann *et al.,* 2000). Insufficient potassium levels inhibit water stress induced stomata closure and resistance to low water conditions. This can be one of the main causes of dehydration in plants growing in low potassium soils (Benlloch-Gonzalez *et al.,* 2007).

Dehydration in plants results in elevated levels of ABA, which triggers the synthesis of some proteins responsible for tolerance. Most of the drought stress-induced genes studied to date are induced by ABA and many aspects of stress response are also mediated by the ABA-dependent mechanisms (Shinozaki and Yamaguchi-Shinozaki, 2000). ABA also acts as a long distance chemical signal, which can provide information on the water avalability in the soil. ABA plays an important role in regulating the droughted plants in response to perturbations at the root even when xylem ABA concentrations are not increased (Wilkinson and Davies, 2002).

2.5.2 Biochemical responses to drought induced water deficit

When plants are subjected to unfavourable weather conditions including drought, they respond by synthesising compatible solutes like glycinebetaine, osmolytes such as proline, soluble sugars such as trehalose and mannitol, compartmentalisation of ions and scavenging of reactive oxygen species (Zhu, 2002). Accumulation of these molecules allows plant to take up additional water from the environment for buffering the effects of reduced water levels in the plant, they also do not intefere with biochemical reactions in a plant (Jaleel *et al.,* 2007). The amino acid Proline occurs widely in higher plants and accumulates in larger amounts than other amino acids in response to environmental stress (Ashraf and Harris, 2004; Irigoyen *et al*., 1992; Kohl *et al*., 1991). In response to drought, proline accumulation occurs in the cytosol where it adjusts cytoplasmic osmosis. Proline is synthesized through L-glutamate pathway in plants (Szabados and Savoure, 2009) (Fig.6).

Drought induced accumulation of proline has been related with the increased contents of its precursors including; glutamic acid, arginine and ornithine. The main precursor of

proline in plants is glutamic acid (Ashraf and Foolad, 2007). Proline accumulation has been correlated with the stress tolerance and its concentration has been shown to be higher in stress tolerant than stress sensitive plants (Ashraf and Foolad, 2007). Proline plays an essential role in plant responses to water stress by helping in regulation of osmotic adjustment, scavenging of free radicles, acting as a stabilizer of sub-cellular structures, serving as a sink for energy and also as a stress signal. Proline is also involved in reducing the photo-damage in the thylakoid membranes by scavenging for reactive oxygen species, confers protection to membranes and proteins and reduces denaturation of enzymes (Kavi-Kishor *et al.,* 1995). Proline also serves as a nitrogen storage product in plant tissues (Hare *et al.,* 1998). Other than protecting of plants during stress conditions, proline breakdown upon relief of stress provides sufficient reducing agent that support mitochondrial oxidative phospohorylation and generation of ATP for recovery from stress and repair of stress induced damages.

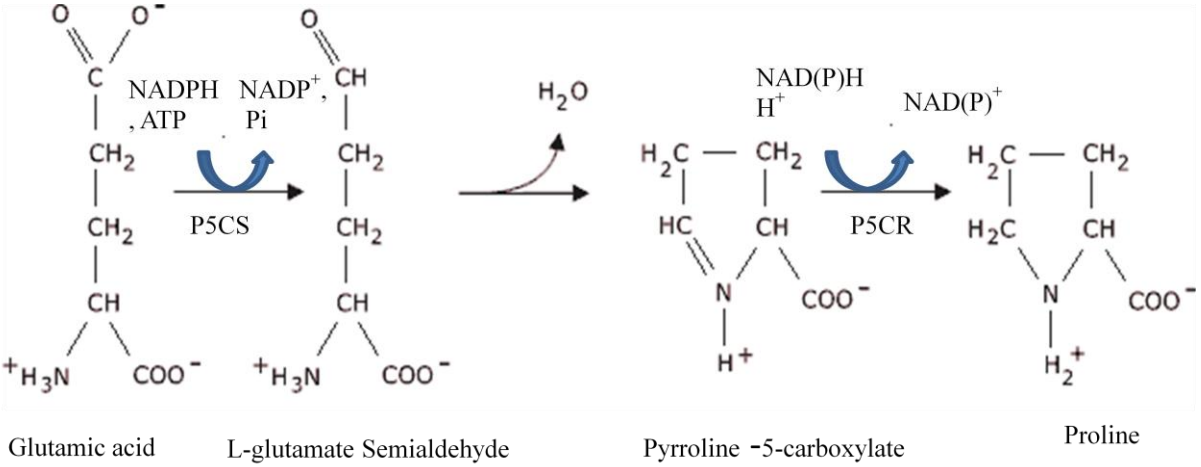


Figure 6; Biosynthetic pathway of proline, (Szabados and Savoure, 2009); P5CS and P5CR are the pyrroline-5-carboxylate synthase and pyrroline-5-carboxylate reductase, respectively.

Two enzymes pyrroline-5-carboxylate synthatase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) play a critical role in proline biosynthetic pathway. Studies have shown that *P5CS* and *P5CR* genes are expressed when plants are subjected to water stress (Parvaiz and Satyawati, 2008). In the model plant, *A. thaliana*, proline synthetase gene is up regulated in drought stress resulting in resistance to drought while suppression of the gene results in increased susceptibility to drought (Bartels and Sunkar, 2005). Genes responsible for catabolism of proline are suppressed during drought.

Glycine betaine (GB) is an amphoteric compound that is electrically neutral over a wide range of physiological pH values. It is extremely soluble in water but includes a non-polar hydrocarbon moiety that consists of three methyl groups. The molecular features of glycinebetaine allow it to interact with hydrophilic and hydrophobic domains of macromolecules, such as enzymes and protein complexes. In higher plants, glycinebetaine is synthesized in chloroplast and it accumulates in this organelle in response to dehydration stress where it plays a pivotal role in adjustment and protection of thylakoid membrane structure (Ashraf and Foolad, 2007). Glycinebetaine is also essential in protecting the photosynthetic machinery of plants like the ribulose-1,5-bisphosphate carboxylase oxygenase, Rubisco and photosystem II (PSII) complex from inactivation and dissociation into subunits (Sakamoto and Murata, 2000). Glycinebetaine synthesis occurs through a two-step oxidation of choline to betaine aldehyde then to glycinebetaine. The two steps are catalysed by two enzymes choline monoxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). Expression of these two enzymes in the leaves brings about the synthesis of glycinebetaine (Figure 7).

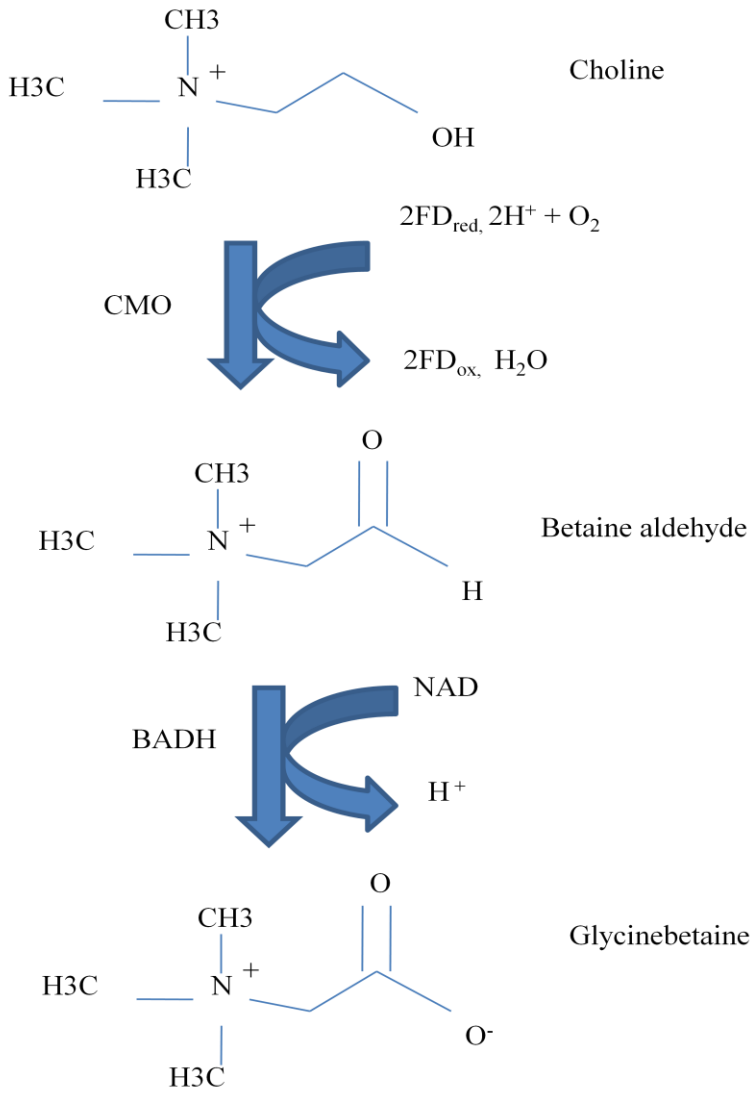


Figure 7: Biosynthetic pathway of glycinebetaine; BADH (Betainealdehyde dehydrogenase), CMO (Choline mono-oxygenase) (Parvaiz and Satyawati, 2008).

Although other pathways such as direct *N*-methylation of glycine is also known, the pathway from choline to glycinebetaine has been identified in all Gb-accumulating plant species (Weretilnyk *et al.*, 1989). When plants are stressed, the genes coding for the two enzymes, *CMO* and *BADH* genes, are up-regulated and expressed in the leaves and roots. In plants that accumulate glycinebetaine in response to stress, the tolerant genotypes accumulate more than the sensitive genotypes. However the relationship is not universal in all plant species, for example in *Citrus limon*, *Triticum agropyron* and *Elymus* the sensitive genotypes accumulate more than the tolerant ones. The relationship between glycinebetaine and stress tolerance is thus likely to be species or genotype specific(Ashraf and Foolad, 2007).

On the other hand, soluble sugars like glucose, fructose and sucrose are often associated with drought tolerance in plants. They play a critical role under water deficit conditions through their the hydroxyl group of that act as water molecule to maintain hydrophilic interaction in the membrane and proteins through hydrogen bonding. They also form a highly viscous layer in the cytoplasm of dehydrated cells reducing the molecular movement and helping to maintain structural stability of macromolecules within a plant (Sun and Leopold, 1997). Studies using transgenic plants have shown that genes involved in metabolism of sugar increases ability of plants to resist water stress (Shinozaki, 2007).

In *Arabidopsis thaliana*, over-expression of galactinol synthetase gene (*AtGolS)* responsible for synthesis of galactinol and raffinose sugars results in drought tolerance (Shinozaki, 2007). Trehalose sugar is also a factor in tolerance to environmental stress conditions, including drought. This is due to its high water retention capacity that helps stabilize proteins and membranes thus enabling cells under dehydration conditions to recover (Thevelein, 1996).

2.5.3 Accumulation of Reactive Oxygen Species

Environmental stresses such as drought stress, heat stress, chilling stress, salt stress and plant diseases induce the accumulation of reactive oxygen species (ROS) that cause diminished plant performance (Grill *et al.,* 2001). The ROS includes; singlet oxygen (O_2^-), superoxide (O_2), hydroxyl (OH^{-1}) and hydrogen peroxide (H_2O_2). Accumulating evidence seems to indicate that oxidative damage to critical cell compounds results from attack by ROS. These damages impact on antioxidant metabolism and results into eventual cell death (Bian and Jiang, 2009; Foyer and Noctor, 2005; Smirnoff, 1993). Plants respond to rising ROS by regulating gene expression and inducing a variety of enzymatic and non-enzymatic

mechanisms that metabolize ROS into less harmful chemical species (Jiang and Huang, 2001). An example of an antioxidant enzyme is the superoxide dismutase (SOD) (Naya *et al.,* 2007; Upadhyaha *et al.,* 2008). The activity of these enzymes increases in plant cells as a response to environmental stresses and they are thus responsible for defense against oxidative stress (Foyer, 2001.; Jiang and Huang, 2001).

2.5.4 Variation in polyphenol profiles

Studies by (Cheruiyot *et al.,* 2007) showed that the level of polyphenols could be a potent indicator of drought tolerance in tea and could hasten the development of water-stress adapting cultivars. In his research, he found out that there is fluctuation in polyphenol levels with changes in soil water content. Water is essential in plant photosynthetic processes and directly influences the synthesis of secondary metabolites. Tea cultivars with stable polyphenols are tolerant to water stress (Cheruiyot *et al.,* 2007). In another study, the level of shoot catechins in tea was found to respond to soil water level shoot catechins in tea was found to respond to soil water level (Cheruiyot *et al.,* 2008b). However the various catechins in tea were not influenced uniformly by the water stress and the difference was attributed to their chemical differences.

2.5.5 Molecular events in plants subjected to water stress

Drought tolerance is a complex quantitative trait regulated by several genes (Shinozaki and Yamaguchi-Shinozaki, 2007). It involves multiple mechanisms that act in combination to avoid or tolerate periods of water deficit. It is well-established that, under water stress, the genes involved in osmotic and redox homeostasis are regulated and hormones such as ABA will participate in the response process. These molecular changes occur within the cell or specific tissue and they also include expression level of LEA dehydrin-type genes, synthesis of molecular chaperones, which help in protecting proteins from degradation, and proteinase responsible for removal of damaged and denatured proteins (Mahajan and Tujeta, 2005). Since plant responses are controlled by the genome, most studies are focused on the molecular response on plant to water-deficit stress (Mohammad and Lin, 2010). To characterize fully the mechanism of tolerance and adaptation to water deficit, a better understanding of genes that are expressed is necessary. There are several classes of genes that are altered in response to water-deficit stress. Among the most frequently altered genes are those involved in gene regulation, signalling and gene products

that support cellular adaptation to water stress (Mohammad and Lin, 2010). Genes with diverse functions are induced and repressed by drought stress. Majority of them are responsible for conferring a certain degree of defences to these stresses while others are responsible for repair of damage caused by drought (Shinozaki and Yamaguchi-Shinozaki, 2007). The drought induced genes in the plant *Arabidopsis thaliana* model are classified into two main categories comprising of functional proteins like osmotin, antifreeze, mRNA binding proteins, proteases, late embryogenesis abundant proteins, enzymes for osmolyte biosynthesis and water channel proteins. Genes that encode aquaporins (Fray *et al.*, 1994) are induced by drought alone, while others are induced by drought and ABA. Regulatory proteins on the other hand are involved in signal transduction and stress responsive gene expression include transcription factors, protein phosphatases, enzymes involved in lipid metabolism and protein kinases, (Mohammad and Lin, 2010). Stress inducible genes for variety of transcription factors contain typical DNA binding motifs like zinc finger and bZIP(Shinozaki and Yamaguchi-Shinozaki, 1997). These genes are thought to function not only in protecting the cells from water deficit by production of important metabolic proteins but also help in the regulation of genes for signal transduction in drought response stress (Ingram and Bartels, 1996).The induced genes are involved in morphological, physiological and molecular adaptation of plant to support growth. Drought repressed genes are also essential in survival and development of plants during water deficit (Ricardo and O'connell, 2005). Regulation of expression of stress related genes is largely by transcription factors that bind to specific cis-acting element in the promoter region of the target genes (Paul *et al.*, 2012). To identify stress functional genes therefore require the wide analysis of their transcript.

2.6 Transcriptome analysis

The complete set of messenger RNA (mRNA) and non-coding RNA (ncRNA) transcript produced by a particular cell comprises the transcriptome. The mRNA are the coding transcripts that are linked to the different cell types that express different sets of genes with different functions under varying condition. The correlation of cellular fate and function with gene expression patterns has thus been of prime interest to molecular biologists for decades. The studies on plant transcripts not only help elucidate the trancriptomes themselves but also help in the search for candidate genes modulated by stress. Various technologies including; Microarray, Nothern blotting, Sanger sequencing, Expressed sequence tag and

next generation sequencing technologies, have been developed for use on whole genome and transcriptome analysis in living organism.

2.6.1 Candidate gene approaches

Northern blot analysis was the first candidate gene-based approach to be used. However the technique had several limitations like low through-put, large amount of RNA required and hence few known transcripts detected at a time. The technique also required the use of radioactivity. The limitation of Northern-blot analysis led to the use of Reverse transcription quantitative PCR. The method gave better throughput and reduced amount of RNA used. However the transcripts detected were in hundreds and not wide scale.

2.6.2 Microarray Technology

The development of microarrays allowed simultaneous characterization of expression levels of thousands of known or putative transcripts (Schena *et al.,* 1995). The gene expression data from this technique are useful in elucidating the mechanisms of biological processes by identifying genes into functional pathways (Venkatesh *et al.,* 2008). In tea, this technique has been used to study the different genes expressed by the first and the second leaf flush. The study revealed that several genes are up and down regulated. Advances in this technique have also enabled other transcriptomics applications, such as the detection of noncoding RNAs, singlenucleotide polymorphisms (SNPs), and alternative splicing events (Mockler *et al.,* 2005). Despite their power to measure the expression of thousands of genes simultaneously, this method has limitations; notably the inability to detect novel transcripts and also to study the coding sequence of detected transcripts.

2.6.3 Sequence-Based Approaches to Studying Transcriptomes

Use of sequencing technologies to unravel the genes that are modulated by drought helps in understanding of biochemical and physiological basis of stress response in plants. Such information is essential in rationally manipulating and optimizing tolerance traits for improved productivity. DNA sequencing approaches to transcriptome analysis is advantageous in that it directly determines the identity and the abundance of a transcript. Transcriptome sequencing studies have evolved from determining the sequence of individual

cDNA clones (Stone *et al.,* 1985) to construction of cDNA sequencing libraries representing portions of the species transcriptome (Seki *et al.*, 2002).

2.6.4 Sanger sequencing technology

The development of Sanger sequencing technology provided a landmark in DNA sequencing. The Sanger sequencing is based on the electrophoretic separation of chain-termination products resulting from sequencing reactions (Voelkerding *et al.,* 2009). It has primarily been applied in novel transcript discovery (Seki *et al.,* 2002). The technology was used in the sequencing of the first human genome which was completed in 2003 after a 13-year effort. However, the use of the Sanger technology is limited by high cost involved; for example the complete human genome costed around $2.7 billion (Voelkerding *et al.,* 2009). The cloning step is complex and it results in low coverage that is insufficient to comprehensively characterize whole transcriptomes of multicellular species. The EST sequencing using Sanger method has been greatly applied in transcript discovery.

2.6.5 Expressed Sequence Tag

The expressed sequence tag (EST) referring to short and single pass sequence reads from mRNA (Adams *et al.,* 1991) has been used as the core technology in transcript discovery since they represent a section of gene expressed by tissue of plant and during certain stage of development (Mekhedov *et al.,* 2000). The development of EST was aimed at addressing the cost limitation of FLcDNA sequencing by introducing a less complete, less accurate, yet cheaper approach to the detection of expressed transcripts than was possible with sequencing FLcDNAs (Boguski, 1995). Expressed Sequence Tags (EST) provide an effective method for discovering new functional genes in plant (Bausher *et al.,* 2003; Liu *et al.,* 1995; Yamamoto and Sasaki, 1997). They help in understanding genes involved in plant growth (Sterky *et al.,* 1998) secondary metabolism (Park *et al.,* 2004), biochemical pathways (Lange *et al.,* 2000) as well as response to biotic and abiotic stress factors (Sugui and Deising, 2002). Sequencing and analysis of EST is one of the primary tools for discovery of novel genes, especially in non-model plants (Zhou *et al.,* 2012). The technique is also useful as a resource for functional genomics experiment like the gene expression analysis using microarrays (Taniguchi *et al.,* 2012). The number of genes expressed by a plant during a lifetime is estimated to be between 15,000 and 60,000. EST sequencing has been used in tea where it was established that photosynthesis related proteins were the most prevalent in tender shoots since they help make enough energy and substance for activities like growth,

cell division, elongation and differentiation (Chen *et al.,* 2005). As of September 2012, 47,400 ESTs, had been deposited in gene bank. With respect to drought, *dr1*, *dr2* and *dr3* drought responsive ESTs from leaf tissues have been reported using differential display technique (Sharma and Kumar, 2005), and another 572 EST from young roots of drought tolerant tea cultivars discovered using suppression substractive hybridization (SSH) technique (Das *et al.,* 2012). In another study, analysis of ESTs with more than three sequences, identified genes responsible for cell rescue, defense, cellular transport, metabolism, energy, protein synthesis, cell cycle and DNA processing, signal transduction, transcription and biogenesis of cellular components in drought stressed tea (Muoki *et al.,* 2012). In a drought tolerant cultivar, chaperones and defense related genes, traumatin like proteins, chitinase and heat shock proteins, were over expressed in leaf tissues (Muoki *et al.,* 2012). As a result of this technique, sequencing of cDNA library clones, generation and analysis of ESTs of *Camellia sinensis* provide mRNA expression profile and a rapid, low cost and efficient way to identify functional genes. Other than the tea ESTs, 1 Genome Survey Sequence, 2 sequences reads archives, and 770 proteins have been deposited in genebank.

Despite the decrease in cost, EST sequencing with the Sanger method was still too expensive and labour intensive for routine use. It also has low throughput, and lacks quantitation of expressed genes. There were also limitations resulting from bacterial cloning biasness; for example vector contamination, over representation of preferentially cloned sequence and lack of adequate representation of inherently un-clonable transcript (Morozova *et al.,* 2009; Mortazavi *et al.,* 2008; Simon *et al.,* 2009). Moreover, due to the low redundancy of sequencing reads, EST data are not suitable for estimating transcript abundance. Other than the inefficiency of previous technologies, the little genomic information available on tea is due to distinctness of tea from other taxa, owing to its; perennial nature, high inbreeding depression, unavailability of mutants of different biotic and abiotic stress and a large genome size of 4.0 Gigabases (Tanaka and Taniguchi, 2006). The above limitations provide opportunity for the use of advanced sequencing technologies referred to as next-generation sequencing (NGS) techniques.

2.6.6 New-Generation Sequencing Methods

For over 30 years, the Sanger sequencing technology has been the dominant approach for DNA sequencing but with the advent of high-through put sequencing technologies the Sanger technology has now been overtaken by other more esoteric and efficient methods. The

high-through put sequencing technologies are now referred to as next-generation sequencing technologies. The NGS technologies employ a common principle of massively parallel sequencing of amplified or single DNA molecules. The first application of next-generation sequencing technologies was in 2008 in which the entire human genome was sequenced within five months at a cost of $1.5 million as compared to the Sanger which took 13 years at a cost of $2.7 billion. The NGS technologies have thus rendered the sequencing process faster and cheaper (Zhou *et al.,* 2012). They are also labour efficient, less complex and with improved transcript coverage particularly in large scale genomic projects (Yann and Juan, 2010). The techniques do not require bacterial cloning of cDNAs; instead the cDNAs are sequenced directly to generate short reads (Shi *et al.,* 2011). The sensitivity of the technique allows detection of low abundant transcripts. RNA-sequencing is not restricted to detecting transcripts corresponding to existing genomic sequences and hence can be used for non-model organisms whose genomes are yet to be determined or those with limited genomic information (Kristiansson *et al.,* 2009; Meyer *et al.,* 2009; Vera *et al.,* 2008; Wang *et al.,* 2010).

There are three major high-throughput sequencing technologies that are in use today. They include; llumina/Solexa, Applied Biosystems SOLiD and Roche/454 pyrosequencing. The Illumina/Solexa sequencing technology was first used in a tea transcriptome study to unravel genes responsible for major metabolic pathways (Shi *et al.,* 2011). The 454/Roche genome sequencers also called pyrosequencers (Ahmadian *et al.,* 2006) are the most commonly used technology for *de novo* sequencing and analysis of trancriptome in organisms whose genomes have not been fully sequenced (Zhou *et al.,* 2012) including tea. The term pyrosequencer has been used because the technology is based on chemiluminescent detection of pyrophosphate released during DNA polymerase mediated incorporation of deoxynucleosided triphosphate (Voelkerding *et al.,* 2009). The general requirement for generation of sequences through the use of pyrosequencers is that the samples library fragments are end-repaired, ligated to adapted oligonucleotide, and hybridized to individual beads containing sequence complementary to adaptor oligonucleotide. The beads are compartmentalized into water-in-oil microvesicles for amplification of single DNA molecule during emulsion PCR before loading onto picotiter well plate, Figure, 8. The picotiter functions as a flow cell where interactive pyrosequencing is performed by successive addition of the four nucleotide bases. Incorporation of a nucleotide in a well containing amplified template produce pyrophosphate and luminescence which is transmitted and recorded on a

device camera as image which is further analyzed for their signal and filtered based on quality before translating into linear sequence the strength of the signal is dependent on the number of nucleotides incorporated.

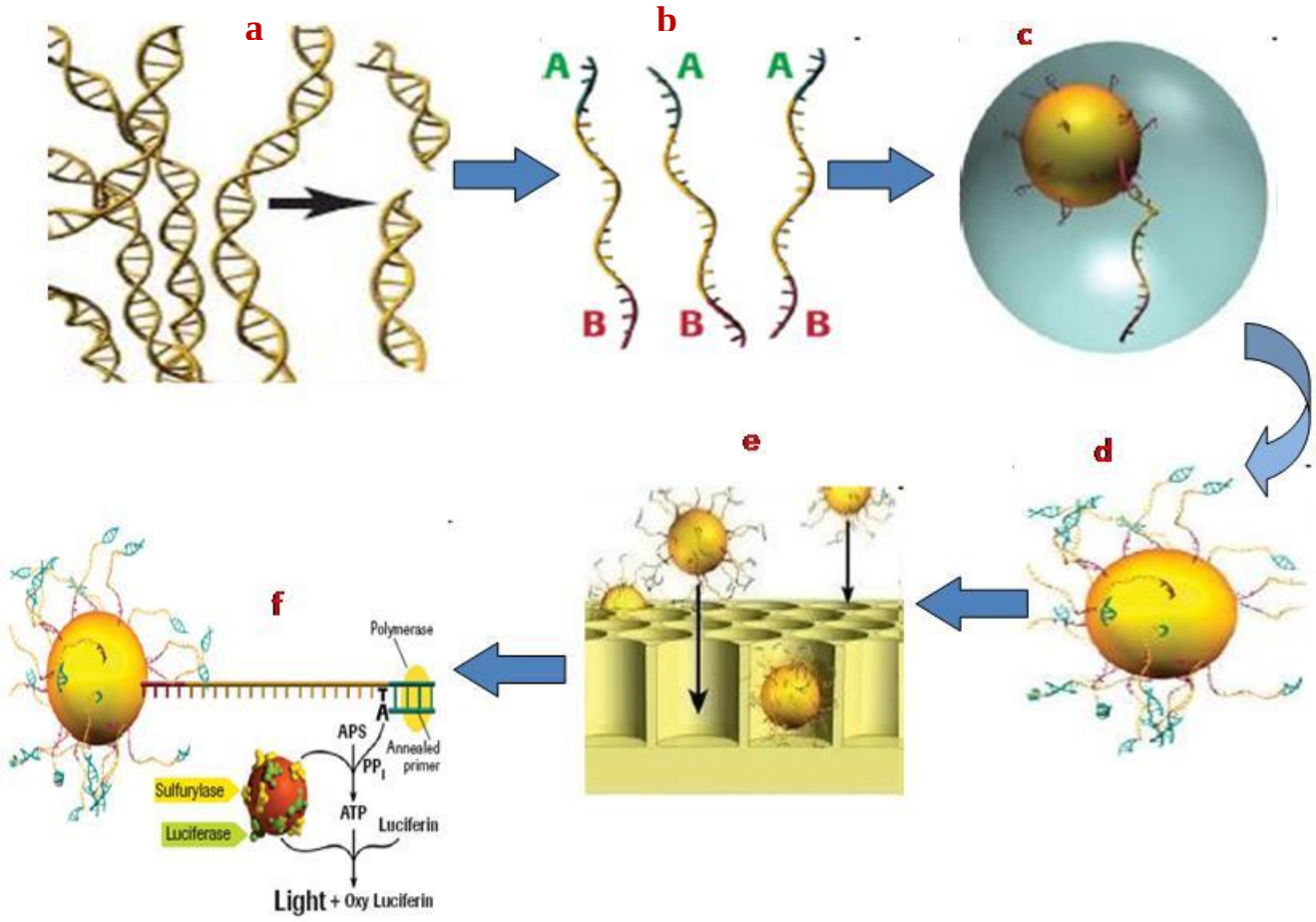


Figure 8; Diagram showing the pyrosquencing process. (a) Fragmentation of sample DNA to several hundred base pairs in length. (b) Adaptors ligation to both ends of the fragments and separated to single strands. (c) Hybridization of individual fragments with a water-in-oil emulsion to form a microreactor such that each fragment can be amplified emulsion PCR (emPCR). (d) The emPCR amplifies each fragment to several million copies of DNA template. After amplification, the emulsion shell is broken, DNA is denatured and beads carrying DNA are enriched. (e) The PicoTiterPlate is loaded such that one fragment carrying bead will occupy a well and smaller beads with the enzymes necessary for sequencing. (f) Sequencing accomplished by synthesizing the complementary strands of the bead attached templates. The four bases (ATGC) are sequentially washed over the PicoTiterPlate. The incorporation of a new base triggers the release of inorganic pyrophosphate which is

converted to ATP with adenosine 5′-phosphosulfate (APS) and ATP sulfurylase. The ATP is used by luciferase in the metabolism of luciferin into oxyluciferin thus producing light signal which is captured by a CCD camera in the equipment (Rothberg and Leamon, 2008).

The 454 technology can generate upto a million reads with average lengths of about 400 base pairs (bp) at 95% accuracy (Zhou *et al.,* 2012).

Examples of plant species in which the 454 pyrosequencing has been used include *Artemisia annua* (Wang *et al.,* 2009), *Eucalyptus grandis* (Novaes *et al.,* 2008).

In the present study, the physiological and biochemical responses of tea to water deficit were determined and the data correlated to determine the relationship between the two. On the other hand molecular characterization was done to identify candidate genes responsible for the changes in physiological and biochemical parameters and hence responsible for drought tolerance in tea.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Experimental materials

The tea cultivars used in this study were obtained from a tea nursery established at the Tea Research Foundation of Kenya, Kericho County, Kenya. Eight tea cultivars from the three main varietal types, Cambod, China and Assam, were selected based on their tolerance and susceptibility to drought (Kamunya *et al*., 2009.) The cultivars included; EPK C12, EPK TN14/3, TRFK 306, TRFCA SFS150, TRFK 303/216, AHP S15/10, TRFK 301/4 and 301/5. Their special attributes are presented in Table 1.

Table 1: Attributes of the cultivars used in the experiment

Cultivar	Varietal type and Origin	Special atributes
TRFCA SFS150	Assam (Malawian selection)	Drought, cold and pest tolerant
EPK C12	Chinery (Kenyan selection)	Drought tolerant and susceptible to mites.
EPK TN14/3	Chinery (Kenyan selection)	Drought tolerant and susceptible to mites
TRFK 301/5	Cambod (Kenyan selection)	High yielding and drought tolerant
TRFK 306	Assam (Kenyan selection)	Antioxidant rich and drought tolerant
AHP S15/10	Assam (Kenyan selection)	High yielding, highly pubescent but susceptible to drought.
TRFK 301/4	Cambod (Kenyan selection)	High yielding but drought susceptible
TRFK 303/216	Assam (Kenyan selection)	Susceptible to drought.

3.2 Experimental Set-up and Design

The experiment was carried out in a rain-out shelter at the Tea Research Foundation of Kenya (TRFK) located in Kericho county at latitude 0° 22'S, longitude 35°21'E and altitude of 2180m a.m.s.l. The rain out shelter was constructed as described by (Cheruiyot *et al*., 2008a). The 18 months old seedlings teas that were developed through vegetative propagation in the nursery were transplanted into 1000-gauge black polythene tubes measuring 0.3m in diameter by 0.3m in depth. The potted plants were arranged according to treatments in a completely randomized design and replicated three times giving a total of 72 experimental units. The plants were watered uniformly to field capacity for two weeks after which watering was progressively reduced on weekly basis to respective treatment levels. The three soil moisture content treatments subjected to the eight cultivars were 34%v/v (high soil moisture), 26%v/v (moderate soil moisture) and 18%v/v (low soil moisture). These soil water regimes were based on calculations determined by (Cheruiyot *et al*., 2008a). Each experimental plot was maintained at the predetermined soil moisture content during the experimental period. The control (non-droughted), at 34% soil moisture content, was watered throughout the experiment. The soil moisture content was routinely determined using a time domain reflectometer (TDR) soil moisture meter (TRIME-FM-2-Eijkelkamp Agrisearch Equipment, The Netherlands) according to the manufacturer's instructions, and maintained at desired levels by watering. SMC (% v/v) measurement was done twice daily at 1000hrs and 1500hrs and maintained within ±2% of the treatment level.

The growing conditions in the rain out shelter were also monitored by determining, temperature and relative humidity using maximum and minimum thermometer and hygrometer respectively.

3.3 Determination of leaf relative water content, shoot water potential and shoot growth

The plant water status and shoot growth were determined using the methods described by (Cheruiyot *et al*., 2008a). The leaf relative water content was determined by measuring the fresh weght($fr._{wt}$) of the third leaf immediately after harvesting, The leaves were then saturated in deionized and distilled water for 24 hours, after which they were blotted dry and turgid weight ($t._{wt}$) taken. The leaves were finally oven dried at 70° C for another 24 hours and dry weight ($d._{wt}$) measured. The leaf relative water content (RWC) was then calculated using the formula;

RWC% = ($Fr._{wt}$-$d._{wt}$/ $T._{wt}$ - $d._{wt}$)*100 formular, where $Fr._{wt}$ = fresh weight, $d._{wt}$ = dry weight and $T._{wt}$ = Turgid weight

The shoot water potential was determined using a pressure chamber (PMS Instruments, Co., Corvallis, OR., USA). A single leafy shoot was sealed in a pressure chamber with the cut end protruding outside the chamber and exposed to atmospheric pressure. Pressure was applied to the chamber from a tank of compressed nitrogen gas until xylem sap appeared at the cut end of the shoot. The amount of pressure required to force water out of the leaf cells into the xylem and up to the cut surface was considered as approximately equal to the water potential originally existing in the cells.

Shoot growth was determined by measuring the shoot extension on weekly basis using a vernier caliper. Two shoots from each experimental unit were selected randomly and tagged on the stem below the first opened leaf. The first measurement (initial shoot length, I_l) was measured from the node below the tag to the upper most visible node. Subsequent measurements were done weekly for 12 weeks (Final shoot length, F_l). Shoot growth was finally calculated on the basis of increase in shoot length (F_l - I_l) over time (t).

3.4 Determination of stomatal conductance (Gs) and Evapotranspiration (E) and net photosynthesis (P)

The gas exchange parameters were determined by measuring on the top most fully expanded leaf using a portable photosynthesis system, (TPS-2 Portable Photosynthesis system by PP systems Inc. USA). The measurements were done between 1030hrs and 1300hrs on mostly sunny days.

3.5 Determination of glycinebetaine profiles in tea

Glycinebetaine was determined using a modified (Grieve and Grattan, 1983) method. Dried and finely ground leaves, (0.5g), were shaken in 20ml of distilled water in an incubator at 25ºC for 24hrs. The filtrate was mixed with H_2SO_4 in the ratio 1:1. Aliquots of 0.5ml of this mixture was put in ice water for 1 hr before 0.2ml of a cold potassium iodide-iodine was added. The mixture was then kept at 2ºC for 16hrs. After the expiry of this period, the samples were centrifuged at 12,000rpm for 15 minutes at 0ºC. The supernatant was carefully aspirated and the periodite crystals were dissolved in 5ml of 1,2-dichloroethane. The mixture

was vigorously vortexed to effect solubility in the solvent, then left to stand for 2-2.5hrs before measuring the optical density at 365 nm using a Jenway 6505 UV-visible spectrophotometer (UK). Reference standards of glycinebetaine, (Cat. no. B3501 by Sigma, UK) were prepared in 2N sulphuric acid. The concentrations of glycinebetaine were calculated against the standard linear curve as follows:

GB in nmol·mg^{-1} FW or in µmol·g^{-1} FW = Absorbance /slope*$Vol_{extract}$/$Vol_{aliquot}$*1/DW.

Where Absorbance was the reading at wavelength 365 nm of the extract, slope (expressed as absorbance·nmol^{-1}) determined by linear regression, $Vol_{extract}$ was the total volume of the extract, $Vol_{aliquot}$ was the volume of extract assayed and DW (expressed in mg) was the amount of plant material extracted. It was confirmed that Absorbance was within the linear range.

3.6 Determination of proline accumulation in tea

Proline was determined using the method of (Bates *et al*., 1973). Fresh leaves (1g) were homogenized in 10 ml of 3% sulfosalicylic acid (Cat. No. S3147 by Sigma, UK) and the homogenate filtered. The filtrates (2 ml) were treated with 2ml acid ninhydrin (Cat. No. 151173 by Sigma, UK) and 2ml of glacial acetic acid (Cat. No. 33209 by Sigma, UK), then 4 ml of toluene was added and absorbance taken at optical density of 520 nm using a CE 393 UV-spectrophotometer (UK). The proline concentration was determined from a standard curve (Supplementary 1) generated from a proline standard reagent. Leaf proline levels were calculated on fresh weight basis using the formula below:-

µmole proline g^{-1} fresh weight = (µg proline ml^{-1} x ml of toluene/115.5)/(g of sample)

3.7 Sampling for total RNA isolation

Fresh shoots were harvested from the growing plants in the rain-out shelter after 12 weeks of exposure to the soil water treatments. The third and the fourth leaves were harvested and immediately dropped in liquid nitrogen before transporting them to the laboratory for storage at -80ºC.

3.8 Extraction and quantification of total RNA and mRNA

The freeze dried leaf (100mg) were ground into fine powder in liquid nitrogen using a pestle and mortar. Total RNA was extracted from the powdered samples using the ZR Plant

RNA Miniprep Kit (Cat. No. R2024, USA) according to the manufacturer's instruction. The wash buffer used was prepared in 95% ethanol. Purity and concentration of RNA was assessed by determining the absorbance at 260/280 nm using a 2000-NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The quality/intactness of the RNA extracted was also checked by denaturing in a buffer containing formamide and electrophoresing on a 1% agarose gel. The mRNA was isolated from the total RNA using a mRNA isolation kit (version 8, Roche, Germany) according to the manufacturer's instructions while the rest was stored at -80ºC. The integrity of the mRNA was also assessed using a NanoDrop spectrophotometer.

3.9 Preparation of cDNA library for transcriptome sequencing

The cDNA library was synthesized using a cDNA Rapid Library Preparation kit (GS FLX Titanium Series, Roche, Germany) according to manufacturer's instruction. The mRNA was fragmented into smaller pieces at 70°C for 30 seconds in a fragmentation buffer and denatured by adding Roche 'random' primers. The fragments were reverse-transcribed to synthesize first strand cDNA using AMV reverse transcriptase (Roche, Germany). Subsequently, the second strand cDNA was synthesized using second strand enzyme (Roche, Germany) and T4 DNA polymerase (Roche, Germany). The cDNA fragments were end repaired using T4 DNA polymerase, taq polymerase and polynucleotide kinase before ligation of adaptors with MID adaptor and ligase (Roche, Germany). The products were purified to remove fragments less than 50 bp long using Individual Sample Cleanup (ISC) sizing solution. The cDNA library was then quantified and assessed for quality using TBS 380 Fluorometer (Turner Biosystems, USA) and Agilent Bioanalyzer High Sensitivity DNA chip (Agilent technologies, Germany), respectively.

3.10 emPCR amplification

The emulsion PCR (emPCR) amplification was done using the emPCR Kit GS FLX Titanium series (Roche, Germany). The adapter containing quality cDNA was mixed with capture beads, PCR reagents and emulsion oil and run using the program;1 x 94^0C for 4 minutes , 50 x 94^0C for 30seconds, 58^0C for 4.5 minutes, 68^0C for 30 seconds, 10^0C on hold. After the PCR reaction the beads were checked for emulsion breakage (distinct layer with clear middle layer). The broken emulsion were discarded while intact emulsion were used for bead recovery. Those beads that did not hold DNA were eliminated while beads holding

more than one type of DNA were recovered and washed. To ensure that only the beads carrying the amplified DNA was used in the sequencing, the recovered beads were enriched by hybridization of the biotinylated enrichment primer to the adaptor of each amplified DNA template to which it is complemetary in its binding to streptavidin-coated magnetic beads. The beads carrying the amplified DNA were separated from the null and poorly amplified beads using magnetic particle separator. The DNA library beads were separated from the magnetic beads by melting the amplification products away from the enrichment primer, leaving a population of bead-bound single-stranded template DNA fragments (immobilized and amplified DNA library). The final step in the emPCR amplification process was the annealing of sequencing primer to the amplified DNA template to form a library of clonally amplified DNA fragments ready for loading onto a picoTiter plate and sequencing.

3.11 454 Sequencing

Sequencing was done from both the 5' and 3' end using the 454 sequencing technique. Sequencing was done for cDNA from the most drought tolerant and susceptible cultivars under both the well watered and non-watered soil conditions. Sequencing was completed using a GS FLX Titanium Sequencing kit according to the manufacturers' instruction. The DNA-capture beads were loaded onto PicoTiterPlate™ such that each well contained single DNA beads. Sequencing reagents comprising of dNTP buffers, sodium chlorite tablet, Apyrase, Ppiase, inhibitor TW reagent, enzyme beads, bleach and DTT were sequentially flowed over the plate. The sequencer automatically performed and monitored the sequencing reactions in all the wells of the PicoTiterPlate simultaneously. The raw output of the sequencing process consisted of a set of digital images (PIF files) from which the sequence of the DNA library fragments were read. The processing of the images to sequences and base-calling calculation were performed by the 454 data processing pipeline in which raw reads were obtained.

3.12 Physiological and Biochemical data analysis

All the experiments were conducted using three biological independent replicates. Statistics were carried out using a GENSTAT statistical software, Version 10.3. Analysis of variance (ANOVA) was used to test for the difference in response of cultivars to different water stress conditions. The probability limit was set at $P \leq 0.05$ for significant confidence

interval. Correlation analysis was also used to determine the phenotypic relationships between physiological and biochemical parameters.

3.13 Sequence analysis

3.13.1 Quality control and *De novo* assembly of sequencing reads

Before assembly, the raw reads were filtered to obtain the high-quality clean reads by removing adaptor sequences, duplication sequences, the reads containing more than 10% "N" rate (the "N" character representing ambiguous bases in reads), and low-quality reads containing more than 50% bases with Q-value < 20. The Q-value is the quality score assigned to each base and is defined by the equation;

$Q = -10\log_{10}(e)$;

Where "**e"** is the probability of a base call being wrong. The higher the score, the lower the probability of a wrong base call during sequencing. *De novo* assembly of the clean reads was performed using the Newbler program (version 1.03).

All high-quality reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the accession number SRX485271.

3.13.2 Functional annotation and classification of contigs

All the assembled contigs longer than 100 bp were annotated by assigning Gene Ontology (GO) terms and putative metabolic pathways to them based on sequence similarity with previously identified genes annotated in the Arabidopsis proteome.

Functional categorization by Gene Ontology terms was carried out based on BLASTX hits from Arabidopsis protein datasets of NR database using Blast2GO software (version 2.3.5, http://www.blast2go.de/) with E-value threshold of 10^{-5}. The KEGG pathways annotation was performed by sequence comparisons against the Kyoto Encyclopedia of Genes and Genomes database using BLASTX algorithm (E-value threshold: 10^{-5}). The sequences were categorized based on functions homologous to Arabidopsis. The roles of the transcripts identified with known and putative function were assigned categories of plant genes.

CHAPTER FOUR
RESULTS

4.1 Growth conditions in the rain-out shelter

The maximum and minimum temperature in the rain-out shelter ranged from 18.5 ºC to 32.0ºC, while the relative humidity ranged between 44 and 96%. The soil used for planting had the following physical and chemical properties; Clay-loam in texture, 4ppm of Phosphorous, 249ppm of Potassium, 147ppm of Calcium, 43ppm of Magnesium, 34ppm of Manganese and pH of 4.0.

4.2 Effects of water stress on physiological parameters in tea

4.2.1 Leaf relative water content

The (leaf relative water content) LRWC differed significantly ($F_{(2,46)}$ =256.34, P<0.001) with the SMC subjected to the test tea cultivars. All the test cultivars used in the study responded to reduced soil moisture content by lowering the leaf relative water content as shown in Figure 9. The mean leaf relative water content differed significantly ($F_{(7,46)}$ =2.89, P<0.001) among the test cultivars. The LRWC under high SMC ranged between 78% and 88% for cultivars AHP S15/10 and EPK C12, respectively. At the moderate level of SMC, it was 67-83% for cultivars EPK C12 and TRFCA SFS150, respectively while at the low SMC, it ranged between 46.7 and 67% for cultivars TRFK 301/5 and AHP S15/10, respectively. The difference in LRWC between the high and moderate SMC was very small in cultivars TRFCA SFS150 (1.3%) and AHP S15/10 (2.0%), while cultivar EPK C12 had the highest difference in LRWC at 21%. On the other hand, cultivar EPK C12 had the lowest (6.0%) difference in LRWC between the moderate and low SMC. Cultivars EPK TN14/3, TRFK 301/5 and TRFCA SFS150 showed significantly higher differences of 31%, 29% and 27%, respectively, between the two treatments. The interaction between the test tea cultivars and soil moisture content differed significantly ($F_{(14,46)}$ =4.45, P<0.001).

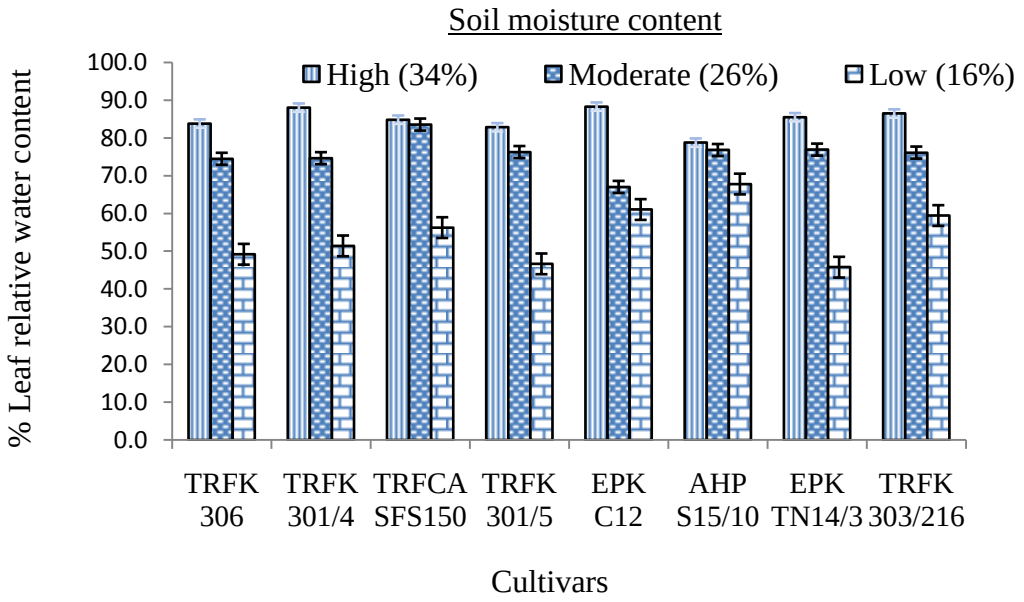


Figure 9: Effect of decreasing soil moisture content on leaf relative water content (%) among different tea cultivars. Bars represent the standard error of the mean.

4.2.2 Shoot water potential

The present study showed a significant decline in shoot water potential (SWP) with decrease in soil moisture content (Table 2). The levels were -0.8, -0.9 and -1.1Mpa for the high, moderate and low soil moisture content, respectively. The SWP values were notably negative. The three soil moisture content levels subjected to the tea cultivars differed significantly ($F_{(2,46)}$ =166.3, P<0.001) with respect to plant response. All the tea cultivars used in the present study responded to decline in SMC by lowering the shoot water potential. Shoot water potential also differed significantly ($F_{(7,46)}$ =33.79, P<0.001) amongst the test cultivars at the different soil water regimes. Under stress condition (low soil moisture content), cultivar AHP S15/10 which is drought susceptible had the least shoot water potential (-1.2MPa) while the drought tolerant cultivar EPK C12 had the highest SWP (-0.9MPa). This status was repeated in the unstressed condition (high SMC) at -0.9MPa and -0.6MPa, respectively. The cultivars responded differently to changing SMC, the difference in SWP between the high and moderate SMC was significantly (P<0.05) high (0.25, 0.36 and 050Mpa) for cultivars TRFK 301/4, TRFK 301/5 and TRFK 306, respectively as compared to other test cultivars. There was no significant difference for cultivars EPK C12, TRFK 303/216 and TRFCA SFS150 under the same conditions. The interaction between cultivar response and soil moisture content was significant, ($F_{(14,46)}$ =11.73, P<0.001).

Table 2: Effects of different soil moisture content levels on shoot water potential (MPa) of different tea cultivars.

	Tea cultivars							
	TRFK 306	TRFK 301/4	TRFCA SFS150	TRFK 301/5	EPK C12	AHP S15/10	EPK TN14/3	TRFK 303/216
Treatment (% SMC)								
Low (18%)	-1.15^{a}	-1.16^{a}	-1.19^{e}	-1.13^{i}	-0.93^{f}	-1.21^{i}	-1.23^{f}	-1.08^{f}
Moderate (26%)	-0.65^{b}	-0.91^{b}	-1.16^{e}	-0.77^{f}	-0.92^{f}	-1.03^{i}	-1.03^{f}	-1.05^{f}
High (34%)	-0.69^{b}	-0.91^{b}	-0.88^{f}	-0.75^{f}	-0.62^{i}	-0.94^{f}	-0.86^{j}	-0.92^{i}

^{ab} *Means within a column followed by the same letter are not significantly different at P≤0.05 by the LSD test.*

4.2.3 Shoot growth rate

The rate of shoot growth in all the test tea cultivars was reduced with decline in soil moisture content. The average growth rate ranged from 1.8, 0.9 and 0.3mm/day for the high, moderate and low SMC treatments, respectively. The three SMC treatments subjected to the test cultivars elicited significantly different ($F_{(2,46)}$ =578.01, P<0.001) shoot growth rates.. All the test cultivars responded to decline in SMC through a decrease in shoot growth rate as shown in figure 10. The rate of shoot growth also differed significantly, ($F_{(7,46)}$ =30.75, P<0.001), amongst the test cultivars. Cultivar TRFK 306 had the highest growth rate (3.31mm/day) under high soil moisture content while cultivar TRFK 303/216 had the least shoot growth rate (0.87mm/day). At moderate SMC, cultivar AHP S15/10 showed the highest growth rate of 1.38mm/day while TRFK 303/216 maintained the least growth rate of 0.57 mm/day. Under low soil moisture content, cultivar EPK C12 showed the highest growth rate whereas cultivar AHP S15/10 showed the lowest shoot growth rate. The rate of growth was comparable for cultivar TRFK 303/216 and TRFK 306. The difference in growth rate between high and moderate SMC was high for cultivars TRFK 306 and TRFK 301/4 while it was low for cultivar EPK TN14/3. The difference in growth rate between the moderate and low SMC was highest for cultivar AHP S15/10. The interaction between cultivars and SMC was significant ($F_{(14,46)}$ =21.67, P<0.001).

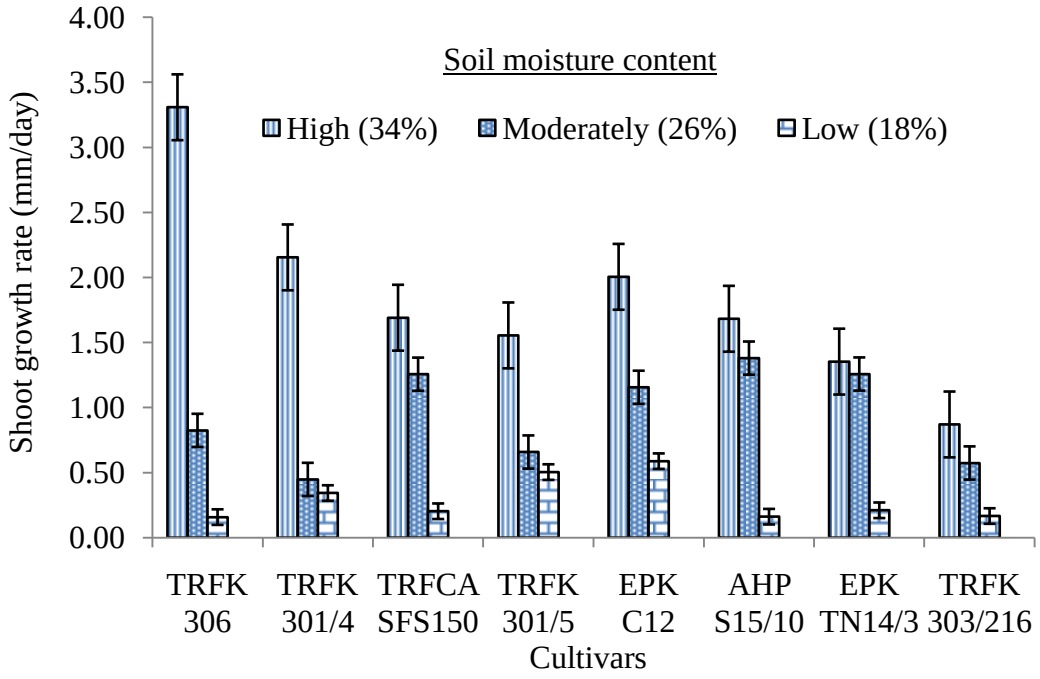


Figure 10: Effects of soil moisture content on shoot growth rate (mm/day) of tea cultivars. Bars represent the standard error of the mean.

4.2.4 Stomata conductance

Stomata conductance was significantly reduced with decline in soil moisture content. The stomatal conductance ranged from 301, 276 and 256µmol-m-$^{2\ -}$s for the high, moderate and low SMC treatments, respectively. The three treatments (SMC) differed significantly ($F_{(2,46)}$ =13.20, P<0.001). Except for cultivar TRFK 301/4, all the tea cultivars studied also differed significantly ($F_{(7,46)}$ =6.38, P<0.001) in their stomatal conductance due to varying SMC. Under low soil moisture content, cultivar EPK C12 had the highest stomatal conductance of 290µmol^{-}m-$^{2-}$s whereas TRFK 306 had the least of 235µmol^{-}m-$^{2-}$s. Under high soil moisture content, cultivar EPK C12 exhibited the highest stomatal conductance of 370µmol^{-}m-$^{2-}$s while TRFK 301/4 had the least of 245µmol^{-}m-$^{2-}$s. The interaction between the two factors (cultivar and %SMC-treatment) was however not significant. The difference in stomatal conductance between the various treatment (high, moderate and low SMC) also varied with the test tea cultivars. The difference between the high and moderate SMC was significantly higher in cultivars, TRFK 301/5, EPK C12 and AHP S15/10. However, there was no significant difference in the other test tea cultivars. The difference in stomatal conductance between the moderate and low SMC was also not significant for cultivars TRFK 306 and EPK C12 while the rest of the cultivars showed significantly higher differences.

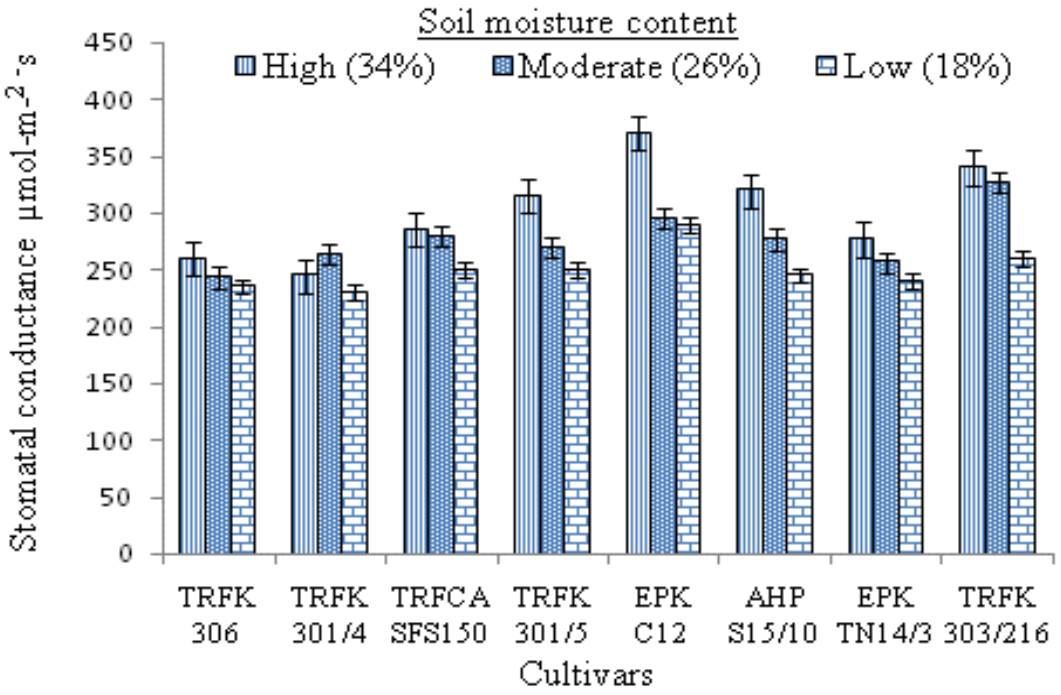


Figure 11: Changes in stomatal conductance (µmol-m^{-2}s) in different tea cultivars in response to changing soil moisture content. Bars represent the standard error of the mean.

4.2.5 Evapotranspiration

The evapotranspiration rate declined with reducing soil moisture content (Table 3). The mean evaprotranspiration rates were, 2.2, 2.0 and 1.9mmol-m-^{2}s for the high, moderate and low SMC treatments, respectively. The three different SMC treatments differed significantly ($F_{(2,46)}$ =5.62, P<0.001) with respect to tea plant response. The rates of evapotranspiration differed significantly, ($F_{(7,46)}$ =3.83, P<0.01) amongst the test tea cultivars. At low soil moisture content, the transpiration response varied between cultivars with cultivar EPK C12 having the highest rate of 2.2mmol-m-^{2}s while cultivar EPK TN14/3 exhibiting the lowest rate of 1.7mmol-m-^{2}s. At high soil moisture content, cultivar AHP S15/10 showed the highest evapotranspiration rate of 2.5mmol-m-^{2}s while cultivar TRFK 301/4 had the least rate of 2.0 mmol-m-^{2}s. Except for cultivars TRFK 303/216, EPK TN14/3 and TRFK 301/5, all the other cultivars exhibited significantly (P<0.05) large differences in evapotranspiration rate between the high and moderate SMC treatments. At the moderate and lower SMC treatments, cultivars TRFK 303/216, EPK TN14/3, EPK C12 and AHP S15/10 exhibited significantly (P<0.05) large differences in their rates of evapotranspiration..

Table 3: Effects of soil moisture content on rates of evapo-transpiration (mmol-m^{-2} $^{-}$s) in tea cultivars.

	TRFK 306	TRFK 301/4	TRFCA SFS 150	TRFK 301/5	EPK C12	AHP S15/10	EPK TN14/3	TRFK 303/216
Treatment (% SMC)								
High (34%)	2.1^{a}	2.0^{b}	2.1^{d}	2.1^{e}	2.4^{g}	2.5^{i}	2.1^{k}	2.5^{a}
Moderate (26%)	2.1^{a}	1.7^{c}	2.0^{d}	1.9^{f}	2.4^{g}	2.4^{i}	1.9^{l}	2.2^{b}
Low (18%)	2.0^{a}	1.8^{c}	2.0^{d}	1.9^{f}	2.2^{h}	2.1^{j}	1.7^{m}	2.0^{c}

^{ab} *Means within a column followed by the same letter are not significantly different at P≤0.05 by the LSD test.*

4.2.6 Net photosynthesis

The net photosynthetic rate also declined significantly with decreasing soil moisture content. The net photosynthetic rate differed significantly ($F_{(2,46)}$ =65.19, P<0.001) amongst the three SMC treatments subjected to the test cultivars with the highest mean photosynthetic rate maintained by the tea plants subjected to high soil moisture content. The average net photosynthesis was 21.17, 15.66 and 7.48µmol-m-^{2} $^{-}$s for the high, moderate and low SMC treatments, respectively. All the cultivars used in the study exhibited a decline in net photosynthetic rate with decline in SMC as shown in figure 12. The results exhibited significant differences ($F_{(7,46)}$ =2.12, P<0.05) amongst cultivars in response to different SMC levels. At the high SMC level, cultivar EPK C12 showed the highest net photosynthetic rate of 24.3µmol-m-^{2} $^{-}$s while TRFK 301/4 showed the lowest 17.7 µmol-m-^{2} $^{-}$s. At the low soil moisture content, cultivar TRFCA SFS150 had the highest net photosynthetic rate of 12.5µmol-m-^{2} $^{-}$s while cultivar TRFK 303/216 showed the lowest rate of 5.28µmol-m-^{2} $^{-}$s. At moderate SMC, cultivars EPK TN14/3 and AHP S15/10 exhibited the highest net photosynthetic rates whereas TRFK 301/4 had the lowest. The net photosynthetic rate of cultivar TRFK 301/4 was comparable at both the moderate and low SMC. For cultivars AHP S15/10, EPK TN14/3 and EPK C12 there was a significant (P<0.05) variation between the two SMC treatments. However, cultivars AHP S15/10 and EPK TN14/3 exhibited the least difference in net photosynthetic rate at the high and moderate SMC. Except for cultivar TRFK 301/5 all the other test tea cultivars showed significantly (P<0.05) high differences in

net photosynthetic rate between the moderate and low SMC. The interaction between the SMC and the test cultivars was significant ($F_{(14,46)}$ =1.90, P<0.01).

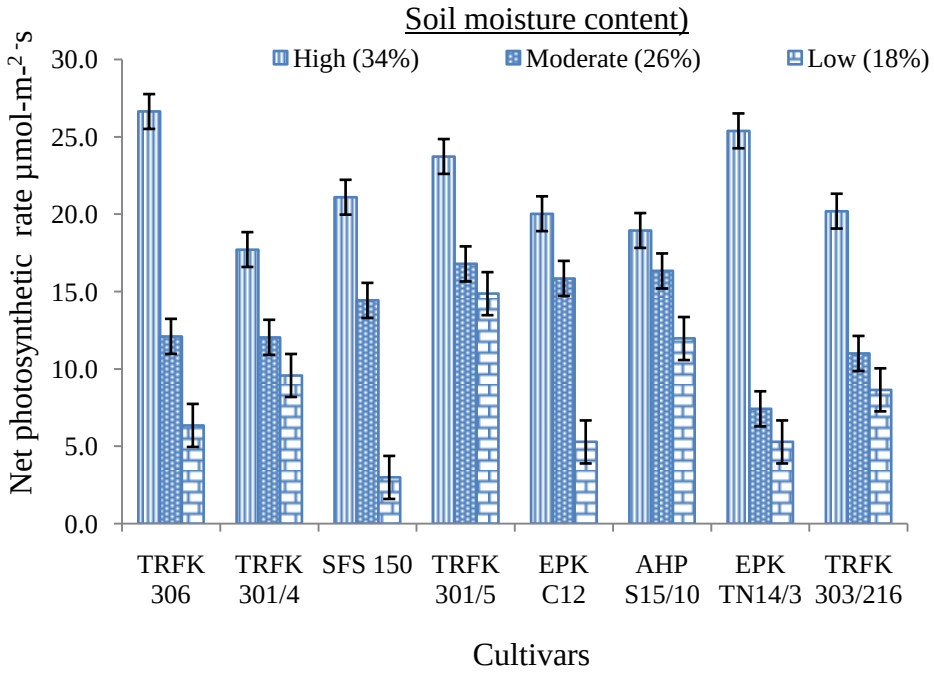


Figure 12: Effects of soil moisture content on net photosynthetic rate (µmol-m^{-2-}s) in different tea cultivars. Bars represent standard error of the mean.

4.3 Effects of water stress on Proline and Glycinebetaine levels in tea

4.3.1 Proline levels

Unlike the other physiological parameters studied, decreasing SMC induced the accumulation of leaf proline in the test tea cultivars. The leaf proline levels differed significantly ($F_{(2,46)}$ =253.7, P<0.001) with the SMC (high, moderate and low). The average leaf proline levels varied from, 0.104, 0.131 and 0.244µmol/g FW for the high, moderate and low SMC, respectively. All the test cultivars also showed significant and steady rise in proline profiles with decline in SMC as shown in figure 13. There was significant difference ($F_{(7,46)}$ =93.73 P<0.001) in leaf proline levels amongst the test tea cultivars. Clone TRFK 306 had the highest mean level of proline of 0.53, 0.22 and 0.21 µmol/g FW under the low, medium and high soil moisture content, respectively. Cultivar AHP S15/10 had the lowest proline level of between 0.05 and 0.06 µmol/g FW under the high and medium soil moisture content, respectively. Cultivar EPK C12 had no significant difference in proline content at the

different SMC. Other than cultivars TRFK 301/5 and TRFK 303/216, all the others exhibited a steady and significant rise in proline levels with decline in SMC. The interaction between cultivar response and water treatment was significant ($F_{(14,46)}$ =24.08, P<0.001).

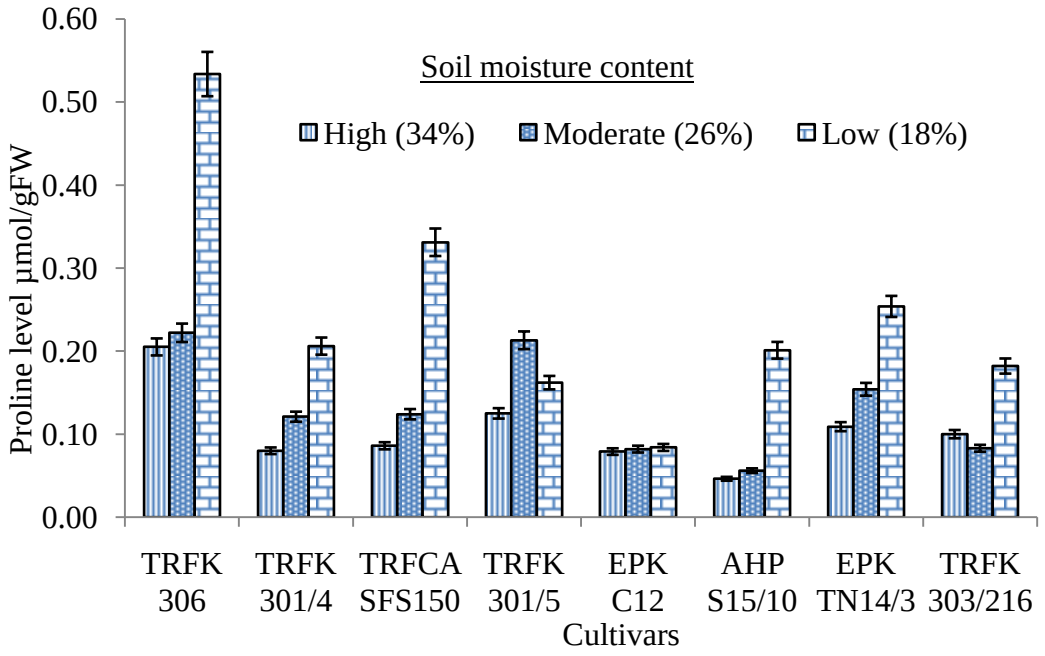


Figure 13: Leaf proline levels (µmol/g FW) of different tea cultivars grown under different soil moisture regimes. Bars represent standard error of mean.

4.3.2 Glycinebetaine levels

Like proline, leaf glycinebetaine content increased in the test tea cultivars with decline in SMC (figure 14). The variation in glycinebetaine levels was significantly ($F_{(2,46)}$ =21.16, P<0.001) different among the treatments. The mean glycinebetaine levels observed were 1.5, 1.7 and 2.0mmole/g DW for the high, medium and low soil moisture content, respectively. The assayed tea cultivars also differed significantly, ($F_{(2,46)}$ =4.97, P<0.001) in leaf glycinebetaine levels. Cultivar AHP S15/10 had the highest (2.5mMole/g DW) level of glycinebetaine under low soil moisture content while TRFK 301/5 had the lowest levels (1.57mMole/g DW) a situation that was replicated in all the other treatments. Under high soil moisture content, cultivar TRFCA SFS150 had the highest leaf glycinebetaine content, (1.71mMole/g DW). At moderate SMC cultivar EPK TN14/3 accumulated the highest amount of leaf glycinebetaine. The difference in glycinebetaine level between the high and moderate SMC treatments was significantly low in cultivars TRFK 306 and TRFK 303/216, and high for cultivars EPK TN14/3 and TRFK 301/4. There was significantly higher

difference (P<0.05) in glycinebetaine levels between the moderate and low SMC for cultivars AHP S15/10, TRFK 303/216 and EPK C12. Cultivars TRFK 301/5 and TRFK 301/4 showed the least difference in glycinebetaine levels between the moderate and low SMC treatments. The interaction between test tea cultivars and soil moisture content was significant ($F_{(14,46)}$ =2.03, P<0.05).

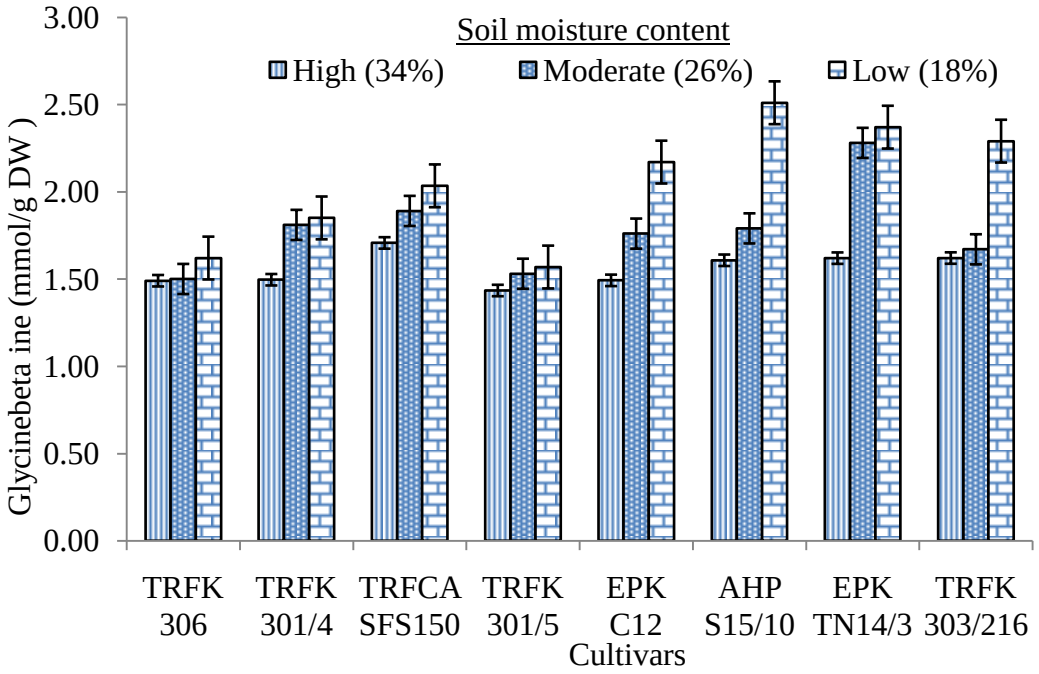


Figure 14: Variation in leaf glycinebetaine levels (mMol/g DW) of tea cultivars at different soil moisture treatments. Bars represent standard error of mean.

4.4 Correlation coefficient analysis of physiological and biochemical parameters

The phenotypic correlation coefficient (r) between the various physiological and biochemical parameters are presented in Table 4. The leaf relative water content was positively correlated to shoot growth, stomatal conductance, evapotranspiration rate and the net photosynthetic rate. The shoot water potential was also significantly and positively correlated to shoot growth, stomatal conductance and net photosynthetic rate. Shoot water potential was however not significantly correlated to evapotranspiration. The rate of shoot growth was positively and significantly correlated with the net photosynthetic rate. There was also a positive correlation between stomatal conductance and evapotranspiration together with net photosynthetic rate. All the physiological parameters were negatively correlated with the two assayed biochemical parameters (leaf proline and glycinebetaine content). The two biochemical parameters were positively correlated to each other though not significantly.

Table 4; Correlation coefficient between the physiological and biochemical parameters of water stress in tea

	Lrwc	Swp	Sg	G_s	E_e	Pn	Prl
Swp	0.6802***						
Sg	0.7239***	0.6471***					
G_s	0.4309*	0.4196*	0.3075^{NS}				
E_e	0.3926*	0.3376^{NS}	0.3516*	0.7141***			
P_n	0.7526***	0.6593**	0.7689***	0.4588*	0.3934*		
Prl	-0.5253**	-0.3496*	-0.4374*	-0.5994**	-0.4871*	-0.489*	
Gb	-0.5009*	-0.6031**	-0.5161**	-0.4455*	-0.3094^{NS}	-0.6562**	0.218^{NS}

NB: Lrwc = Leaf Relative water content, Sg = Shoot growth, Prl= proline, G_s= stomatal conductance and P_n = net Photosynthetic rate, E_e = Evapotranspiration, Swp = shoot water potential, Gb = Glycinebetaine. *, **, and *** signify significance levels at $P < 0.05$, 0.01, and 0.001, respectively.

4.5 Molecular analysis

4.5.1 Quality and concentration of total RNA and mRNA

The quality and quantity of total RNA and mRNA isolated are shown in Table 5. The results showed the absorbance ratio $A_{260/280,}$ ranging between 1.92- 2.0 and $A_{260/230}$ ratio ranging between 1.59 and 1.8 for total RNA extracted. The concentration of total RNA isolated ranged from 583.9ng/µl and 1274ng/µl at low SMC for cultivar TRFCA SFS150 and AHP S15/10, respectively. The mRNA isolated from the total RNA had a concentration ranging from 34.1 to 49.2ng/µl. The 260/280 absorbance ratio for assessing quality ranged between 1.8 and 2.0.

Table 5; Concentration and quality of total RNA and mRNA isolated form tea leaves.

Sample	Nucleic acid	Conc. (ng/ µl)	260/280	260/230
AHP S15/10 (High SMC)	Total RNA	942.3	2.0	1.8
	mRNA	35.8	1.8	1.3
AHP S15/10 (Low SMC)	Total RNA	583.9	2.0	1.7
	mRNA	34.1	1.8	1.3
TRFCA SFS150 (High SMC)	Total RNA	916.4	1.9	1.6
	mRNA	49.2	1.9	1.3
TRFCA SFS150 (Low SMC)	Total RNA	1274	2.0	1.9
	mRNA	45.4	2.0	1.7

4.5.2 cDNA synthesis

The cDNA libraries synthesised from the isolated mRNA produced thick band between 600 and 1200 bp as shown in Figure 15.

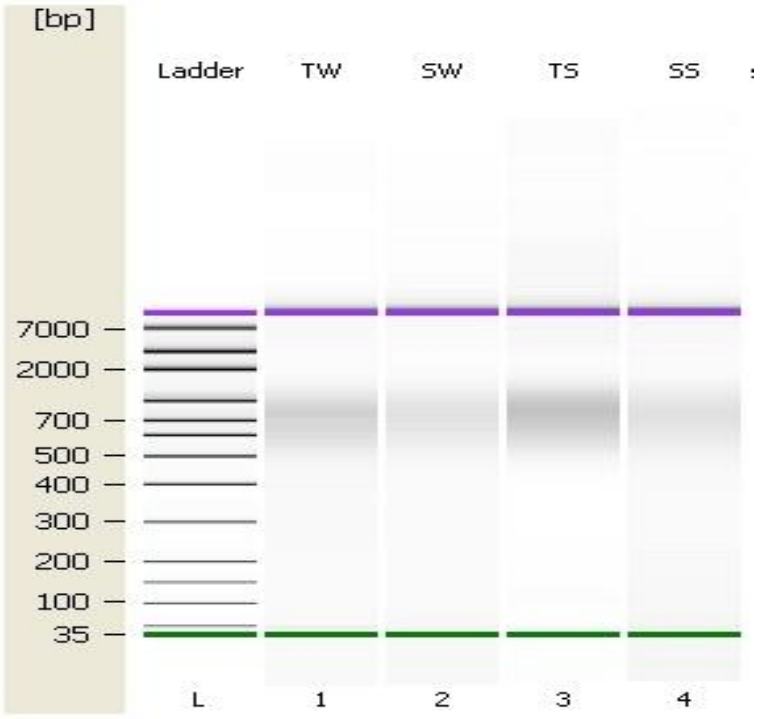


Figure15: Gel-like image of the cDNA library samples as run on an Agilent Bioanalyzer High sensitivity DNA chip. The initials; TW = TRFCA SFS150 (Watered), TS= TRFCA SFS150 (stressed), SW= AHP S15/10 (Watered), SS= AHP S15/10 (stressed) are the four libraries synthesised for use in sequencing. The top and bottom distinct band are the upper and lower markers used.

4.5.3 Summary statistics of raw reads

The sequencing of the cDNA libraries constructed from the two varieties under stressed and unstressed conditions produced 232,385 reads. The reads length ranged from 40 -1143bp with an average of 369bp. The test tea cultivars produced more reads at low soil moisture content than at high SMC in both cultivars as shown in Table 6. Cultivar TRFCA SFS150 produced the lowest amount of reads at High SMC.

Table 6; Summary statistics of raw reads generated using 454 pyrosequencer

Cultivar	Treatment	No. of reads
TRFCA SFS150	High SMC	38
TRFCA SFS150	Low SMC	47363
AHP S15/10	High SMC	26592
AHP S15/10	Low SMC	158392
Read length range		40 - 1143bp
Average read length		369bp

4.5.4 Quality control report of sequences

After the removal of low quality reads, adaptors and sequences less than 50bp, the quality checks showed that all the sequences from the four libraries had Phred-like quality scores greater than 25 (Q25) level equivalent to 0.01 error probability of a base being called incorrectly as shown in Figure 16. This meant that there was minimal chance of a base being called incorrectly.

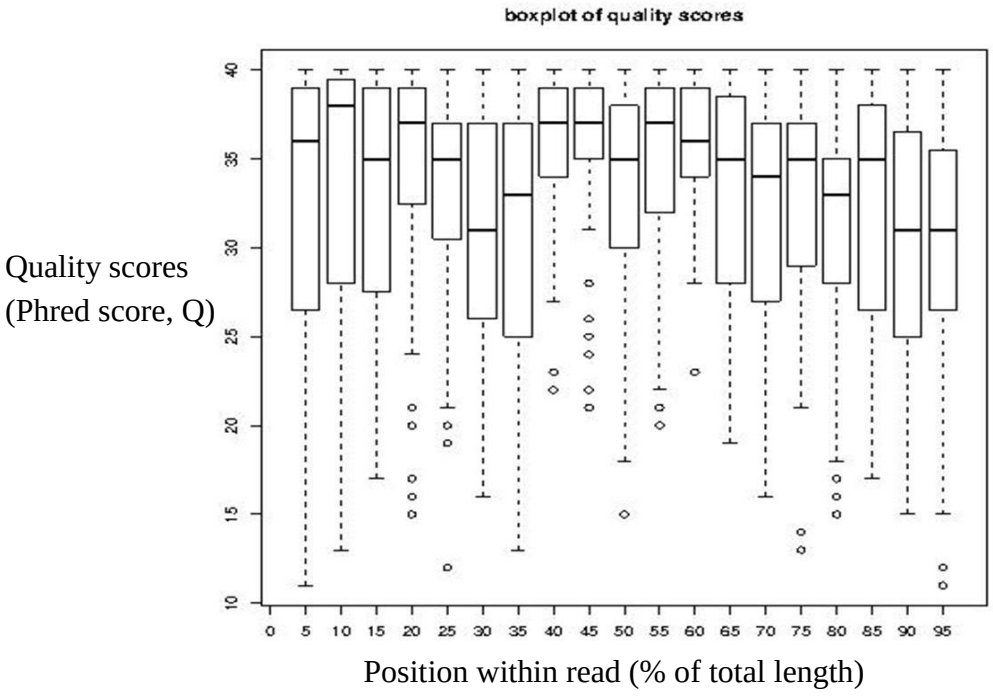


Figure 16; Box plot showing quality scores of trimmed sequence. The Y-axis shows the quality scores referred to as phred scores (Q) which is equivalent to the probability of errors in a particular base. In the scale used, quality score, Q10, means the probability of an incorrect base call is 1 in 10, Q20 = 1 in 100, Q30 = 1 in 1000. The lowest score was Q25. The X-axis shows the position within the read (0-100% of the total length of read).

4.5.5 Summary statistics of assembled sequences

The preprocessed sequences were assembled into 460 contigs. The contig length ranged from 100-2,466bp with majority of the contigs falling between 100-500bp as shown in Figure 17. The mean length of the contigs was 250bp with 13 contigs being greater than 1kb. The total number of bases in all the contigs was 115,177 with a GC content of 43.9%.

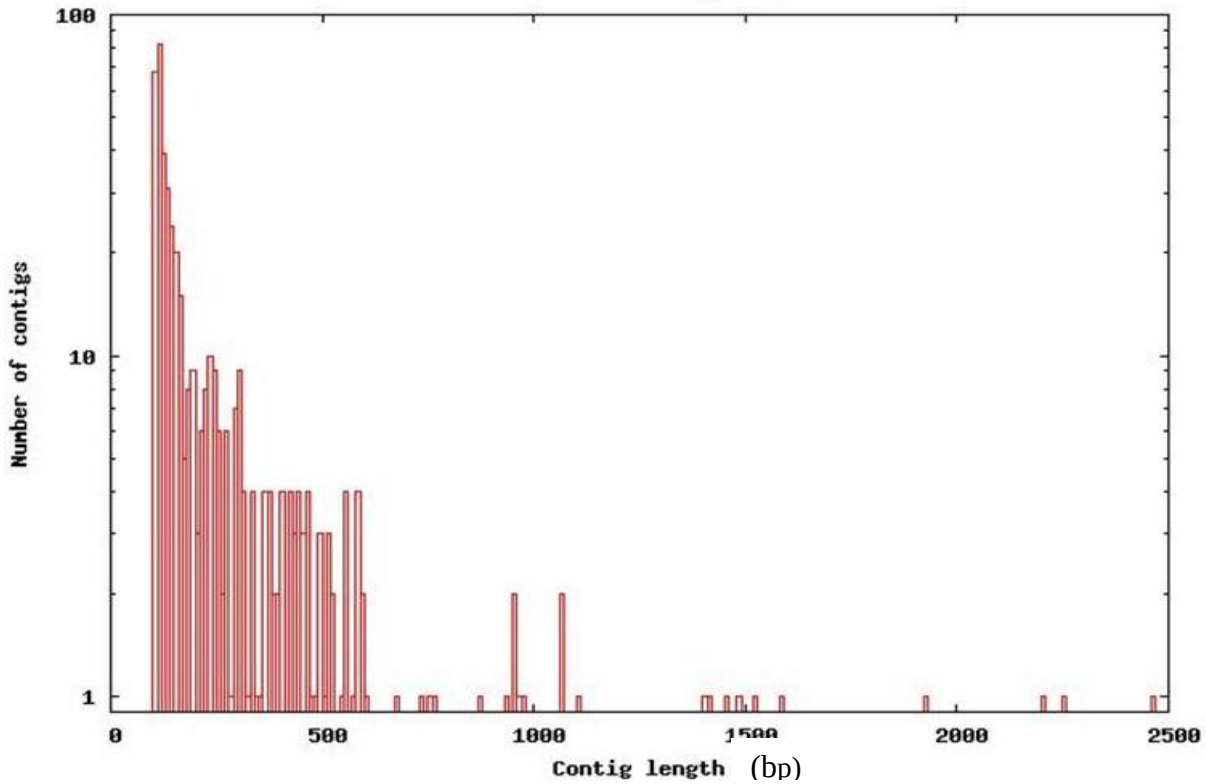


Figure 17; Size distribution of the contigs generated by *de novo* assembly of the filtered and trimmed 454 pyrosequence reads.

4.5.6 Gene functional categories

The contigs were categorized into three broad categories (biological processes, cellular component and molecular functions) as established for the *Arabidopsis* proteome (Figures 18a, b, c, respectively). In the biological process category, the 'metabolic processes' related genes were dominant (20%), followed by 'multicellular organismal development' (15%) and 'cellular processes' (12%). It was also notable that 10% of the genes in this category represented genes related to 'stimulus response'. The 'secretion' related genes, (0.4%), were the least in this category. In the cellular component category, genes assigned to the intracellular region accounted for the largest group (78%) followed by cell part (2%) whereas genes of the extracellular region were the least (1%). In the molecular function category, the highest percentage was covered by binding related genes (43%), followed by the catalytic activity related genes (27%), Nucleic acid binding (10%) and structural molecule activity related genes (10%).

The signal transduction (2%) and transporter activity (2%) related genes were the least in this category of genes.

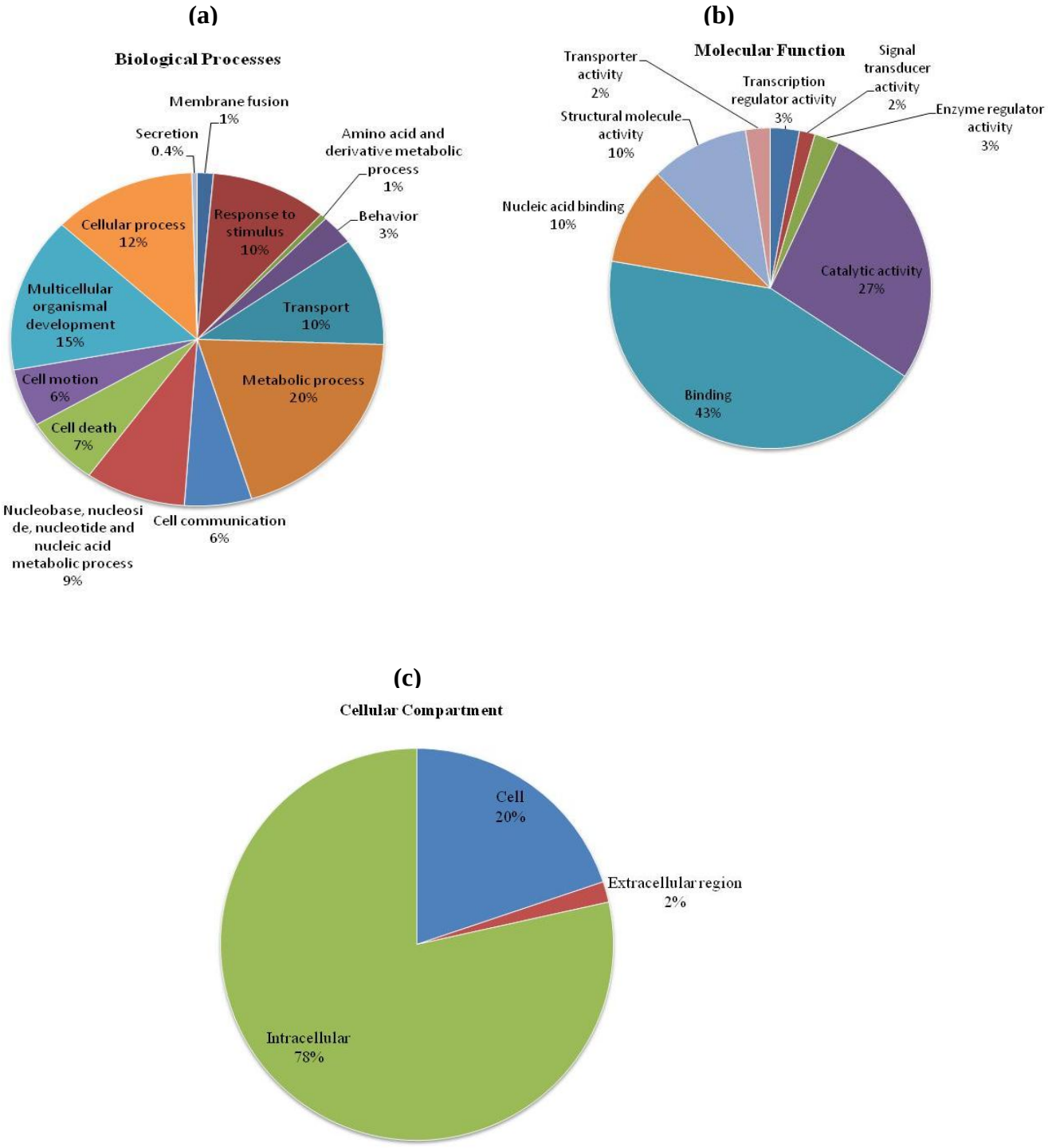


Figure 18: Gene ontology (GO) classification of *Camellia sinensis* contigs as summarized into three main categories; **(a)** Biological processes, **(b)** Molecular functions and **(c)** Cellular components. The % shows the proportion of genes related to various activities within the three main functional categories of genes.

The most dominant biological pathways that were active in the leaf of *C. sinensis* from the present study are presented in Figure 19. The pathways with the most representation of the contigs were related to 'oxidative phosphorylation' and photosynthetic processes 53% and 31%, respectively. "RNA synthesis' related contigs accounted for 8% while 'nitrogen metabolism', 'Ribosome', 'carbohydrate metabolism' and 'energy metabolism' accounted for the least number of contigs.

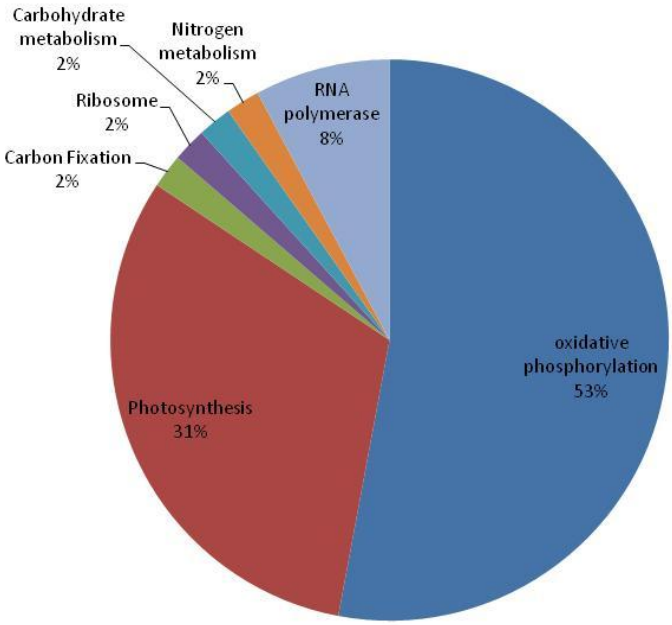
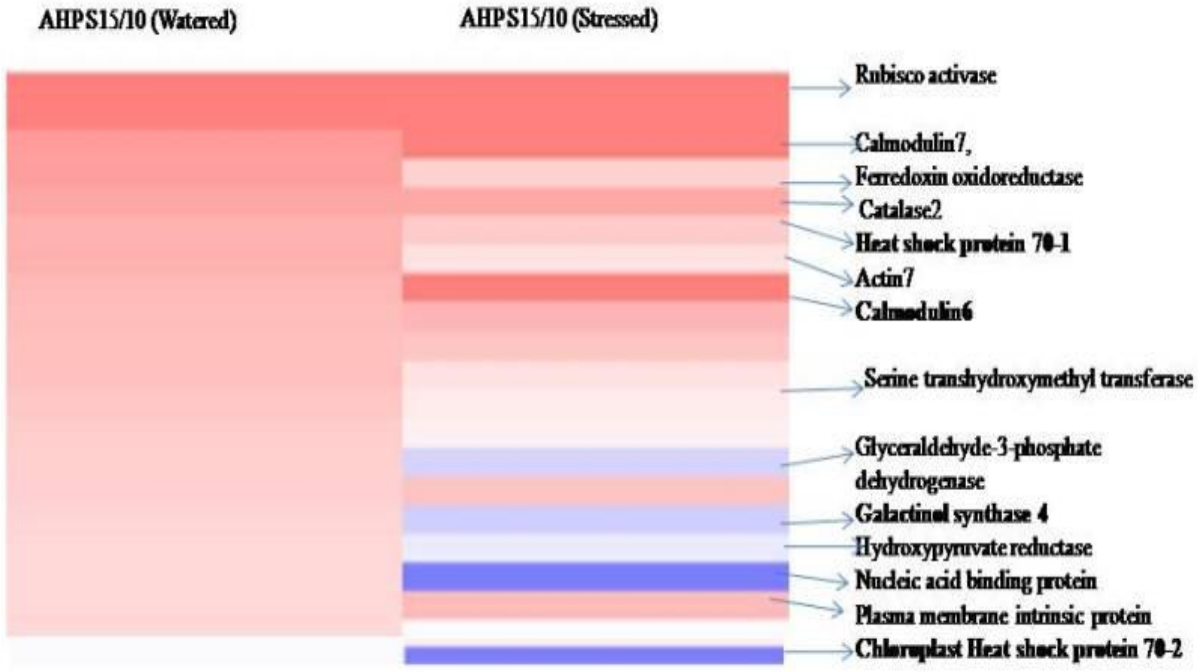


Figure 19: The Biologically active pathways in the leaf transcriptome of tea

4.5.7 Responsive genes to water deficit in tea.

The potential water stress responsive genes presented in a form of heat map is shown in figure 20. The classification of the genes was based on sequence similarity to those in *Arabidopsis* proteome. The drought sensitive Cultivar AHP S15/10 showed genes responsible for defense against drought that had been repressed at low soil moisture content (stressed). The stressed tea plants showed the repression of Heat shock protein related genes (*cpHsc70-1 and 2),* and Galactinol synthase related gene (*GolS4*) as compared to the unstressed plant. However, the 'calmodulin' gene (*CAM6*), signal inducer, was induced (Figure 20). Other genes that were minimally expressed under the stress conditions as compared to control conditions were the nucleic acid binding protein and glyceraldehydes-3-phosphate dehydrogenase.



Legend;

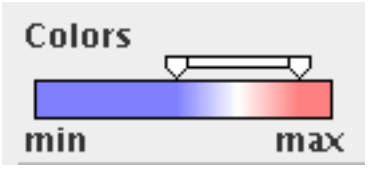
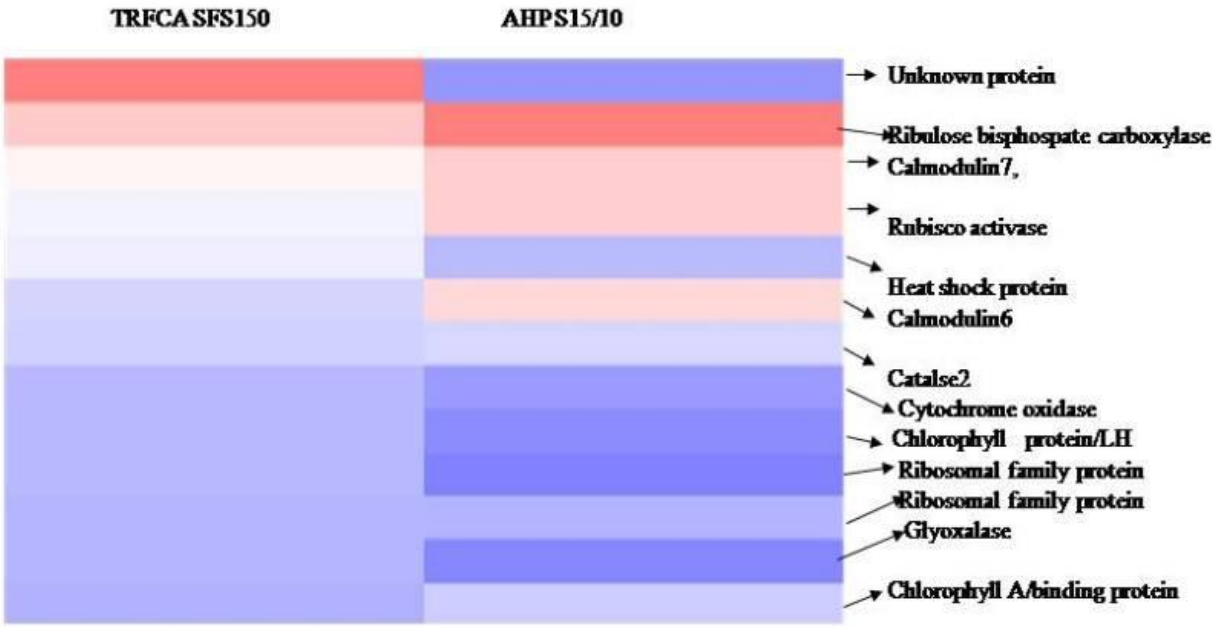


Figure 20: Heat map of expression pattern of genes in the susceptible cultivar (AHP S15/10) with response to water deficit.

However, analysis of potential genes in the stressed tolerant and susceptible cultivar showed various genes expressed and or repressed, Figure 21.



Legend;

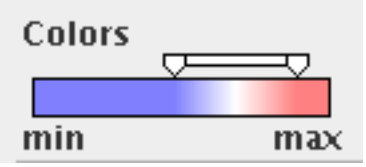


Figure 21: Heat map of expression pattern of genes in the tolerant cultivar TRFCA SFS150 and susceptible AHP S15/10 in response to water deficit.

The signalling genes, *CAM7* and *CAM6,* were induced in the susceptible cultivar under water stress conditions. Other than calmodulin like proteins, another signalling gene related to calcium dependent protein kinase 'CDPK' was also upregulated in the water stressed plant. The transcript related to defence against effects of drought like *heat shock protein was* also expressed at higher levels in the tolerant cultivar TRFCA SFS150 as compared to the susceptible cultivar, AHP S15/10. Other notably expressed genes were those related to photosynthetic processes such as rubisco activase and the Ribulose bisphosphate carboxylase. Individual analysis on transcripts from the four libraries showed that transcripts related to Calatase *(Cat2)*, Peroxidase family protein *(PRXR1,* and Superoxide dismutase *(SOD2)* were also expressed by the test tea cultivars. The tolerant cultivar, TRFCA SFS150, expressed all the three antioxidant molecules whereas the susceptible cultivar, AHP S15/10, expressed the catalase and peroxidases activity only. The level of expression differed with cultivar, for example the *SOD2* was expressed in the tolerant cultivar, TRFCA SFS150, and not in the susceptible cultivar AHP S15/10.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Effects of drought on leaf relative water content and shoot water potential in tea

In this study, the mean leaf relative water content (Lrwc) declined with reduced soil moisture content. Maximum mean leaf relative water contents were maintained at high soil moisture content. The decline in Lrwc can be attributed to deficiency in soil water and hence the inability of the plant roots tissues to take-up adequate water for translocation to the leafy parts of the plant. Although all the test tea plants responded by lowering their Lrwc with decline in SMC, the reduction varied with the cultivar. This can be attributed to varying response in tea cultivars to water deficit. The results generated in this study corroborated earlier observations reported in tea by (Waheed *et al.,* 2012). The minimal reduction in leaf relative water content serves as an adaptive strategy in the tea plant (Chakraborty *et al.,* 2002). To withstand soil drying due to decline in SMC, the tea plants responded by lowering their water potential to values less than that of the soil. Cultivar response showed that the tolerant cultivars maintained higher SWP than the sensitive cultivars. Similar observations have been reported in earlier studies on tea. This can be attributed to the fact that high shoot water potential in drought tolerant cultivars helps maintain an appreciable rate of photosynthesis and equally high water use efficiency (Damayanthi *et al.,* 2010).

5.2 Effects of drought on shoot growth rate

The rate of shoot growth in the present study was greatly affected by the reducing soil moisture content. The significant difference in shoot growth rate amongst cultivars suggests that the effect of water deficit varies depending on sensitivity of plant genotype to stress. The change in growth rate can be attributed to a reduction in cyclin-dependent kinase activity which results in slower cell division (Shilpi and Narendra, 2005). Cell growth is considered the most sensitive physiological process during water deficit conditions (Shakeel *et al.,* 2011), and the decline in soil moisture content in the present study suggests that water deficit contributes to decline in shoot growth rate. The decline in growth rate in the present study can also be attributed to inhibition of cell elongation by the interruption of water flow from the xylem to elongating cells due to decline in shoot water potential (Shakeel *et al.,* 2011). The present study also showed a highly significant positive correlation between shoot growth

rate and the net photosynthetic. This implies that the higher the photosynthetic rate, the higher the rate of growth and this can be attributed to accumulation of carbon in the actively growing shoots of plants. The decline in both growth rate and net photosynthesis can also be attributed to reduced carbon uptake due to stomatal limitation. Studies have shown that nearly all plant species show growth response to elevated CO_2 (Miko, 2011). However, in the present study cultivar TRFK 303/216 maintained high photosynthetic rate at both low and high SMC but the same was not replicated in growth rate. This can be attributed to the fact that additional carbon can only be converted into useful plant tissues if nutrients especially nitrogen are available (Miko, 2011). Limited nutrients availability means that even additional carbon cannot be used to sustain growth despite initial enhancement of photosynthetic carbon gain.

5.3 Effects of drought on gas exchange parameters

In the present study, the significant decline in stomatal conductance with decreasing soil moisture content can be attributed to stomatal regulation by water availability. This was corroborated by a positive correlation between the leaf relative water content and stomatal conductance. Since stomata control of water loss is considered the first response by plants to water deficit (MansWeld and Atkinson, 1990), the response by the test tea cultivars to reduce stomatal conductance under soil water deficit conditions, suggests a mechanism used by the tea plants to minimize water loss through the leaf. Earlier studies have shown that soil moisture stress induces anatomical changes in stomata which in turn reduces leaf stomatal density resulting to reduced stomatal conductance (Waheed *et al.*, 2012). This observed decline in stomatal conductance in the present study may be attributed to anatomical changes in leaf tissue. This was however not verified.

The decline in stomatal conductance in the present study varied significantly among the cultivars with the most drought sensitive cultivar having a higher decline as compared to drought tolerant one. This suggests that changes in stomatal conductance are dependent on sensitivity of the plant variety to water stress. Similar observations have been made in studies using tea cultivars of Sri Lankan origin (Damayanthi *et al.*, 2010).

The decline in net photosynthetic rate with SMC in the present study also suggests that tea plants are rain-fed crops and a resulting water deficit interferes with the plant photosynthetic process. This observation is corroborated by the highly significant positive correlation

between leaf relative water content and photosynthetic rate. The effects of reduced SMC on net photosynthesis can thus be attributed to stomatal limitations which results in carbon dioxide deficit in the chloroplast (Mafakheri *et al.,* 2010). Cellular changes in plant water status causes a reduction in stomatal conductance which in turn restricts carbon dioxide availability in the assimilation sites in chloroplast and hence reduction in photosynthetic rate (Yordanov *et al.,* 2003). The significant and positive correlation between stomatal conductance and net photosynthesis in this study corroborated the linear relationship between the two parameters. The decline in net photosynthetic rate with increased soil water deficit in the test cultivars can also be attributed to impairment of the photosynthetic apparatus which causes resistance in the flow of CO_2 from the mesophyll cell to the chloroplast stroma hence resulting in decreased chloroplast activity (Damayanthi *et al.,* 2010).

Studies have also shown that photosynthetic capacity of leaves depend on the amount and characteristics of photosynthetic components such as Rubisco (ribulose-1,5-bisphosphate carboxylase / oxygenase) which is responsible for carbon dioxide assimilation (Mohotti and Lawlor, 2001). Reduction in net photosynthesis in water stressed plants is thus ascribed to reduction in activity of Rubisco (ribulose-1,5-bisphosphate carboxylase / oxygenase) (Bota *et al.,* 2004a). The reduction in net photosynthetic rate in droughted tea plants the present study may also be attributed to the above observation.

The evapotranspiration rate was also reduced as the tea plants were subjected to decreasing soil moisture content. Varying evapotranspiration rates as a result of decline in SMC can also be attributed to cultivar specificity. Results from this study corroborate those made in other studies on tea (Damayanthi *et al.,* 2010). Results obtained in this study further revealed a significant positive correlation between transpiration and leaf relative water content suggesting that SMC affects the rate of evapotranspiration. Reduction in transpiration allows the tea plant to conserve water when subjected to drought conditions. This observation corroborates results obtained in similar studies with different plant species; *Amaranthus* sp. (Liu and Stutzel, 2002), *Vigna subterranean* (Jorgensen *et al.,* 2011). Results from this study also indicated that decline in stomatal conductance caused a reduction in transpiration rate Which supports the view that stomata play a significant role in regulation of water loss through the leaf. Similar observations have been made in *Phaseolus vulgaris* (Crus de Carvalho *et al.,* 1998), *Coffea arabica* (Damatta, 2004) and *Vigna unguiculata* (Crus de Carvalho *et al.,* 1998).

5.4 Effects of drought on proline and glycinebetaine accumulation

Data obtained in this study revealed that leaf proline levels in tea significantly increased with decline in soil moisture content. The significant negative correlation between leaf proline levels and leaf relative water content indicates that decline in water content as a result of drought induces the accumulation of proline. There was also a significant and negative correlation between shoot water potential and proline accumulation. This observation suggests that accumulation of proline lowers the water potentials in order to allow for additional water to be taken up from the environment to assist in buffering against the immediate effect of water shortages within the plant (Yamada *et al.,* 2005). Data obtained in this study also revealed that levels of proline varied significantly amongst test tea cultivars which may be ascribed to differing cultivar response to water stress. Research has for example shown that plant varieties tolerant to water stress accumulate more proline during water stress conditions as compared to the less tolerant cultivars (Upadhyaha *et al.,* 2008). From this study, the tea cultivar TRFK 306 had the highest level of leaf proline while under low soil moisture content. This biochemical character of cultivar TRFK 306 coupled to an earlier observation that the variety changes leaf colour from purple to green during drought periods (unpublished) could be indicative of the cultivar's tolerance to water stress. Studies in tobacco have shown that the over expression of pyrroline-5-carboxylate synthase gene leads to an increase in proline level and improved growth under drought condition (Parvaiz and Satyawati, 2008).

Besides proline, the tea plants used in the present study accumulated more glycinebetaine (Gb) at low soil moisture content. Glycinebetaine accumulation varied significantly amongst cultivars and this can be attributed to cultivar specificity. However, the levels of glycinebetaine were generally high in the water stressed plants as compared with the unstressed plants. Studies have shown that Gb accumulation is associated with specific cultivar, stage of growth and the level of droughts stress (Ashraf and Foolad, 2007). Cultivar AHP S15/10 accumulated the highest amount of Gb under water stressed conditions, whereas cultivar TRFK 301/5 accumulated the least under the same conditions. The two cultivars are notably drought susceptible. This observation seems to suggest that Gb accumulation is not a good marker for water stress tolerance in tea cultivars. The positive correlation between glycinebetaine and proline levels though not significant, also suggests that an increase in glycinebetaine level leads to an increase in proline levels and vice versa. This observation corroborates earlier reports that glycinebetaine synthesis enhances the protection of activity

of enzymes including those associated with sugar and amino acid metabolism leading to an increase in total soluble sugars and free amino acids like proline (Quan *et al.*, 2004).

5.5 Gene ontology assignments and KEGG pathway

In this study, Gene ontology (GO) categories associated with contig annotations as derived from sequence homology to *A. thaliana* gene, showed that majority of contigs in the biological processes were associated with metabolic processes, cellular development and response to stimulus. This indicates that the diverse metabolic processes are active in the *C. sinensis* leaf, and a variety of metabolites are synthesized in the leaf. The dominance of contigs associated with cellular development and response to stimulus is an indication that whereas the plants under high SMC are actively growing, the stressed plants induce stimulus response related genes in order to mediate signaling as a result of exposure to water deficit and other drought related incidences like heat.

Majority of the contigs were assigned to metabolic pathways, which included categories such as carbohydrate metabolism, energy metabolism/oxidative phosphorylation and photosynthesis. A significant proportion of the contigs were related to photosynthetic processes. This can be attributed to the fact that the leaf is the main photosynthetic site in a tea plant. Carbohydrate metabolism and energy metabolism were also represented in the KEGG pathway indicating that many active metabolic processes occurred in tea leaves. The leaf acts as the main organ for complex carbohydrate synthesis and energy conversion in plants (Wu *et al.*, 2012).

5.6 Drought responsive genes in tea, *Camellia sinensis*

During drought, the tea plants up regulated or down regulated several genes to mitigate against cellular damage. The genes varied from signaling to defence related genes. In this study, the signaling gene calmoduline like protein was found to be up regulated at various levels in the stressed tea plants. It is hypothesized that this is to allow the tea plants under stress to transduce calcium ion signals that activate major pathways by which extracellular signals such as growth factors, hormones and abiotic stress stimuli are converted into intracellular response (Munnik and Meijer, 2001). Calmodulin like protein was expressed at a higher level in the susceptible cultivar, AHP S15/10, as compared to the tolerant cultivar TRFCA SFS150, under water stress conditions. The induction of this gene at

various levels in the tea cultivars suggest that it plays a significant role in signal transduction during stress.

Transcripts showing homology to galactinol synthase (*Gols*) were also shown to be down regulated in the water stressed susceptible cultivar, AHP S15/10. This implies that the drought adaptation or tolerance of this cultivar to water stress conditions is limited, a trait that had been determined earlier through morphological studies. Galactinol l synthase functions as an osmo-protectant in plants (Nishizawa *et al.,* 2008). It has been shown to be induced in plants subjected to drought such as *Cucumis melo* (Volk *et al.,* 2003), *Coffea arabica L.* and *Arabidopsis thaliana (*Taji and Ohsumi, 2002*).* The induction of *Gols* in these species has been shown to confer some level of drought tolerance (Gupta *et al.,* 2012). The expression of galactinol synthase gene in the tolerant cultivars studied corroborated their role in defence.

Another category of transcripts that showed homology with heat shock proteins were induced in the test cultivars under water stress conditions. The heat shock proteins (*HSP70-1*) were induced in water stressed tea cultivars in this study. Heat shock proteins serve as as intra-cellular chaperones for other proteins and are also involved in plant stress response (Gupta *et al.,* 2012). *HSP* are involved in protection by controlling protein folding and protection of macromolecules and membranes from dehydration during drought (Das *et al.,* 2012). Genes encoding *HSPs* have been reported to be upregulated in tolerant Indian tea cultivars subjected to water stress (Muoki *et al.,* 2012). The results generated from this study, corroborated this observation; with the tolerant cultivar TRFCA SFS150, showing higher levels of HSP70-1 when compared with the less tolerant cultivar, AHP S15/10. This implies that the up regulation of heat shock proteins, *HSp70-1,* confers drought tolerance. Similar observations have been reported in *Pinus* (Heath *et al.,* 2002) and *Apple* (Wisniewki *et al.,* 2008). On the other hand, the inability of cultivar AHP S15/10, to withstand drought can be attributed to the low level of expression of heat shock proteins in the leaf tissues. Such an observation has been reported in *Populus euphratica* (Bogeat-Tribo *et al.,* 2007).

Transcripts showing homology with reactive oxygen scavengers such as peroxidase family protein *(PRXR1),* catalase *(Cat2)* and superoxide dismutase *(SOD)* were also induced in the assayed tea cultivars in this study. The catalase and peroxidase protein were expressed in the tolerant cultivar TRFCA SFS150 and the susceptible AHP S15/10 when exposed to water stress conditions. However the *SOD* was only expressed in the tolerant cultivar, TRFCA SFS150, under the same condition. The accumulation of antioxidant molecules is attributed to their role in scavenging for reactive oxygen species which damage the photosynthetic machinery in plants (Das *et al.,* 2012). Accumulation of antioxidant molecules

such as superoxide dismutase, acts as the first line of cellular defence against oxidative stress by catalyzing the dismutation of $O2^-$ to H_2O_2. The catalases and peroxidases on the other hand catalyse the removal (Chaves *et al.,* 2003) and conversion of H_2O_2 into water (Rossel *et al.,* 2006), respectively. The existence of a balance between *SOD* and other H_2O_2 scavenging enzymes is crucial in maintaining a steady level of oxidant molecules. Expression of *SOD* has been shown to confer tolerance and enhance shoot regeneration in transgenic pepper under water stress conditions (Chatzidimitriadou *et al.,* 2009). The up regulation of *SOD* in the tolerant cultivar TRFCA SFS150 in this study suggests that this antioxidant molecule plays a role in regulating response of tea to drought. The absence of the genes in the susceptible cultivar on the other hand is the probable contributor to its susceptibility to water deficit.

The identified genes which are all DNA-based, are potential targets for developing markers associated with water deficit response in tea. Use of molecular markers in breeding and selection can help in identification of traits of interest at early stages of the breeding cycle and hence reduce the breeding period (Shalini *et al.,* 2007). The advantage of this approach is that molecular markers are not influenced by environmental factors and developmental stage of plant and therefore can be selected for at any stage of the plants phenology and in any environment. They can also be used to screen for resistance to a stress condition in absence of the stress factor (Mphangwe *et al.,* 2013). DNA-based molecular markers have been exploited in breeding programmes of various crops, for example in; rice, apples, eucalyptus and maize. Tea has however not benefited much from this biotechnological advancement. Initially, this approach was considered less applicable to tea because of the limited genetic information that was available in the public domain. Good progress has, however, been made in identifying molecular markers associated with various agronomic traits (Mphangwe *et al*., 2013) including work on quantitative trait loci associated with yield (Kamunya *et al*., 2010) and for diversity studies in tea (Wachira and Waugh, 1995). However, the molecular markers that have been identified in tea are probably still too few considering the big tea genome and therefore necessitate more research work on molecular markers. Development of such markers will help in the identification of resistant tea cultivars at the early stages of breeding without subjecting the plant to water stress, thus facilitating marker assisted selection. Using conventional tea breeding approaches, an elite tea variety can take up to 23 years to be developed but with the use of molecular marker techniques, there is likelihood that this period can be reduced by about 10 years.

5.7 Conclusions

Results from this study revealed that;

i. Physiological responses of tea to soil moisture stress varied among the assayed tea cultivars. However, increase in soil water deficit resulted in decrease in shoot water potential and leaf relative water content of tea cultivars. Shoot growth rate, stomatal conductance, evapotranspiration rate and rate of net photosynthesis also decreased with decreased soil moisture content.

ii. Tea plants generally accumulate proline and glycinebetaine in their leaves as a result of exposure to water stress conditions. Leaf proline is a particularly good maker for tolerance to water stress and can applied as a selection tool in the development of drought tolerant cultivars

iii. Tea plants respond to water deficit through a complex of genes including; Heat shock proteins, calmodulin, antioxidant molecules (catalase, superoxide dismutase and peroxidase), and Galactinol synthase . The drought responsive transcripts identified between the tolerant (TRFCA SFS150) and susceptible cultivars (AHP S15/10) can be regarded as targets potentially associated with drought tolerance in tea plants.

5.8 Recommendations

Arising from the findings of this study, the following recommendations are made;

i. Leaf proline content should be used as a potential marker in clonal selection for drought tolerance in tea.

ii. The following identified candidate genes; Heat shock proteins *(HSP70-1,and 2),* calmodulin *(CAM6 and 7),*, antioxidant molecules like catalase (*Cat*), superoxide dismutase (*SoD*) and peroxidase (*Pox1*), and Galactinol synthase (*Gols4*) should be used to develop markers for use in marker assisted selection of water deficit tolerant tea cultivars.

iii. The present study provides a baseline for further research on drought responsive genes in tea and there is need therefore to determine systematic response of tea plants to drought from perception of stress to wilting.

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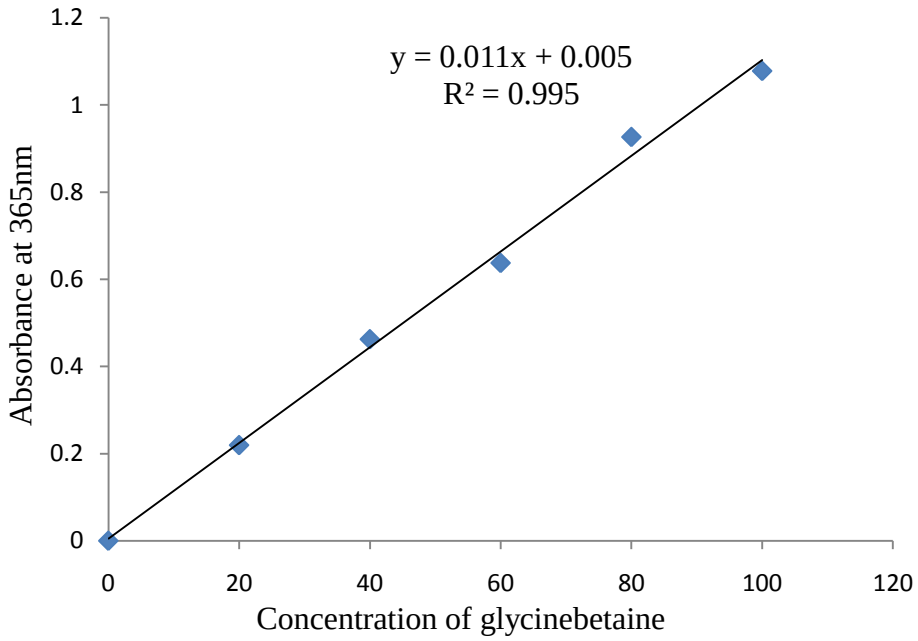
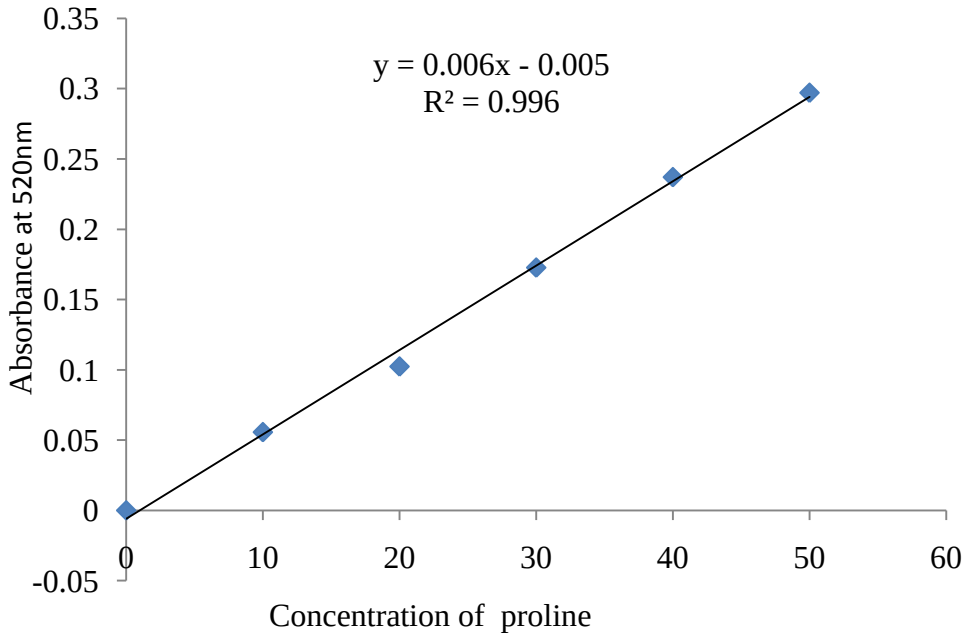
APPENDICES

Appendix 1; Standard calibration curve of glycinebetaine and proline





Appendix 2; Analysis of Variance tables

Analysis of variance table of leaf relative content

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	52.83	26.42	0.95	
rep.*Units* stratum					
clone	7	561.87	80.27	2.89	0.014
Treatment	2	14245.90	7122.95	256.34	<.001
Clone.treatment	14	1732.81	123.77	4.45	<.001
Residual	46	1278.20	27.79		
Total	71	17871.61			

Least significant differences of means (5% level) $F_{(2,46)}$ =256.34, P<0.001)

Table	clone	treatment	clone treatment
rep.	9	24	3
d.f.	46	46	46
l.s.d.	5.002	3.063	8.664

Stratum standard errors and coefficients of variation

Variate: data

Stratum	d.f.	s.e.	cv%
rep	2	1.049	1.5
rep.*Units*	46	5.271	7.7

Analysis of variance of Shoot water potential

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	0.019401	0.009700	2.25	
rep.*Units* stratum					
clone	7	1.020105	0.145729	33.79	<.001
treatment	2	1.434367	0.717184	166.30	<.001
clone.treatment	14	0.708494	0.050607	11.73	<.001
Residual	46	0.198383	0.004313		
Total	71	3.380750			

Least significant differences of means (5% level)

Table	clone	treatment	clone treatment
rep.	9	24	3
d.f.	46	46	46
l.s.d.	0.062	0.038	0.108

Stratum standard errors and coefficients of variation

Stratum	d.f.	s.e.	cv%
rep	2	0.020	2.1
rep.*Units*	46	0.066	6.8

Analysis of variance of shoot growth measurement

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	0.02258	0.01129	0.31	
rep.*Units* stratum					
clone	7	7.95177	1.13597	30.75	<.001
treatment	2	42.70749	21.35375	578.01	<.001
clone.treatment	14	11.20580	0.80041	21.67	<.001
Residual	46	1.69939	0.03694		
Total	71	63.58704			

Least significant differences of means (5% level)

Table	clone	treatment	clone treatment
rep.	9	24	3
d.f.	46	46	46
l.s.d.	0.182	0.112	0.316

Stratum standard errors and coefficients of variation

Stratum	d.f.	s.e.	cv%
rep	2	0.022	2.0
rep.*Units*	46	0.192	17.3

Analysis of variance of stomatal conductance

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	5106.2	2553.1	2.74	
rep.*Units* stratum					
clone	7	41670.8	5953.0	6.38	<.001
treatment	2	24639.6	12319.8	13.20	<.001
clone.treatment	14	20293.8	1449.6	1.55	0.130
Residual	46	42927.1	933.2		
Total	71	134637.5			

Least significant differences of means (5% level)

Table	clone	treatment	clone treatment
rep.	9	24	3
d.f.	46	46	46
l.s.d.	29.0	17.8	50.2

Stratum standard errors and coefficients of variation

Variate: data

Stratum	d.f.	s.e.	cv%
rep	2	10.3	3.7
rep.*Units*	46	30.5	11.0

Analysis of variance of Transpiration

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	0.81316	0.40658	4.21	
rep.*Units* stratum					
clone	7	2.58990	0.36999	3.83	0.002
treatment	2	1.08524	0.54262	5.62	0.007
clone.treatment	14	0.50387	0.03599	0.37	0.976
Residual	46	4.43864	0.09649		
Total	71	9.43081			

Least significant differences of means (5% level)

Table	clone	treatment	clone
rep.	9	24	3
d.f.	46	46	46
l.s.d.	0.295	0.180	0.511

Stratum standard errors and coefficients of variation

Variate: data

Stratum	d.f.	s.e.	cv%
rep	2	0.130	6.2
rep.*Units*	46	0.311	14.9

Analysis of variance of Net photosynthesis

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	74.90	37.45	2.23	
rep.*Units* stratum					
clone	7	249.31	35.62	2.12	0.060
treat	2	2188.02	1094.01	65.19	<.001
clone.treat	14	445.23	31.80	1.90	0.053
Residual	46	771.92	16.78		
Total	71	3729.39			

Least significant differences of means (5% level)

Table	clone	treat	clone
rep.	9	24	3
d.f.	46	46	46
l.s.d.	3.887	2.380	6.733

Stratum standard errors and coefficients of variation

Stratum	d.f.	s.e.	cv%
rep	2	1.249	8.4
rep.*Units*	46	4.096	27.5

Analysis of variance of proline profiles

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	0.0019910	0.0009955	1.91	
rep.*Units* stratum					
clone	7	0.3420850	0.0488693	93.73	<.001
treatment	2	0.2645544	0.1322772	253.70	<.001
clone.treatment	14	0.1757942	0.0125567	24.08	<.001
Residual	46	0.0239840	0.0005214		
Total	71	0.8084087			

Least significant differences of means (5% level)

Table	clone	treatment	clone
rep.	9	24	3
d.f.	46	46	46
l.s.d.	0.0217	0.0133	0.0375

Stratum standard errors and coefficients of variation

Variate: data

Stratum	d.f.	s.e.	cv%
rep	2	0.0064	4.0
rep.*Units*	46	0.0228	14.3

Analysis of variance of glycinebetaine levels

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	0.30099	0.15050	2.52	
rep.*Units* stratum					
treat	2	2.52706	1.26353	21.16	<.001
clone	7	2.07877	0.29697	4.97	<.001
treat.clone	14	1.69952	0.12139	2.03	0.036
Residual	46	2.74707	0.05972		
Total	71	9.35341			

Least significant differences of means (5% level)

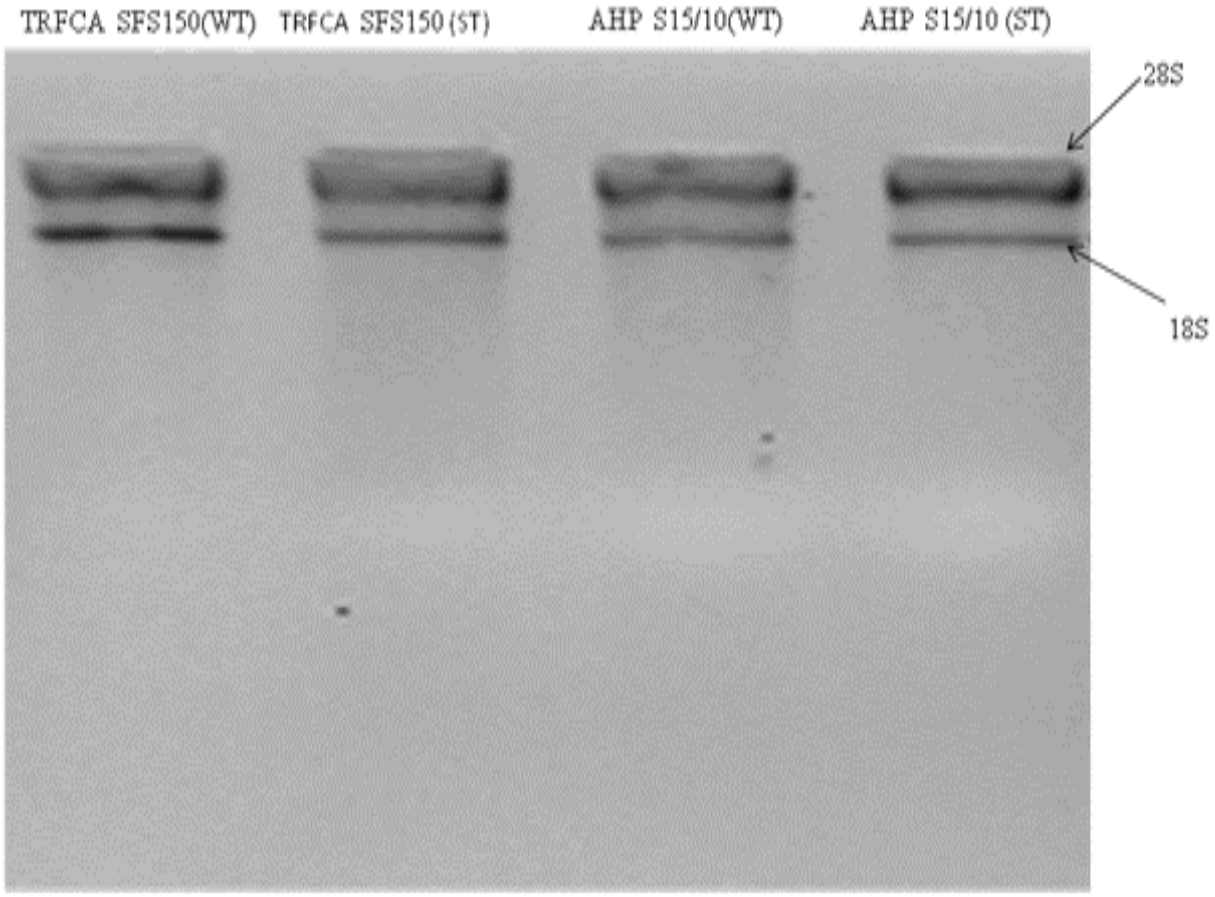
Table	treat	clone	treat
rep.	24	9	3
d.f.	46	46	46
l.s.d.	0.142	0.232	0.402

Stratum standard errors and coefficients of variation

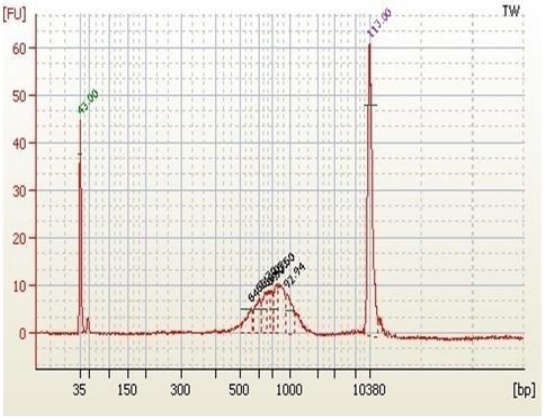
Variate: data

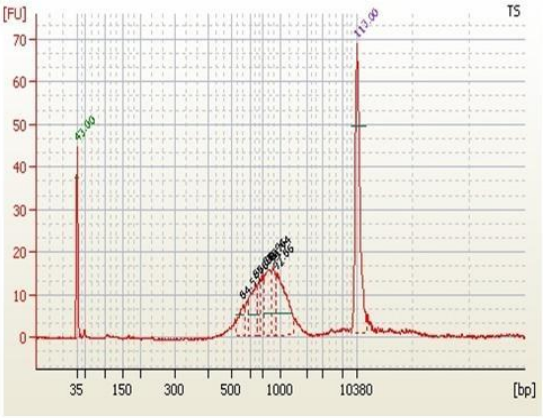
Stratum	d.f.	s.e.	cv%
rep	2	0.079	4.4
rep.*Units*	46	0.244	13.7

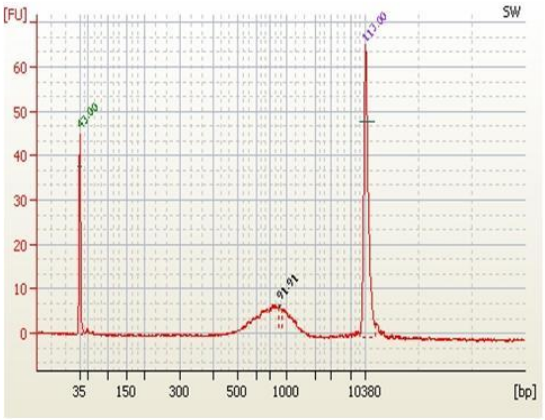
Appendix 2; Gel electrophoresis image of total RNA. ST; stressed condition, WT; watered condition

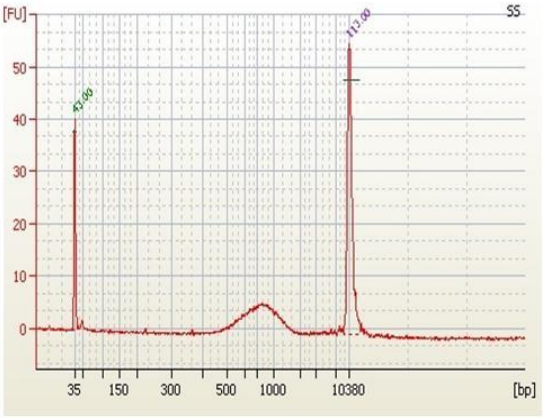


Appendix 3 ; Trace representation of cDNA library from 4 tea samples as assessed on an Agilent Bioanalyzer High sensitivity DNA chip. TW = TRFCA SFS150 (Watered), TS= TRFCA SFS150 (stressed), SW= AHP S15/10 (Watered), SS= AHP S15/10 (stressed)









Appendix 4; Summary statistics of assembled contigs

Statistics for contig lengths:	
Min contig length:	100
Max contig length:	2,466
Mean contig length:	250.38
Standard deviation of contig length:	288.67
Median contig length:	141
N50 contig length:	332
Statistics for numbers of contigs:	
Number of contigs:	460
Number of contigs>=1kb:	13
Number of contigs in N50:	85
Statistics for bases in the contigs:	
Number of bases in all contigs:	115,177
Number of bases in contigs>=1kb:	20,936
GC Content of contigs:	43.91%
Simple Dinucleotide repeats:	
Number of contigs with over 70% dinucleotode repeats:	0.00 % (0 contigs)
AT:	0.00 % (0 contigs)
CG:	0.00 % (0 contigs)
AC:	0.00 % (0 contigs)
TG:	0.00 % (0 contigs)
AG:	0.00 % (0 contigs)
TC:	0.00 % (0 contigs)
Simple mononucleotide repeats:	
Number of contigs with over 50% mononucleotode repeats:	0.00 % (0 contigs)
AA:	0.00 % (0 contigs)
TT:	0.00 % (0 contigs)
CC:	0.00 % (0 contigs)
GG:	0.00 % (0 contigs)

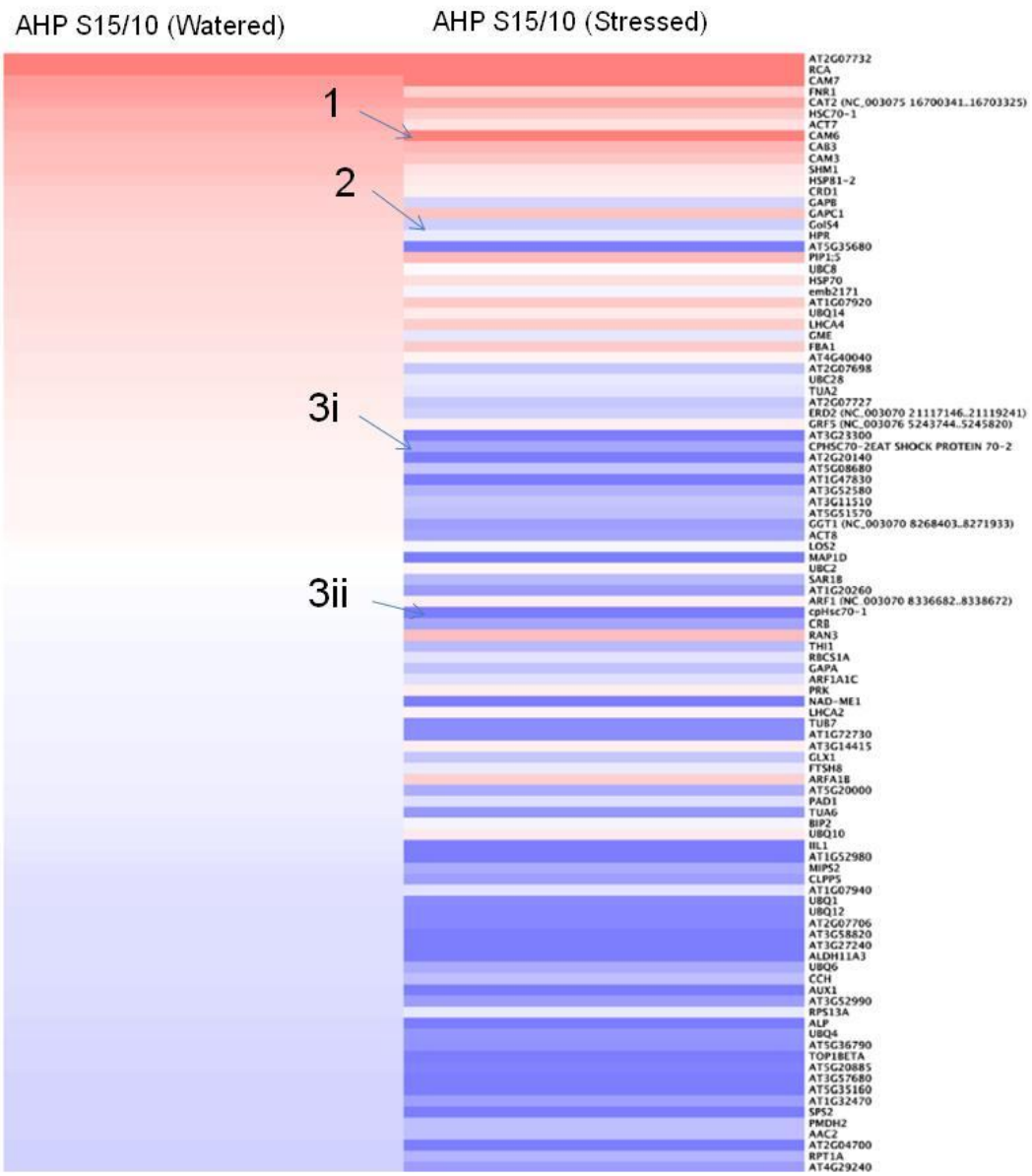


Figure showing expression pattern of genes in the susceptible cultivar (AHP S15/10) with response to drought. 1; Calmoduline like protein (*CAM6*), 2; Galactinol synthase (*GolS4*), 3i; Chloroplast *heat shock protein (cpHSC70-2*), 3ii; Chloroplast heat shock protein (*cpHSC70-1*).

Manuscripts 1

Responses of tea *(Camellia sinensis)* to drought stress (In-press JHSB)

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SUMMARY

The physiological and biochemical responses of tea *(Camellia sinensis)* to water stress were determined on eight cultivars raised in a rain-out shelter. The plants were subjected to three levels of water treatment consisting of 34%, 26% and 18% soil moisture content and arranged in a randomized complete block design with three replications. The assayed tea cultivars differed significantly ($P \leq 0.05$) in their responses to water stress. Imposition of water deficit conditions for 3 months to the 18-months-old tea seedling caused a significant decline in shoot growth, leaf relative water content, shoot water potential and gas exchange parameters of stomatal conductance, evapotranspiration rate and net photosynthetic rate. The water stressed tea plants accumulated leaf proline and glycinebetaine. Proline accumulation was particularly shown to be significantly high in drought tolerant cultivars and thus it may be a potential marker for drought tolerance in tea.

THE JOURNAL OF HORTICULTURAL SCIENCE & BIOTECHNOLOGY

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Manuscripts 2

Transcriptome-based identification of drought tolerance genes in the tea plant, *Camellia sinensis* (In-press)

Maritim T., Wamalwa M., Mireji P., Mwendia, C. , Kamunya S., Stomeo F., Muoki, R., Michuki, G. , Skilton, R., Kyalo, M., M. Karori, Wachira F. N*

ABSTRACT

Tea is among the most widely consumed beverages worldwide and the leading agricultural export of Kenya. Among the abiotic stresses experienced in rural areas, where tea farming is based, drought is the most predominant. Developing drought-adapted tea cultivars depends on gaining a deeper understanding of the genetic mechanisms underlying drought tolerance in this species, about which little is currently known. In this study, we used high-throughput sequencing to generate and compare expression data for 18-month old seedlings from two tea cultivars, TRFCA SFS150 (drought tolerant) and AHP S15/10 (drought susceptible), subjected to both drought (18% soil moisture content) and control conditions. Plants were exposed for three months using a randomized complete block design with three replicates, after which fresh shoots were harvested and total RNA extracted. Isolated mRNA was reverse transcribed and sequenced to produce 232,853 raw reads, which were quality-filtered, trimmed and assembled into 460 long transcripts (contigs). Contigs were annotated using BLAST to search against similar proteins in the *Arabidopsis* proteome. Based on Gene Ontology analysis, drought-related genes, including heat shock proteins, *HSP70,* antioxidant molecules such as superoxide dismutase (SOD), catalase (cat) and peroxidase (*PoX*), signal tranducers, calmoduline-like protein (*Cam7),* and galactinol synthase (*Gols4*), were induced in plants exposed to drought. The expression of HSP70 and SOD were enhanced in the tolerant cultivar TRFCA SFS150 as compared to the susceptible cultivar AHP S15/10 under drought condition. In conclusion, many of the observed changes in expression occurred at loci with known functional links to physiological and biochemical features of drought response. These genes provide important targets for future work on the development of molecular markers and functional genomics in *Camellia sinensis* and an understanding of the molecular mechanisms underlying drought tolerance in plants.