DROUGHT RESPONSIVE GENES IN TEA CULTIVARS GROWN IN KENYA

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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

We confirm that this thesis has our approval to be presented for examination as per the Egerton University regulations.

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DEDICATION

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

ACKNOWLEDGEMENT

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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. However, tea growing areas in Kenya experience abiotic stresses with drought been the most predominant. Tea plants tolerate drought 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. Use of sequencing technologies provide a fast, cost effective, and reliable approach to generate large expression datasets for functional genomic analysis in plants exposed to drought. In the present study, 18-months old seedling tea from eight cultivars, tolerant and susceptible to drought, 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 block design with three replications. After three months of exposure to treatment, physiological parameters (leaf water status, shoot growth and gas exchange parameters), biochemical parameters (proline and glycinebetaine levels) were determined. The data generated were subjected to two-way anova using Genstat. For molecular analysis, total RNA extracted was from tolerant and susceptible cultivars under stressed and unstressed conditions. The extracts were used to isolate mRNA which was reverse transcribed to complementary DNA. The sequences/reads generated from the cDNA libraries using 454 GLX sequencer were analysed *in silico* using bioinformatics tool. There was a reduction in shoot growth, leaf relative water content, shoot water potential and gas exchange parameters that varied significantly, $P<0.05$, amongst the cultivar and treatment in all the cultivars under study. However, proline and glycinebetaine increased with stress. Cultivar TRFK 306 accumulated the highest, 0.53μmol/g FW, level at low (18%) SMC whereas TN14-3 accumulated the least, <0.1 μmol/g FW, amounts. Glycinebetaine accumulation was high, 2.4mMoles/g DW, in AHP S15/10 and lowest (1.4 mMoles/g DW) in TRFK 301/5 under low soil moisture content. Accumulation of the two osmolytes also varied significantly ($P<0.05$) amongst treatment with the lowest soil moisture content inducing higher accumulation of the two biochemicals analysed. The 232,853 high quality raw reads generated from the sequencer were quality-filtered, trimmed and assembled into 460 long transcripts (contigs). The contigs were annotated by BLAST similarity search against proteins in *Arabidopsis* proteome. Based on Gene ontology analysis, drought response related transcripts including Heat shock proteins, *Hsp70,* antioxidant molecules such as Superoxide dismutase, catalase and peroxidise, signal tranducers, Calmoduline like protein, and

Galactinol synthase (*Gols4*) were induced in the water stressed plants. In conclusion, molecular changes in tea plants bring about the physiological and biochemical changes. The physiological and biochemical changes observed thus were a clear indicator that water stress response is controlled at the genetic level.

TABLE OF CONTENTS

DECLARATION AND RECOMMENDATION ii
COPYRIGHT iii
DEDICATION iv
ACKNOWLEDGEMENT v
ABSTRACT vi
TABLE OF CONTENTS viii
LIST OF TABLES xii
LIST OF FIGURES xiii
LIST OF ABBREVIATIONS xiv
CHAPTER ONE 1
INTRODUCTION 1
1.1 Background information 1
1.2 Statement of the problem 4
1.3 Objectives 4
1.3.1 General 4
1.3.2 Specific objectives 5
1.4 Hypotheses 5
1.5 Justification of the study 5
1.6 The scope and limitation 5
CHAPTER TWO 6
LITERATURE REVIEW 6
2.1 Tea plant 6
2.2 Economic importance, social and health benefit of tea in Kenya 7
2.3 Factors affecting growth and productivity in tea 7
2.4 Drought in tea 8

2.5 Plant response to drought stress 11
2.5.1Cellular and Physiological responses to drought stress 12
2.5.2 Biochemical responses to drought stress 14
2.5.3 Accumulation of ROS 17
2.5.4 Variation in polyphenol profiles 18
2.5.5 Molecular events in plants subjected to drought 18
2.6 Transcriptome analysis of drought-responsive genes 19
2.6.1 Candidate gene approaches 20
2.6.2 Microarray Technology 20
2.6.3 Sequence-Based Approaches to Studying Transcriptomes 20
2.6.4 Sanger sequencing technology 20
2.6.5 Expressed Sequence Tag 21
2.6.6 New-Generation Sequencing Methods 22
CHAPTER THREE 26
MATERIALS AND METHOD 26
3.1 Experimental materials 26
3.2 Experimental Set-up and Design 27
3.3 Determination of leaf relative water content, shoot water potential and shoot growth 27
3.4 Determination of stomatal conductance (Gs) and Evapotranspiration (E) and net photosynthesis (P) 28
3.5 Determination of glycinebetaine profiles in tea 28
3.6 Determination of proline accumulation in tea 28
3.7 Sampling for total RNA isolation 29
3.8 Extraction and quantification of total RNA and mRNA 29
3.9 Preparation of cDNA library for transcriptome sequencing 29
3.10 emPCR amplification 30

3.11 454 Sequencing 30

3.12 Physiological and Biochemical data analysis 31

3.13 Sequence analysis 31

3.13.1 Quality control and *De novo* assembly of sequencing reads 31

3.13.2 Functional annotation and classification of contigs 31

CHAPTER FOUR 33

RESULTS 33

4.1 Growth conditions in the rain-out shelter 33

4.2 Effects of water stress on physiological parameters in tea 33

4.2.1 Leaf relative water content 33

4.2.2 Shoot water potential 34

4.2.3 Shoot growth rate 35

4.2.4 Stomata conductance 36

4.2.5 Evapotranspiration 37

4.2.6 Net photosynthesis 38

4.3 Effects of water stress on biochemical parameters in tea 39

4.3.1Proline levels 39

4.3.2 Glycinebetaine levels 40

4.4 Correlation coefficient analysis of physiological and biochemical parameters 42

4.5 Molecular analysis 43

4.5.1 Extraction and quantification of total RNA and mRNA in tea 43

4.5.2 cDNA synthesis 43

4.5.3 *De novo* sequencing of the *Camellia sinensis* leaf transcriptome 44

4.5.4 Trimming and quality control of sequences 45

4.5.5 *De novo* assembly of sequence reads 46

4.5.6 Functional annotation by GO and KEGG 47

4.5.7 Expression analysis of genes involved in drought response in tea 49

CHAPTER FIVE **52**

DISCUSSION... **52**

5.1 Effects of drought on leaf relative water content and shoot water potential in tea............52

5.2 Effects of drought on shoot growth rate 52

5.3 Effects of drought on gas exchange parameters 53

5.4 Effects of drought on proline and glycinebetaine accumulation 54

5.5 Gene ontology assignments and KEGG pathway...55

5.6 Drought responsive genes in tea, *Camellia sinensis*..56

5.7 Conclusions..58

5.8 Recommendations..58

REFERENCES **59**

APPENDICES... **72**

LIST OF TABLES

Table 1. Attributes of the cultivars used in the experiment 26
Table 2. Effects of decreasing soil moisture content on shoot water potential of different tea cultivars 35
Table 3. Effects of soil moisture content on transpiration rate 38
Table 4. Correlation coefficient between the physiogical and biochemical parameters of water stress in tea 42
Table 5. Concentration and quality of total RNA and mRNA isolated from tea leaf.............43
Table 6. Summary statistics of raw reads 44

LIST OF FIGURES

Figure 1. Map of tea growing areas in Kenya....................2
Figure 2. Cultivated tea plants under optimum water availability....................9
Figure 3. Cultivated tea plants under minimal (drought) water availability....................9
Figure 4. Response to climatic change in suitability of tea growing regions in Kenya..........10
Figure 5. Physiological, biochemical and molecular responses of plants to drought stress....................11
Figure 6. Biosynthetic pathway of proline....................15
Figure 7. Biosynthetic pathway of glycinebetaine....................16
Figure 8. Diagram showing pyrosquencing process....................24
Figure 9. Effects of decreasing soil moisture content on leaf relative water content amongst different tea cultivars... 34
Figure 10. Effects of soil moisture content on shoot growth amongst different tea cultivars. 36
Figure 11. Changes in Stomatal conductance in different tea cultivars in response to changing soil moisture content....................37
Figure 12. Effect of soil moisture content on net photosynthetic rate in different tea cultivars....................39
Figure 13. Leaf proline levels of different tea cultivars grown under different soil moisture regimes....................40
Figure 14. Variation in leaf glycinebetaine level of tea cultivars at different treatments moisture....................41
Figure 15. Gel-like image of cDNA library sample....................44
Figure 16. Box plot of quality scores....................45
Figure 17. Size distribution of contigs generated by *de vovo* assembly of filtered and trimmed read 46
Figure 18. Gene ontology classification....................48
Figure 19. Biologically active pathways in the leaf transcriptome of tea....................49
Figure 20. Heat map of gene expresion pattern of genes in the susceptible tea cultivar 50

LIST OF ABBREVIATIONS

AAPK	Abscisic acid-activated protein kinase
ATP	Adeneine Triphosphate
APS	Adenosine 5’-Phosphosulfate
BADH	Betaine aldehyde dehydrogenase
BLAST	Basic Local Alignment Search Tools
cDNA	Complementary Deoxyribonucleic acid
CIAT	International Centre for tropical Agriculture
CMO	Choline mono-oxygenase
emPCR	Emulsion polymerase chain reaction
EST	Expressed Sequence Tags
FLcDNA	Full length complementary Deoxynucleic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LRWC	Leaf Relative Water Content
mRNA	Messenger Ribonucleic acid
NCED	9-cisepoxycarotenoid dioxygenase
ncRNA	Non-coding ribonucleic
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

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 and the crop currently covers 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°C (Willson and Clifford, 1992).

Tea contributes 26% of the total foreign exchange earnings annually and 4% of the Gross Domestic Product earnings 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 products (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, antiaging (Khan and Mukhtar, 2007) antioxidant (Karori *et al.,* 2007; Kerio *et al.,* 2013), and antimalarial 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). Tea generally consists of *C. sinensis* var. *sinensis* (Chinary type), *C. sinensis* var. *assamica* (assam type) and *C. sinensis* var. *Assamica* ssp *Lasiocalyx* (Cambod type) varieties that are differentiated by their morphological, biochemical and molecular affinities (Magoma *et al.,* 2003; Wachira and Waugh, 1995; Wachira *et al.,* 2001).

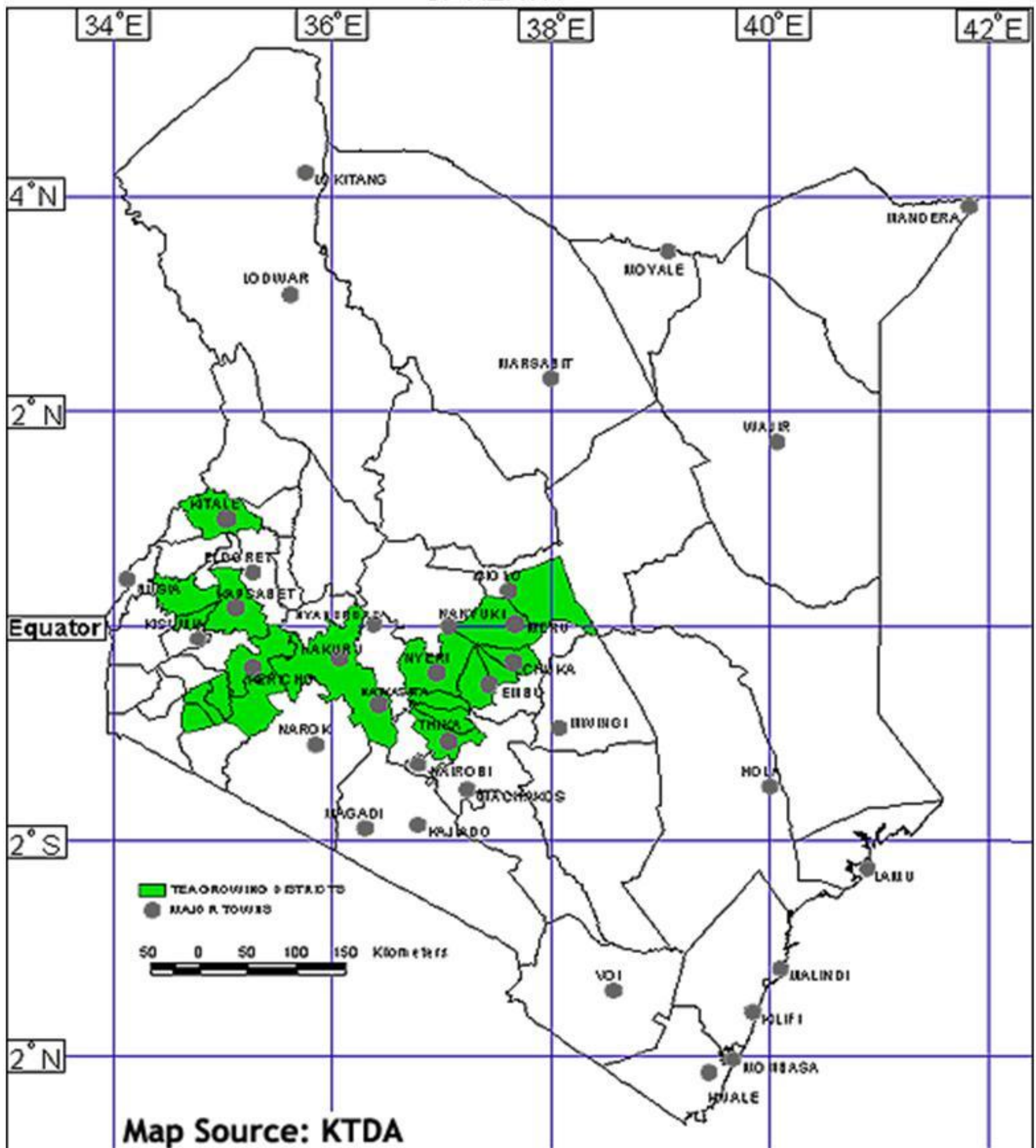


Figure 1; Map of tea growing areas in Kenya.

Tea farming is greatly influenced by environmental conditions, with drought as the most limiting problem 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 and water potential and gas exchange parameters including net photosynthesis, stomatal conductance and evapotranspiration rate (Bota *et al.,* 2004b). Growth rate is also affected by drought (Shakeel *et al.,* 2011). At 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 contributes 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 can 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 drought or water deficit through molecular changes that develop tolerance through expression of specific 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 drought 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. One such technique is the Next Generation Sequencing technologies which comprise of llumina/Solexa, Applied Biosystems SOLiD and Roche/454 pyrosequencing. These techniques are fast, simple, and 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, the technique was used to identify novel genes that are expressed by tea plants upon exposure to drought stress. The new insight is essential for development of plants with enhanced tolerance to drought stress. The present study was designed to determine the physiological, biochemical and molecular responses of tea plant to drought.

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 in future result in some of the traditional tea growing areas in Kenya loosing 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 identify drought responsive genes in tea by sequencing of the leaf transcriptome.

1.3.2 Specific objectives

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

1.4 Hypotheses

1. Water stress does not influence water status and gas exchange parameters of tea plant.
2. Water stress does not influence proline and glycinebetaine profile in tea plant.
3. There are no drought responsive genes in tea plant.

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 season, 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 tea plant 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 crop improvement for 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 are stable polyploid with cultivated tea being diploid (2n=30). Other relatives of tea like the *Camellia irrawadiensis, C. 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; tetraploid (4n=60), hexaploid (6n=90) and triploid (3n=45) have been reported (Wachira and Kiplangat, 1991). Identified polyploidy in tea have better rooting ability than the diploids and hence resistant to environmental stress, however they are poor yielders (Bezbaruah, 1968). They have also been found to be vigorous, and have higher content of total polyphenol than the diploids (Magoma *et al.,* 2000; Wachira, 1994; Wachira and Ngetich, 1999).

Generally, there are *C. sinensis* var. *sinensis* (Chinary type), *C. sinensis* var. *assamica* (assam type) and *C. sinensis* var. *Assamica* ssp *Lasiocalyx* (Cambod type) main varieties of tea that freely interbreed, making the tea plant highly diverse (Willson and Clifford, 1992). The three main taxa can be 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 polyphenol and yield showed that kenyan cultivars were superior in both attributes (Wachira and Kamunya, 2005). This corroborated the earlier findings that Assam varieties are superior compared to chinery varieties

Other wild species of *Camellia* including. *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 polyphenol and antioxidants, saponin, polysaccharide, L. Theanine, pigments and tea water extracts biochemical molecules which contributes to its medicinal value (Bukowski and Percival, 2008; Jia *et al.,* 2005; Khan and Mukhtar, 2007; Monobe *et al.,* 2008). The medicinal properties associated with such biochemical compounds include; antidiabetic, antimicrobial (Koech *et al.,* 2013), anticancer, anti-inflammatory, antiaging properties (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 plethora of abiotic and biotic stress factors. Abiotic stress; low temperature, salinity and drought 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, soil and or atmospheric water deficit resulting from extreme temperature, is the most common abiotic stress that plants encounter. It's 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 drought 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). Risein 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 results in a loss of 14-20% in yields annually and about 6-19% plant mortality. Severe drought often leads to scorching of leave which may lead to dead of crop (Fig. 2,3), thus resulting high cost of infilling.

Figure 2.Cultivatedtea plants under optimum water availability

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

These weather phenomena are increasingly being experienced in tea growing areas as a consequence of global weather change. Reports indicates 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. This area currently experience incidence of hail and frost regularly

Tea producing zones have an optimum altitude of between 1500 and 2100 masl but with global climate change this is expected to increase to altitude between 2000 and 2300 masl by 2050 (Managua, 2011). By this time, areas with altitude 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 variable, temperatures are expected to rise with decreasing altitude and this will have significant impact on tea farming with areas which are fairly suitable for tea farming losing their suitability as shown in figure 4**.**

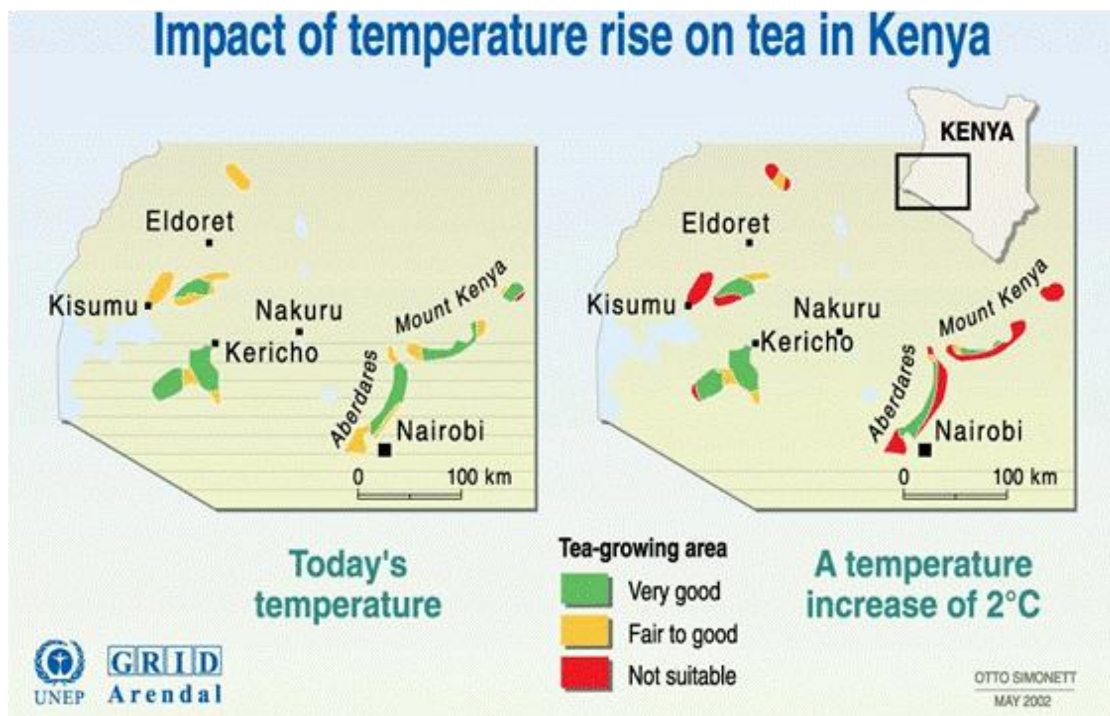


Figure 4. Responses to changing climatic in suitability of tea growing regions in Kenya.

The same incidence of rising temperature has been experienced in Tea Research Agro-meteorological stations, where there has been an annual rise of 0.02℃. Analysis of data on rainfall pattern has also shown that soil water deficit has been increasing by about 3mm annually while rainfall decreases by 5mm annually since 1950s (Cheserek, 2011). The above

environmental conditions cause the plant to respond in different ways to reduce or avoid or adapt to the stress.

2.5 Plant response to drought stress

Plant responses to drought are complex processes involving adaptive changes or deleterious effects and are physiological or biochemical due to the changes in water homeostasis (Chaves *et al.,* 2003; Xu *et al.,* 2009). This brings about decline in chemical activity and loss of turgor in 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 effectsof drought stress and ensure optimal growth condition.

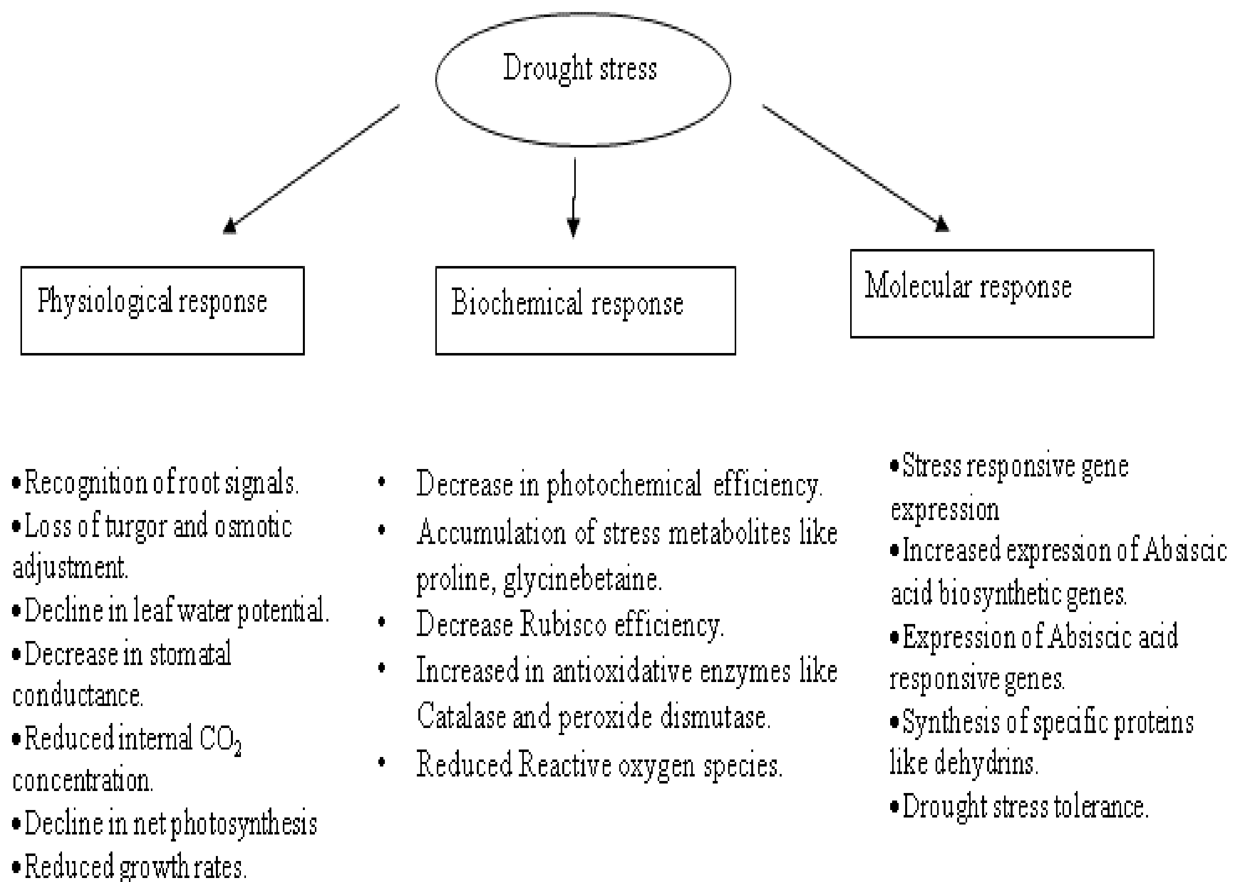


Figure 5; Physiological, biochemical and molecular responses of plants to drought stress.

Understanding how plants respond to drought 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; drought escape by completing plant life cycle before severe water deficit, drought avoidance by enhancing capacity to acquire water by developing root systems (Jackson *et al.,* 2000), drought tolerance mainly through improving osmotic adjustment ability and increasing cell wall elasticity to maintain tissue turgidity, drought resistance by altering metabolic paths for its survival under severe stress for example through increased antioxidant metabolism (Bartoli *et al.,* 1999), drought abandon by removing some parts of plant such as the shedding of older leaves (Chaves *et al.,* 2003), plant evolution under long-term drought condition through genetic mutation and genetic modification (Cheruiyot *et al.,* 2007; Sherrard *et al.,* 2009).

Effects of drought in a plant can occur 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 drought conditions can be used to identify genes and metabolic pathways that are induced as a result of drought. Such plants are referred to as tolerant plants. Tolerant plants can be used to study the physiological, biochemical and molecular responses to drought. The plant species *Arabidopsis thaliana* has particularly been useful as a model plant in determination of stress responsive genes in plants.

2.5.1Cellular and Physiological responses to drought stress

The roots are the primary sites involved in perception of drought in a plant and are asssumed to trigger drought 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 increasing inhibit 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.* 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 stress, 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 affect 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 also play an essential role in physiological response of plants to water stress. It 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 drought 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 overexpression 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 drought acts as a signal for the

initiation of acclimation (Shinozaki and Yamaguchi-Shinozaki, 1997). In response to drought, ABA accumulate 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 drought 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 stress

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 efffects of reduced water levels in the plant they also do not intefere with biochemical reactions in a plant (Jaleel *et al.,* 2007). Proline amino acid occurs widely in higher plants and accumulates in larger amounts than other amino acids in response to environmental stress. In response to drought, proline accumulation occurs in the cytosol where it adjusts cytoplasmic osmosis. Proline is synthesized through L-ornothine and L-glutamate pathway alternative routes in plants (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. It is widely distributed in plants and it accumulates in larger amounts than other amino acids in drought-stressed plants (Ashraf and Harris, 2004; Irigoyen *et al.,* 1992; Kohl *et al.,* 1991). 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 reduce denaturation of enzymes (Kavi-Kishor *et al.,* 1995). Prolineis also a nitrogen storage product in plant tissues (Hare *et al.,* 1998). Other than protecting of plants during stress condition, proline breakdown upon relief of stress provide sufficient reducing agent that support mitochondrial oxidative phospohorylation and generation of ATP for recovery from stress and repair of stress induced damages.

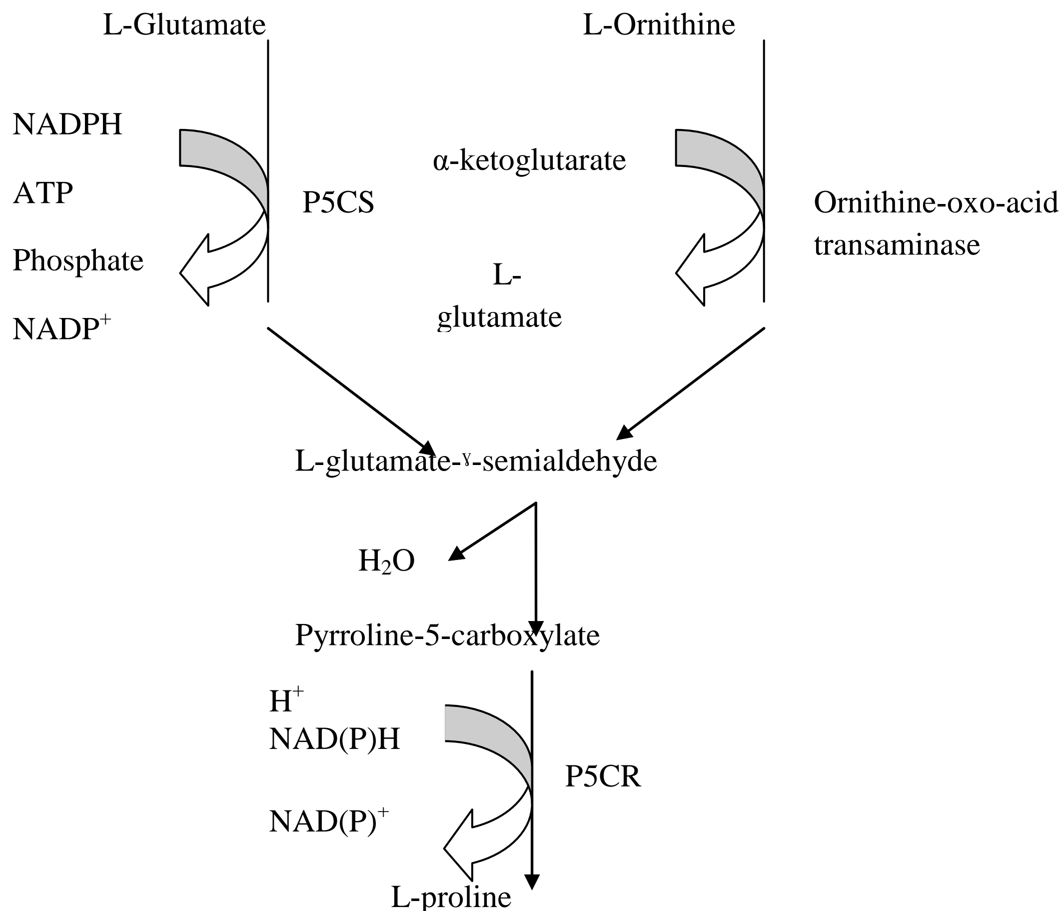


Figure 6; Biosynthetic pathway of proline

Two enzymes pyrroline-5-carboxylate synthatase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) plays 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*, prolinesynthetase gene is upregulated 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. Proline accumulation as a result of drought has not been studied in tea.

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 they accumulate 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 5).

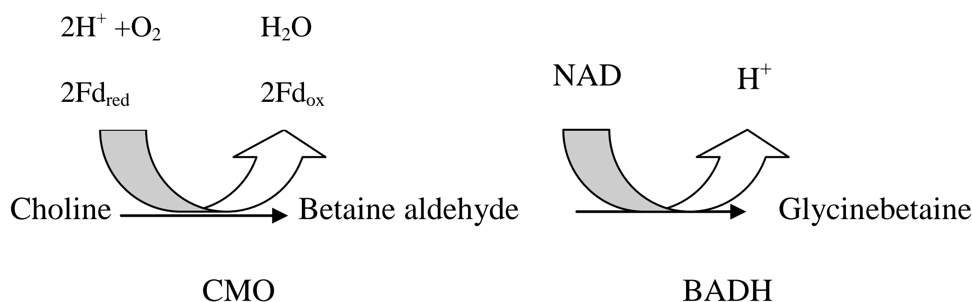


Figure 7: Biosynthetic pathway of glycinebetaine; BADH (Betainealdehyde dehydrogenase), CMO (Choline mono-oxygenase) (Parvaiz and Satyawati, 2008).

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. Their role during droughty conditions include the following; the hydroxyl group of the sugar molecule 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 (*AtGolS2)* responsible for synthesis of galactinol and raffinose sugars resulted in drought tolerance (Shinozaki, 2007). Trehalose sugar is also a factor in tolerance to environmental conditions, including drought, in plants. 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 ROS

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 level 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,(Cheruiyot *et al.,* 2008), also it was found out that the level of shoot catechins in tea responded to soil water level since the later had great influence in shoot growth. However the various catechins in tea were not influenced uniformly by the water stress and the difference was attributed to their chemical difference.

2.5.5 Molecular events in plants subjected to drought

Drought 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 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 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 phosphatises, 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 of drought-responsive genes

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 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 limitation 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 in gene expression profile to study the different genes expressed by the first and the second 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 limitation 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 plant. 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 determine 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 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 result 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) provides 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 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 leave 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 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 labor intensive for routine use, has low throughput, lack of 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, that is; 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 limitation has led to 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 Sanger sequencing technology has been the dominant approach for DNA sequencing but with the advent of high-through put sequencing technologies Sanger technology has been overtaken. 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 human genome project was sequenced within five months at a cost of $1.5 million as compared to the Sanger which took 13 years to sequence the entire human genome at a cost of $2.7 billion. The NGS technologies have thus rendered the sequencing process faster and cheaper generation, (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. Illumina/Solexa sequencing technology has been used in 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.

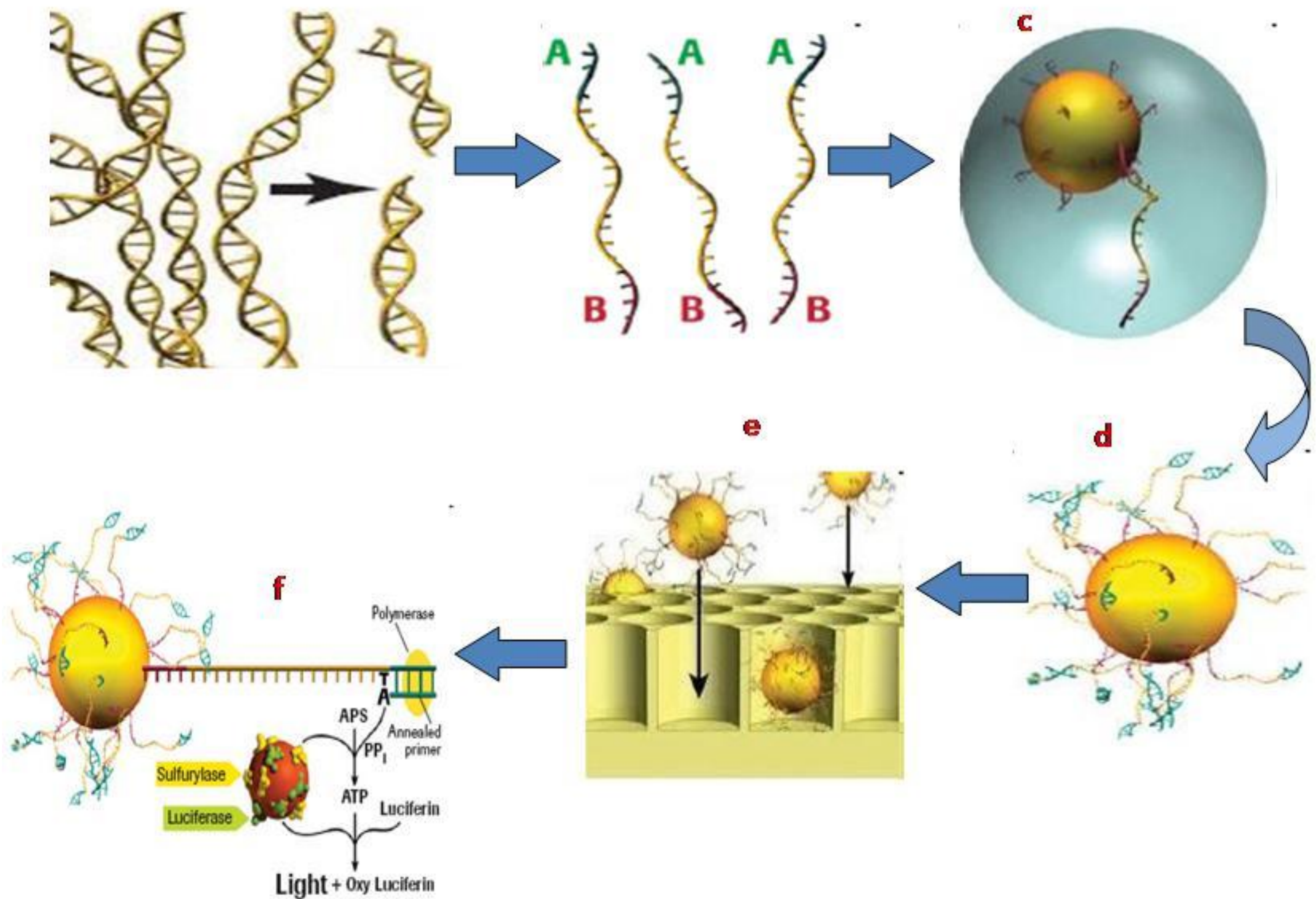


Figure 8; Diagram showing pyrosquencing process. (a) Fragmentation of sample DNA to several hundred base pairs in length. (b) Adaptors ligation to both ends of the fragments and separated to single strands. (c) Hybridization of individual fragments with a water-in-oil emulsion to form a microreactor 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 such plant in which the 454 pyrosequencing has been used include *Artemisia annua* (Wang *et al.,* 2009), *Eucalyptus grandis* (Novaes *et al.,* 2008).The technology can generate upto a million reads with average lengths of about 400 base pairs (bp) at 95% accuracy. In order to identify candidate genes responsible for drought tolerance in tea, transcriptome sequencing of tea leaf was done using the Roche/454 pyrosequencing technology.

CHAPTER THREE
MATERIALS AND METHOD

3.1 Experimental materials

The tea cultivars used in the study were obtained from Tea Research Foundation of Kenya Nursery and they include; 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. The plants were developed through vegetative propagation and raised in the nursery for 18 months and then transferred into 1000-gauge black polythene tubes measuring 0.3m in diameter by 0.3m in depth.

Table 1: Attributes of the cultivars used in the experiment

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

3.2 Experimental Set-up and Design

The experiment was carried out in a rain-out shelter (Cheruiyot *et al.,* 2007). The temperature and relative humidity were monitored using Maximum and minimum thermometer and hygrometer respectively. The potted plants were arranged according to treatments in a completely randomized block design and in three replicates. The plants were well watered uniformly for three weeks then allowed to dry to respective soil moisture content of 34 (high), 26 (moderate) and 18% (low) v/v within a period of 3 weeks. The control (non-droughted), at 34% soil moisture content, was watered throughout the experiment. The soil moisture content was determined using 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.

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

The water status and shoot growth were determined using the procedure described by (Cheruiyot *et al.,* 2007) in which the leaf relative water content of leaves was determined by measuring the fresh weght of the third leaf immediately after harvesting, The leaves were then saturated in deionized and distilled water for 24 hours and oven dried at 70° C for another 24 hours. The leaf relative water content (LRWC) was calculated as follows;

RWC% = (FW-DW/SFW-DW)*100 formular, whereFW= fresh weight, DW= dry weight and SFW = saturated fresh weight

The shoot water potential on the other hand was measured using a pressure chamber (PMS Instruments, Co., Corvallis, OR., USA). In brief 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 appears 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 length on weekly basis using a vernier caliper, and final shoot extension determined after 12 weeks.

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, the TPS-2 Portable Photosynthesis system by PP systems Inc. USA. The measurements were done between 1030hrs and 100hrs on mostly sunny days.

3.5 Determination of glycinebetaine profiles in tea

Glycinebetaine was determined following the Grieve and Grattan (1983) method with some modification. Dried and finely ground plant materials, (0.5g), were shaken in 20ml of distilled water in an incubator at 25°C for 24hrs. The filtrate was mixed with H_2SO_4 in the ratio 1:1. Aliquots of 0.5ml of this mixture were 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 was 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 Jenway 6505 UV-visible spectrophotometer. Reference standard of glycinebetaine (Cat. no. B3501 by Sigma) were prepared in 2N sulphuric acid. The concentrations of glycinebetaine were calculated against the standard curve as follows:

GB in $nmol \cdot mg^{-1}$ FW or in $\mu mol \cdot g^{-1}$ FW = Absorbance /slope*$Vol_{extract}$/$Vol_{aliquot}$*1/DW. Where Absorbance was the reading at wavelength 365 nm of the extract, slope (expressed as $absorbance \cdot nmol^{-1}$) 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 following (Bates *et al.,* 1973) method. Fresh plant material (1g) was homogenized in 10 ml of 3% sulfosalicylic acid (Cat. No. S3147 by Sigma) and the homogenate filtered. The filtrates (2 ml) were treated with 2ml acid ninhydrin (Cat. No. 151173 by Sigma) and 2ml of glacial acetic acid (Cat. No. 33209 by Sigma), then 4 ml of toluene was added and Absorbance taken at optical density of 520 nm using CE. 393 UV-spectrophotometer (Britain). The proline concentration was determined from a standard curve

(Supplementary 1) generated from proline standard reagent. Proline level was calculated in every sample on fresh weight basis as shown 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 upon expression of physiological water stress characteristics. 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 leaf samples using ZR Plant RNA Miniprep Kit (Cat. No. R2024) 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 of the sample at 260/280 nm using a 2000-NanoDrop spectrophotometer by Thermo Fisher Scientific (USA). The quality of the RNA extracted was also checked by denaturing in a buffer containing formamide and run on a 1% agarose gel electrophoresis.Aliquots of RNA were used in the isolation of mRNA using mRNA isolation kit version 8 by Roche (Germany) according to manufacturer's instruction while the rest was stored at -80ºC. The integrity of the mRNA was also assessed using NanoDrop spectrophotometer.

3.9 Preparation of cDNA library for transcriptome sequencing

The cDNA library was synthesized using cDNA Rapid Library Preparation kit (GS FLX Titanium Series) by Roche (Germany) according to manufacturer's instruction. The mRNA was fragmented into smaller pieces at 70°C for 30 seconds in the fragmentation buffer and denatured by adding Roche primers 'random'. The fragments were reverse-transcribed to synthesize first strand cDNA using AMV reverse transcriptase (Roche). Subsequently, the second strand cDNA was synthesized using second strand enzyme (Roche) and T4 DNA polymerase (Roche). 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). 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 by Turner Biosystems (USA) and Agilent Bioanalyzer High Sensitivity DNA chip by Agilent technologies, Germany, respectively.

3.10 emPCR amplification

The emulsion PCR (emPCR) amplification was done using the emPCR KitGS FLX Titanium series by Roche. In brief, 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 do 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 magentic 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 5' and 3' end on 454 sequencing technologies for the most tolerant and susceptible clones under drought and non-droughted condition. This was completed using a GS FLX Titanium Sequencing kit according to the manufacturers' instruction. In brief, the DNA-capture beads were loaded onto PicoTiterPlate™ such that each well contains single DNA beads. Sequencing reagents comprising of dNTP buffers, sodium chlorite tablet, Apyrase, Ppiase, inhibitor TW reagent, enzyme beads, bleach and DTTwere sequentially flowed over the plate. The sequencer automatically performs and monitors the sequencing reactions in all the wells of the PicoTiterPlate simultaneously. The raw output of a sequencing process consists of a set of digital images (PIF files) from which the sequence of the DNA library fragments. The images processing 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

The experiments were conducted using three biological separate replicates. All statistics were carried out using Genstat statistical software, Version 10.3. Analysis of variance was used to test for the difference in response of cultivars to different water stress condition. The probability limit was set at $P \leq 0.05$ for significant confidence interval. Correlation analysis was also used to determine the 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 calls during sequencing. De novo assembly of the clean reads was performed using Newbler program. (version 1.03).

3.13.2 Functional annotation and classification of contigs

All the assembled unigenes (consensuses and singletons) longer than 100 bp were annotated by the assignments of Gene Ontology 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 (GO; *http://www.geneontology.org* 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 generated from the sequencer were trimmed to remove the adaptor sequences and poor quality reads less than 50bp. The sequences were then assembled using Newbler software and the assembled contigs and singlets were subjected to BlastX analysis againts Arabidopsis proteome. The sequences were categorized based on functions

homologous to Arabidopsis. The roles of the transcripts identified with known and putative function were finally 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 and 32ºC respectively while the relative humidity ranged between 44 and 96%. The soil for planting was obtained from a forest virgin land obtained from a depth of between 0-1.5m. The soil 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 mean leaf relative water content declined with decrease in soil moisture content. The levels were 85%, 75% and 55% for high, moderate and low soil moisture content respectively. The LRWC differed significantly ($F_{(2,46)}=256.34$, $P<0.001$) with 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 cultivar AHP S15/10 and EPK C12 respectively. At moderate level of SMC it was 67-83% for cultivar EPK C12 and TRFCA SFS150 respectively while at low SMC, it ranged between 46.7 and 67% for cultivar TRFK 301/5 and AHP S15/10 respectively. The difference in LRWC between high and moderate SMC was very small in cultivar TRFCA SFS150 (1.3%) and AHP S15/10 (2.0%), while cultivar EPK C12 had the highest LRWC at 21%. On the other hand, cultivar EPK C12 had the lowest (6.0%) difference in LRWC between moderate and low SMC. Cultivar EPK TN14/3, TRFK 301/5 and TRFCA SFS150 showed significantly higher variation of 31%, 29% and 27% respectively. The interaction between the test tea cultivars and soil moisture content differed significantly ($F_{(14,46)}=4.45$, $P<0.001$).

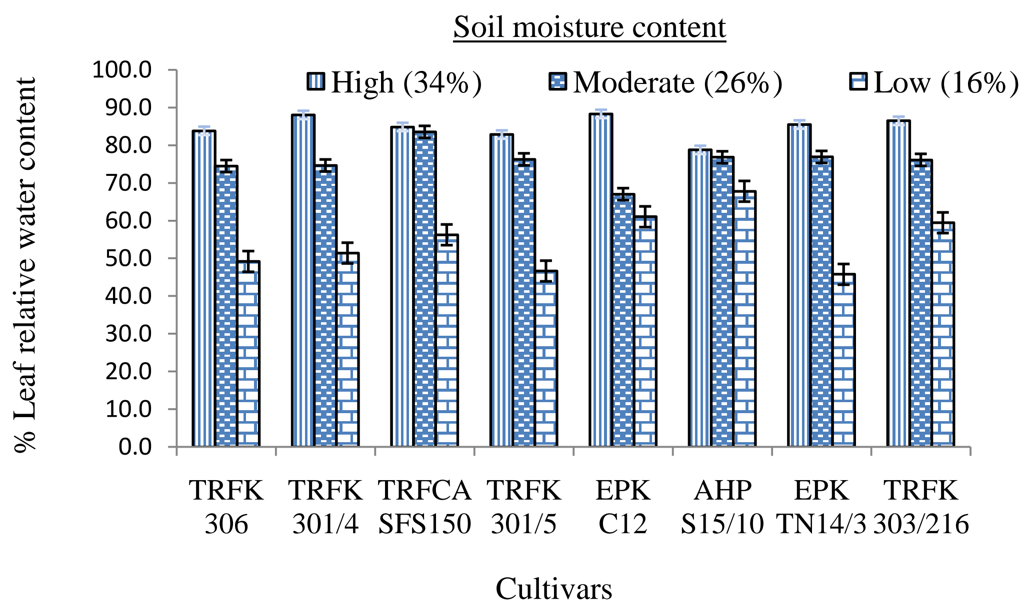


Fig 9: Effect of decreasing soil moisture content on leaf relative water content among different tea cultivars with their respective standard error bars. The high, moderate and low SMC are the different soil moisture contents

4.2.2 Shoot water potential

The present study showed a significant decline in shoot water potential with decrease in soil moisture content. The levels were -0.8, -0.9 and -1.1Mpa for high, moderate and low soil moisture content respectively. The SWP values were notably negative. The three soil moisture content 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 as shown in Table 2. 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 condition The interaction between cultivar response and soil moisture content also varied significantly, ($F_{(14,46)}$ =11.73, P<0.001).

Table 2: Effects of different soil moisture content on shoot water potential 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]

SMC is the % soil moisture content subjected to the test tea cultivars. [ab] Means within a column followed by the same letter are not significantly different at P≤0.05.

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 high, moderate and low SMC respectively. The treatments (high, moderate and low SMC) subjected to the test cultivars differed significantly, ($F_{(2,46)}$ =578.01, P<0.001) in the study. 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 0.57 mm/day. Under low soil moisture content, cultivar EPK C12 showed the highest growth rate whereas cultivar AHP S15/10 showed the least shoot growth rate. The rate of growth was comparable for cultivar TRFK 303/216 and TRFK 306. The changes 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 moderate and low SWC showed that cultivar AHP S15/10 had the highest difference in growth rate. The interaction between cultivar and SMC differed significantly ($F_{(14,46)}$ =21.67, P<0.001).

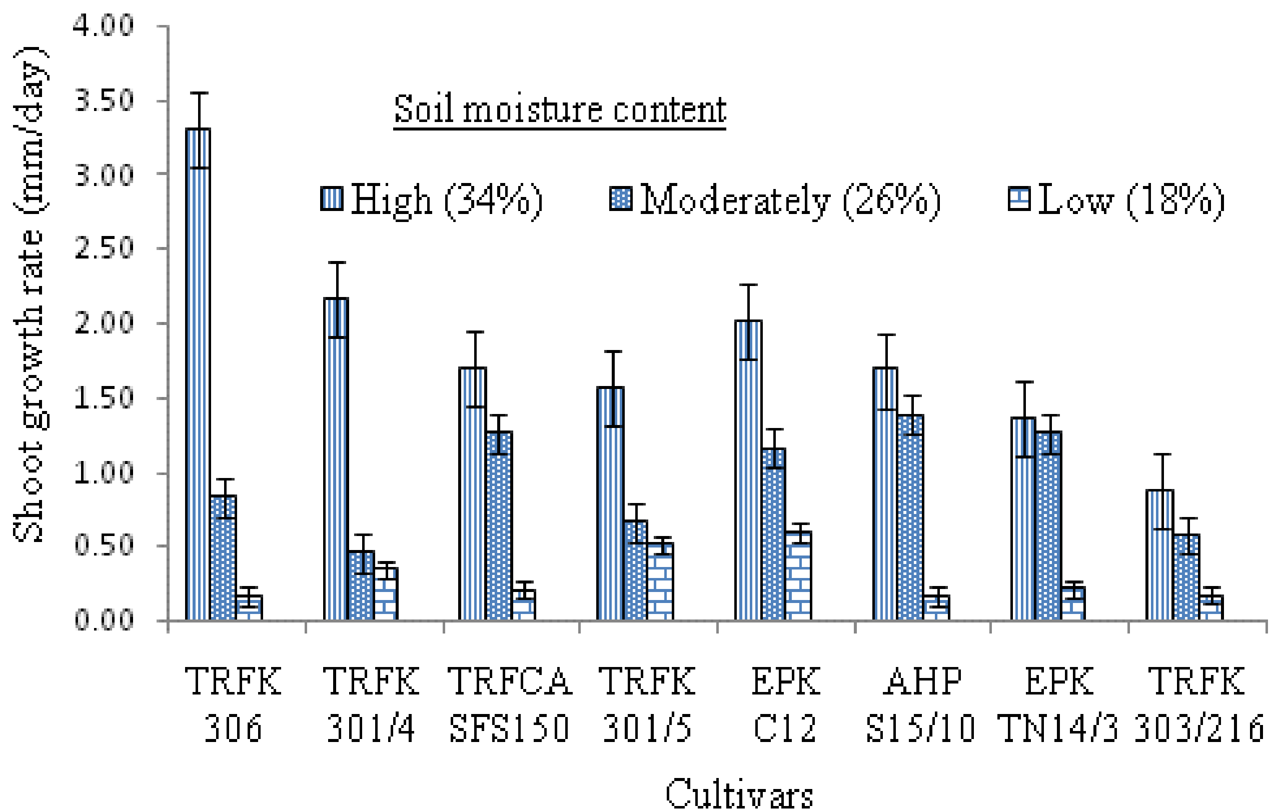


Figure 10: Effects of soil moisture content on shoot growth rate with standard error bars of mean.

4.2.4 Stomata conductance

Stomata conductance was also significantly reduced with decline in soil moisture content. The stomatal conductance ranged from 301, 276 and 256µmol-m-$^{2-}$s for high, moderate and low SMC 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 where as 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) however did not differ significantly. 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 difference ($P<0.05$). The coefficient of variation was 11%.

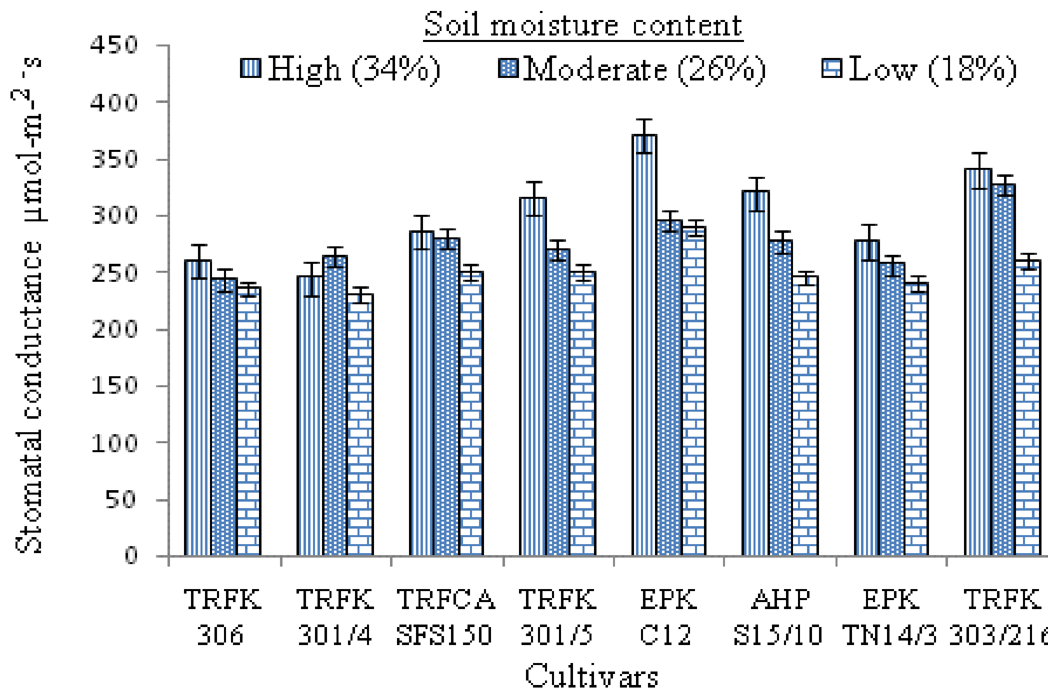


Figure 11: Changes in stomatal conductance in different tea cultivars in response to changing soil moisture content with standard error bars.

4.2.5 Evapotranspiration

The evapotranspiration rate also declined with reducing soil moisture content. The evaprotranspiration rates were, 2.2, 2.0,and 1.9mmol-m-$^{2-}$s for high, moderate and low SMC respectively. The three different SMC differed significantly ($F_{(2,46)}$ =5.62, $P<0.001$) with respect to tea plant response. The tea cultivars used in this study also exhibited reducing pattern in evapotranspiration rate with decline in SMC as shown in Table 3. The rates of evapotranspiration differed significantly, ($F_{(7,46)}$ =3.83, $P<0.01$), amongst the test tea cultivars. At low soil moisture content, transpiration response, varied with cultivar EPK C12 having the highest rate of 2.2mmol-m-$^{2-}$s while cultivar EPK TN14/3 showed the lowest rate of 1.7mmol-m-$^{2-}$s. On the other hand, at high soil moisture content cultivar AHP S15/10 showed the highest 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 significant difference ($P<0.05$) in evapotranspiration rate between

high and moderate SMC. At moderate to lower SMC, the difference was significant for cultivars, TRFK 303/216, EPK TN14/3, EPK C12 and AHP S15/10.

Treatment (% SMC)	TRFK 306	TRFK 301/4	TRFCA SFS 150	TRFK 301/5	EPK C12	AHP S15/10	EPK TN14/3	TRFK 303/216
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]

Table 3: Effects of soil moisture content on evapo-transpiration rate. The cultivars varied significantly with a coefficient of variation of 14%. [ab] *Means within a column followed by the same letter are not significantly different at P≤0.05.*

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 SMC 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 high, moderate and low SMC 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 difference, ($F_{(7,46)}$ =2.12, P<0.05), amongst cultivars in response to different SMC. At high SMC, cultivar EPK C12 showed the highest rate of 24.3µmol-m-^{2} $^{-}$s while TRFK 301/4 showed the lowest 17.7 µmol-m-^{2} $^{-}$s under the same condition. At low soil moisture content, cultivar TRFCA SFS150 had the highest 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 rate whereas TRFK 301/4 had the lowest. The net photosynthetic rate of cultivar TRFK 301/4 was comparable at both moderate and low SMC. For cultivars AHP S15/10, EPK TN14/3 and EPK C12 there was a significant (P<0.05) variation. However, cultivars AHP S15/10 and EPK TN14/3 exhibited the least difference in net photosynthetic rate at high and moderate SMC. Except for cultivar TRFK 301/5 all the other test tea cultivars showed significantly high difference (P<0.05) in net photosynthetic rate between the moderate and low SMC. The interaction between the SMC and the test cultivars also differed significantly, ($F_{(14,46)}$ =1.90, P<0.01).

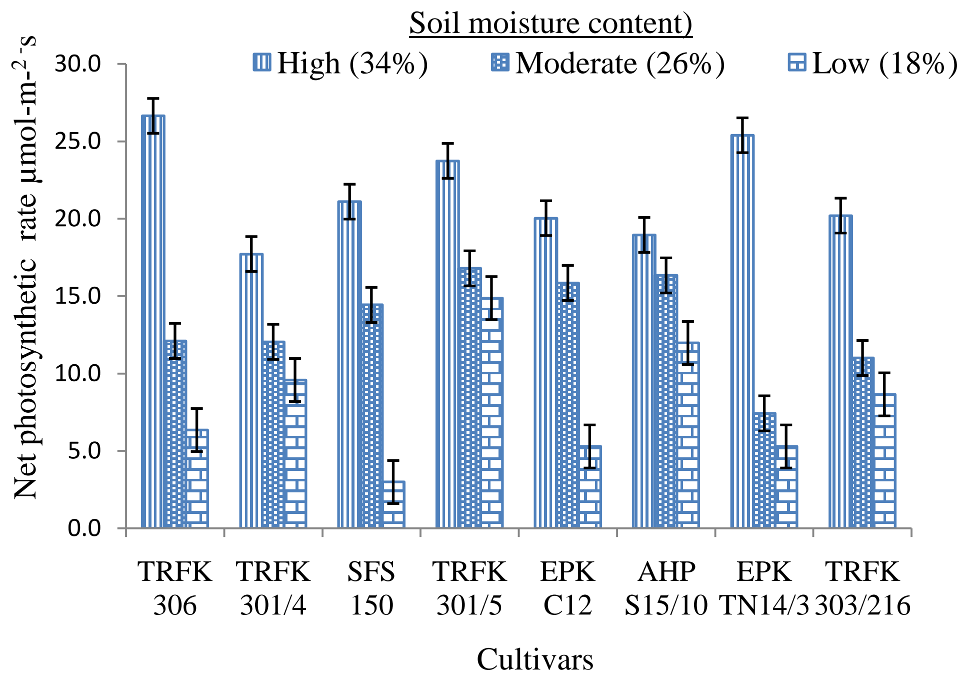


Figure 12: Effects of soil moisture content on net photosynthetic rate in different tea cultivars with their standard error bars

4.3 Effects of water stress on biochemical parameters in tea

4.3.1Proline levels

Unlike the other physiological parameters studied, decreasing SMC induced the accumulation of proline in the present study. 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 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 mol/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 mol/g FW under high and medium soil moisture content respectively. Cultivar EPK C12 had no significant difference in proline content at the different SMC. Other than TRFK 301/5 and TRFK 303/216 all the cultivars exhibited a steady and significant rise in proline levels with decline in SMC. The interaction between cultivars response and water treatment also differed significantly ($F_{(14,46)}$ =24.08, P<0.001).

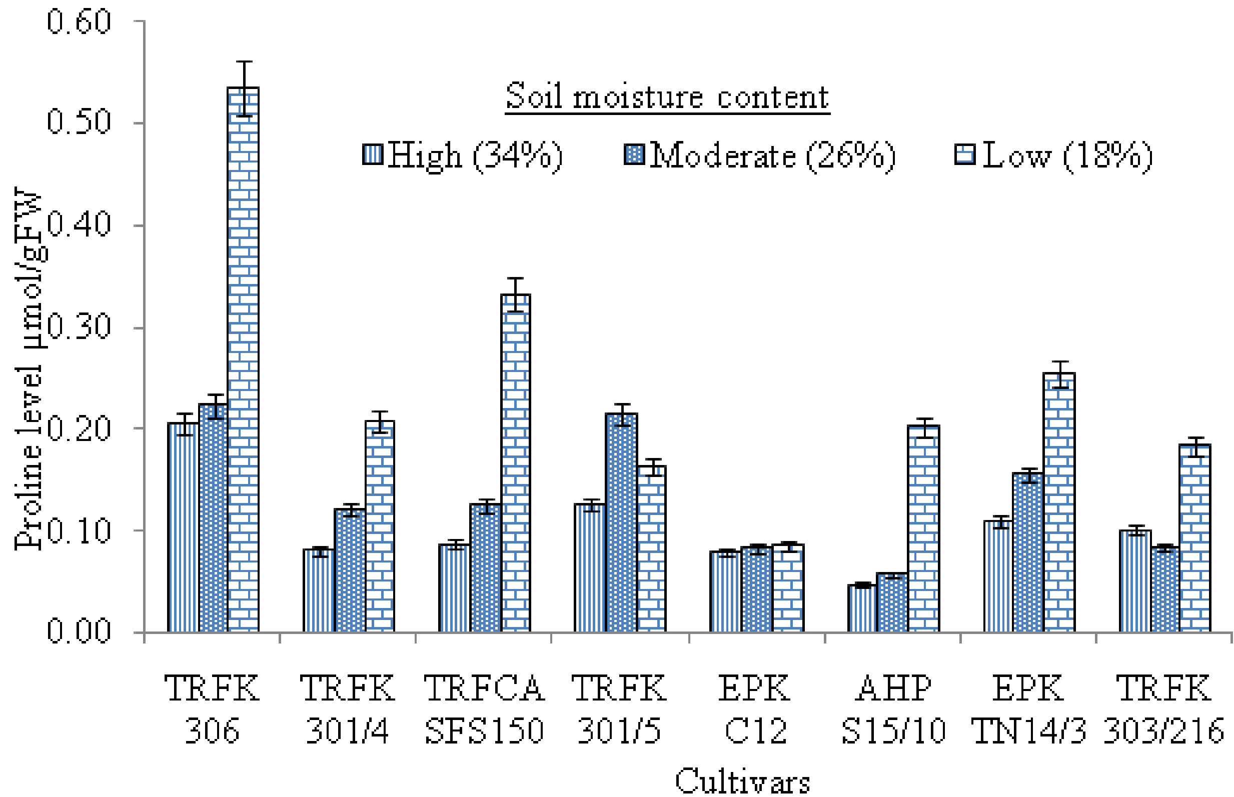


Figure 13: Leaf proline levels of different tea cultivars grown under different soil moisture regimes

4.3.2 Glycinebetaine levels

Like proline, glycinebetaine accumulation in the test tea cultivars, increased with decline in SMC. The variation in glycinebetaine levels was significantly ($F_{(2,46)}$ =21.16, P<0.001) different amongst the treatments (SMC subjected to the test cultivars). The mean glycinebetaine levels observed were 1.5, 1.7 and 2.0mmole/g DW for high, medium and low soil moisture content respectively. The cultivars used in the study also showed a steady rise in leaf glycinebetaine level when subjected to decreasing SMC as shown in figure 14. The different tea cultivars also differed significantly, ($F_{(2,46)}$ =21.16, P<0.001), in leaf glycinebetaine profiles. 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 of 1.57mMole/g DW a condition that was replicated in all the other treatments. Under high soil moisture content, cultivar TRFCA SFS150 had the highest glycinebetaine, 1.71mMole/g DW, levels. At moderate condition cultivar EPK TN14/3 accumulated the highest amount of glycinebetaine. The difference in glycinebetaine level between treatment high and moderate SMC 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 moderate and lower SMC for cultivars AHP S15/10, TRFK 303/216 and TRFK EPK C12. Cultivars TRFK 301/5 and TRFK 301/4 showed the least difference in glycinebetaine levels between moderate and lower SMC. The interaction between test tea cultivars and soil moisture content also differed significantly ($F_{(14,46)}$ =2.03, $P<0.05$).

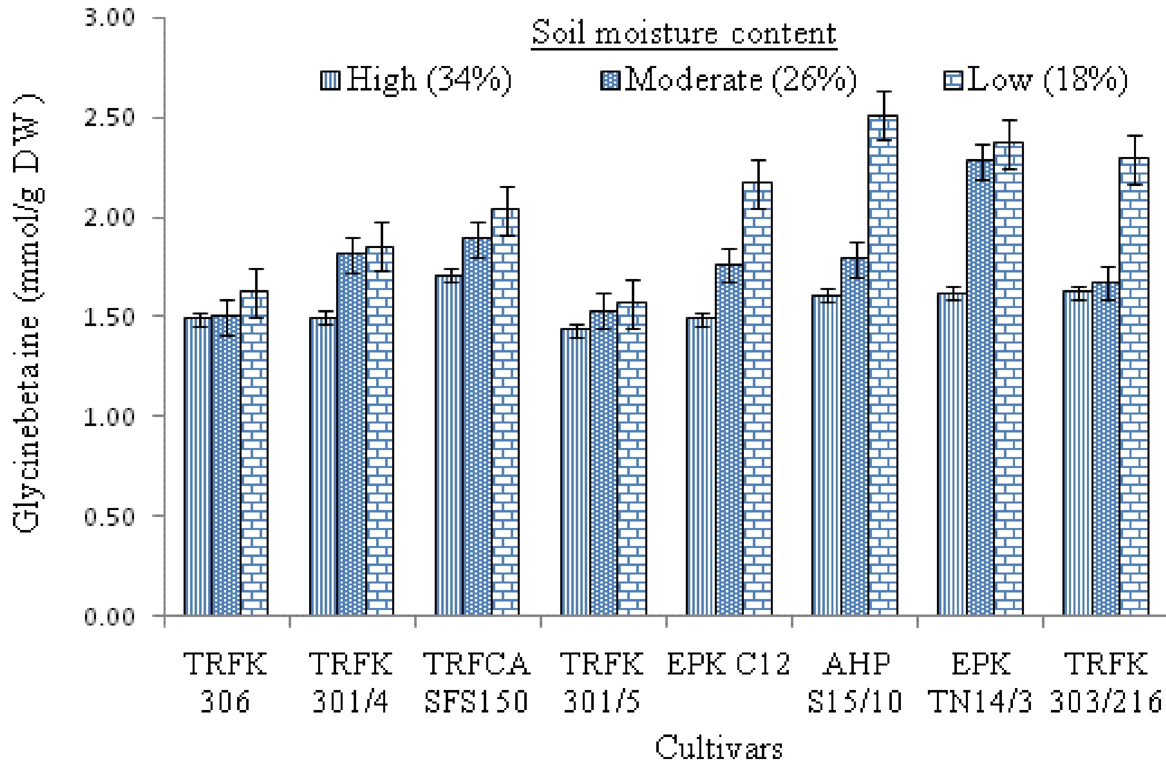


Figure 14: Variation in leaf glycinebetaine levels of tea cultivars at different soil moisture treatments with their standard error bars.

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 and the net photosynthetic rate. The shoot water potential was also significantly and positively correlated to shoot growth, stomatal conductance and net photosynthetic rate. However, it was not significantly correlated to evapotranspiration. The rate of shoot growth was positively and highly significantly correlated with the net photosynthetic rate. There was 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 (proline and glycinebetaine). The two biochemical parameters (proline and glycinebetaine) were positively correlated to each other though the correlation was not significant.

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: L*Rwc = Leaf Relative water content, Sgm = Shoot growth, Prol= 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 Extraction and quantification of total RNA and mRNA in tea

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 ranged 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 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 leaf.

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 betweeen 600 and 1200 bp as shown in Figure 15

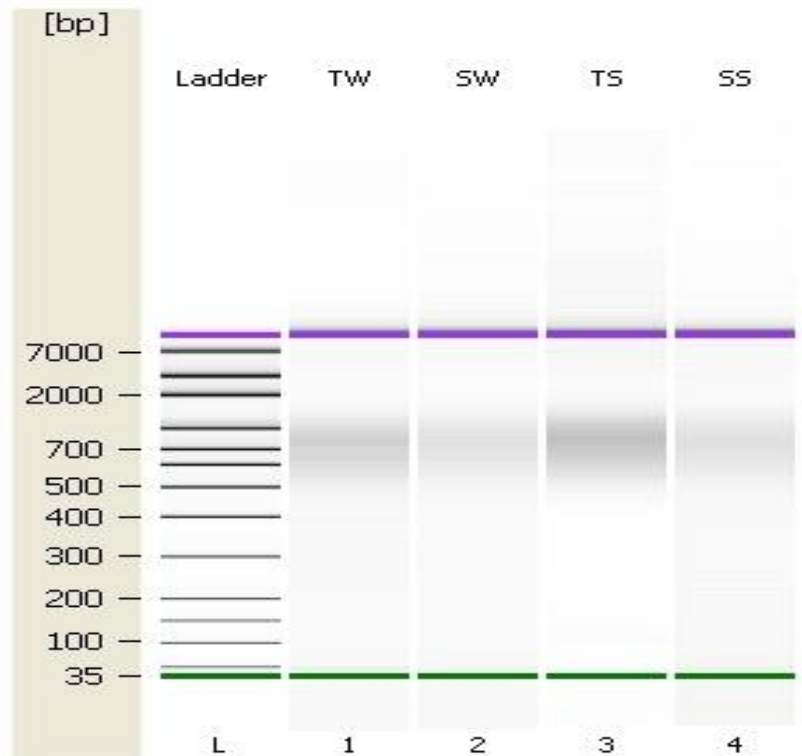


Figure15: Gel-like image of the cDNA library samples as run on an Agilent Bioanalyzer High sensitivity DNA chip. The initials; TW = TRFCA SFS150 (Watered), TS= TRFCA SFS150 (stressed), SW= AHP S15/10 (Watered), SS= AHP S15/10 (stressed) are the four libraries synthesised for use in sequencing. The top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

4.5.3 *De novo* sequencing of the *Camellia sinensis* leaf transcriptome

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 plants produced more reads at low soil moisture content than at high SMC in both cultivars as shown in Table 5. 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 Trimming and quality control 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 been called incorrectly as shown in Figure 16. This meant that there was minimal chance of a base being called incorrectly.

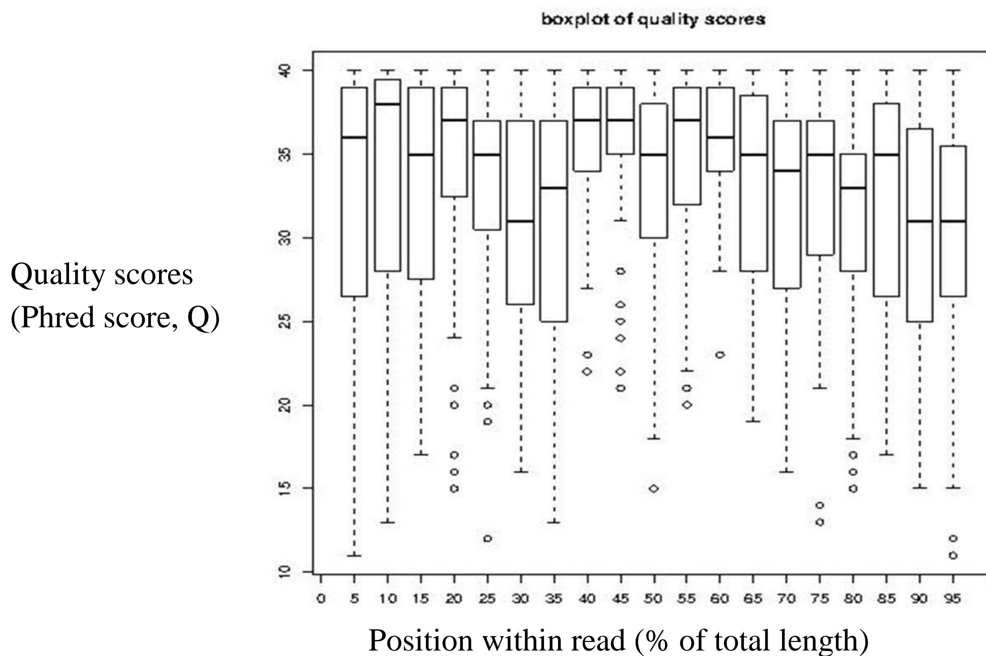


Figure 16; Box plot showing quality scores of trimmed sequence. The Y-axis show the quality scores referred to as phred scores (Q) which is equivalent to the probability of errors in a particular base. In the scale used, quality score, Q10, means the probability of an incorrect base call is 1 in 10, 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 *De novo* assembly of sequence reads

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 21. The mean length of the contigs was 250bp with 13 contigs been greater than 1kb. The total number of bases in all the contigs was 115,177 with a GC content of 43.9%.

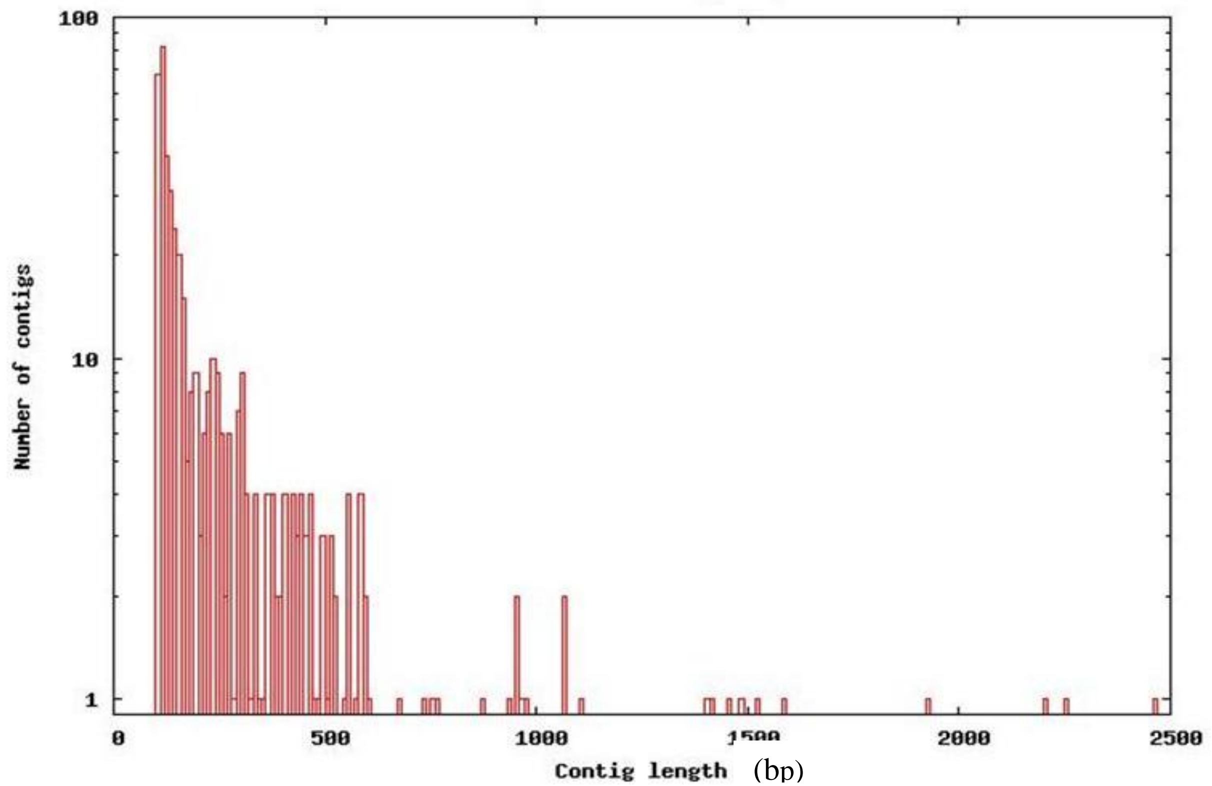


Figure 17; Size distribution of the contigs generated by *de novo* assembly of the filtered and trimmed 454 pyrosequence reads.

4.5.6 Functional annotation by GO and KEGG

The sequences were categorized in to three broad categories (biological processes, cellular component and molecular functions) as established for the *Arabidopsis* proteome, Figure. 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.

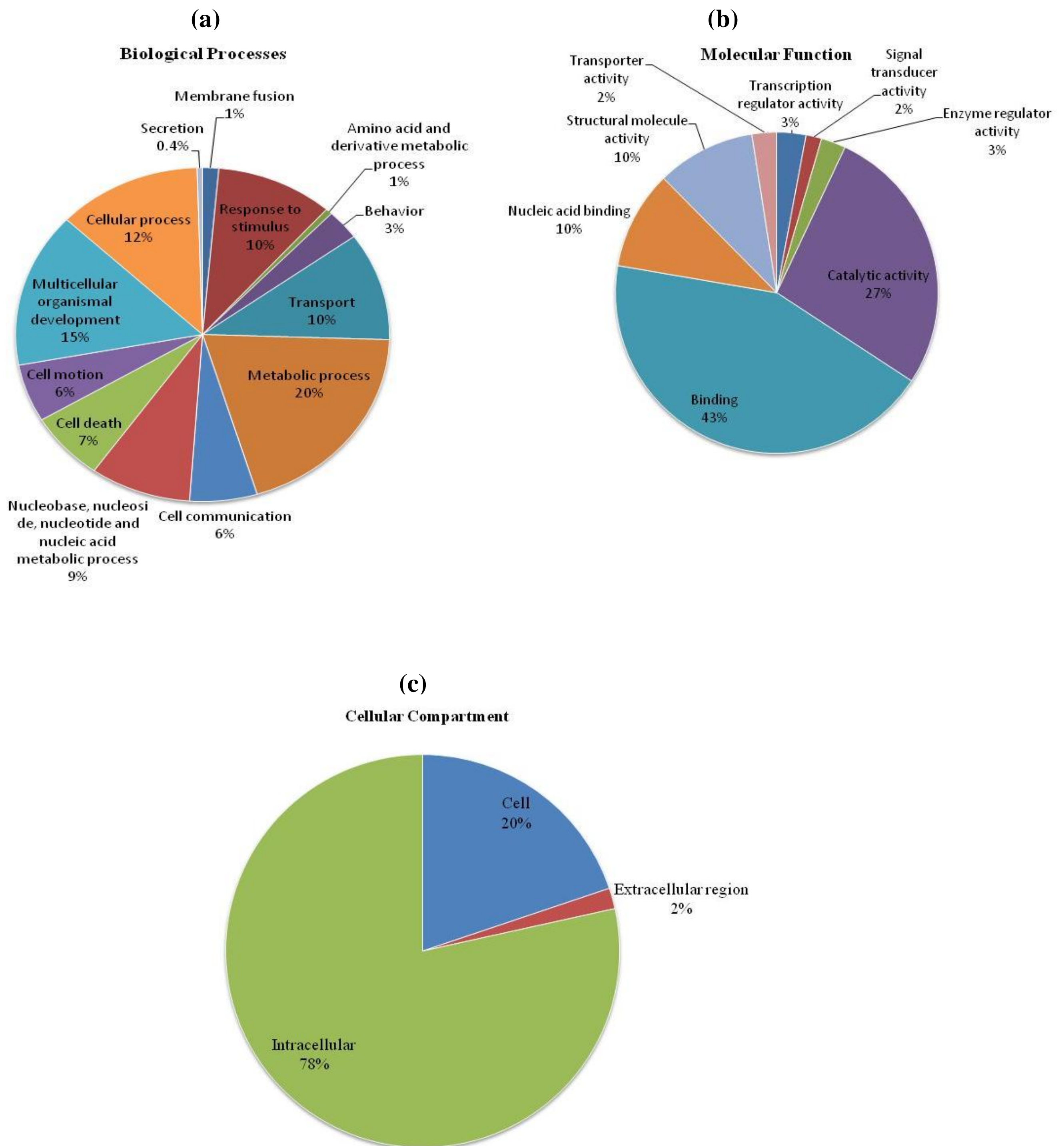


Figure 18: Gene ontology (GO) classification of *Camellia sinensis* contigs as summarized into three main categories; **(a)** Biological process, **(b)** Molecular functions and **(c)** Cellular components. The % shows the proportion of genes related to various activities within the three main functional 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 23. 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.

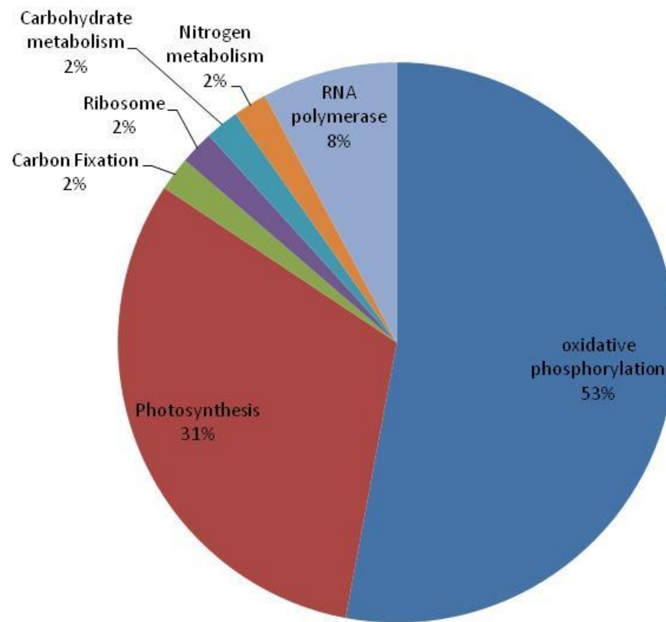
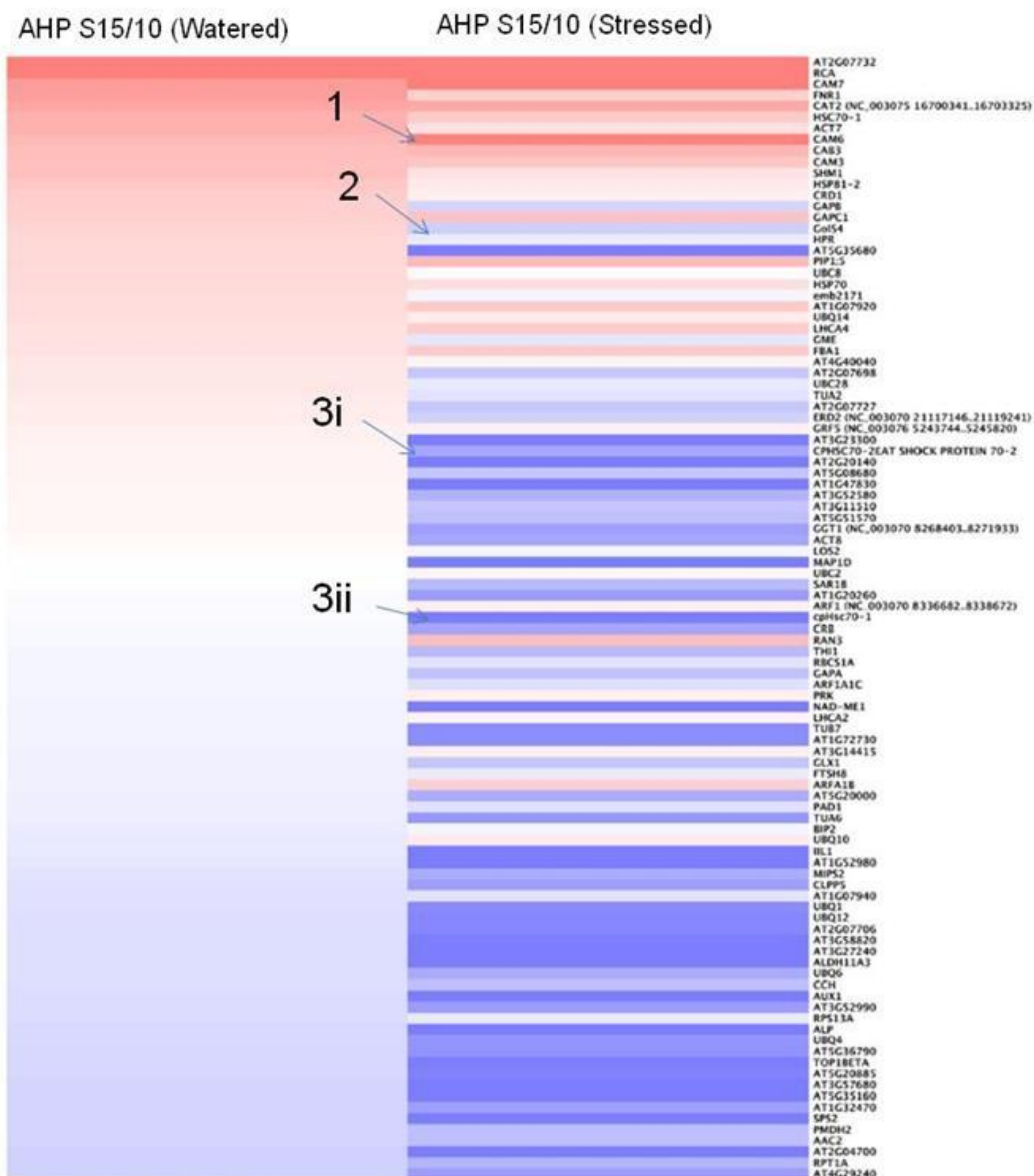


Figure 19: The Biologically active pathways in the leaf transcriptome of tea

4.5.7 Expression analysis of genes involved in drought response in tea

The potential drought responsive genes that mapped to the *Arabidopsis* proteome are presented in a form of heat map as shown in figure 20. The drought sensitive Cultivar AHP S15/10 showed genes responsible for defense against drought 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).



Legend;

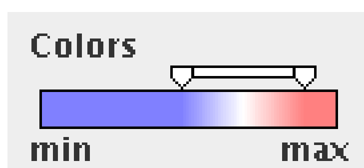


Figure 20: Heat map of expression pattern of genes in the susceptible cultivar (AHP S15/10) with response to drought. 1; Calmoduline like protein (*CAM6*), 2; Galactinol synthase (*GolS4*), 3i; Chloroplast *heat shock protein (cpHSC70-2*), 3ii; Chloroplast heat shock protein (*cpHSC70-1*).

Since the sequences generated from the TRFCA SFS150 library under high soil moisture content was too low, heat map for group comparison at both conditions could not be generated. However, analysis of potential genes in the stressed tolerant and susceptible cultivar showed various genes expressed and or repressed. The signalling genes, *CAM7* and *CAM6,* were induced in all the test cultivars under water stressed condition. The level of expression however differed with cultivar, Susceptible cultivar, AHP S15/10, expressed the calmodulin like protein gene (*CAM7* and *6*) at higher levels than the tolerant cultivar TRFCA SFS150. Other than calmodulin like proteins, another signalling gene related to calcium dependent protein kinase 'CDPK' was also upregulated in the water stressed plants. The transcript related to defence against effects of drought like *HSP70-1, GOls4* were also expressed at higher level in the tolerant cultivar TRFCA SFS150 as compared to the less tolerant cultivar, AHP S15/10.

Other 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 where as the drought sensitive cultivar, AHP S15/10, expressed the catalase and peroxidises 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 less tolerant cultivar.

CHAPTER FIVE

DISCUSSION

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

In the present study, the mean leaf relative water content 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 plants root tissue does not have water to take-up to the leafy part 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 observation reported in tea by (Waheed *et al.,* 2012). The minimal reduction in leaf relative water content serves as an adaptive strategy in 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 and they were 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 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 affect of water deficit varies depending on sensitivity of plant 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). Since cell growth is considered the most sensitive physiological processes during water deficit conditions (Shakeel *et al.,* 2011), the decline in soil moisture content in the present study suggests that water deficit contributes to decline in 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 plant to water deficit (MansWeld and Atkinson, 1990), the respond by the tea cultivars to water deficit by decreasing the stomatal conductance 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 which leads to reduced stomatal conductance (Waheed *et al.,* 2012). This observation suggests that the anatomical changes in leaf tissue can be attributed to decline in stomatal conductance in the present study.

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. This suggests that changes in stomatal conductance are dependent on sensitivity of the plant variety. Similar observation 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 carbondioxide deficit in the chloroplast (Mafakheri *et al.,* 2010). Cellular changes in plant water status causes a reduction in stomatal conductance which in turn restricts carbondioxide 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 process in the test cultivars can also be attributed to impairment of photosynthetic apparatus which causes resistance in the flow of CO_2 from the mesophyll cell to the chloroplast stroma hence resulting decreasing chloroplast activity (Damayanthi *et al.,* 2010).

Studies have also shown that reduction in net photosynthesis in water stressed plants is ascribed to reduction in activity of Rubisco (ribulose-1,5-bisphosphate carboxylase / oxygenase) which contribute to reduced photosynthetic rate (Bota et al., 2004a). The reduction in net photosynthetic rate in the present study may also be attributed to the same 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. The present result corroborated observation made in other studies on tea (Damayanthi *et al.,* 2010). Results obtained in this study revealed a significant positive correlation between transpiration and leaf relative water content. This suggests the effects of water deficit on evapotranspiration. Reduction in transpiration allows the tea plant to conserve water. 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. This supports earlier suggestion that stomata plays 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 level 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 condition as compared to the less tolerant cultivars (Upadhyaha *et al.,* 2008). From this study, the tea cultivar TRFK 306 had the highest level of 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 cultivar's, TRFK 306, tolerance to water stress. The accumulation of proline in tea plant at low soil moisture content can also be attributed to expression of genes responsible for water stress tolerance. Studies using 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 glycinebetaine (GB) during water stress. Glycinebetaine accumulation varied significantly with test cultivars, this can be attributed to cultivar specificity of this biochemical trait. 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 sensitive. 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 level and vice versa. This observation corroborate 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 the present study, Gene ontology (GO) categories associated with contig annotations as derived by homologies to *A. Thaliana* gene showed majority of contigs in the biological processes category to be associated with metabolic processes, cellular development

and response to stimulus. This indicates that the diversifying metabolic processes are active in the *C. sinensis* leaf, and a variety of metabolites are synthesized in the leaf. The dorminancy of contigs associated with cellular development and response to stimulus is an indication that the plants under high SMC are actively growing whereas the stressed plants have induced stimulus response related genes in order to mediate signaling as a result of 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 tea plants upregulate or down regulated several genes to mitigate against cellular damage. The genes vary from signaling to defence related genes. In the present study the signaling gene, calmoduline like protein was found upregulated at various levels in the stressed tea plants. This is mainly to allow the tea plants under stress to transduce calcium ion signals that activate major pathway 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 sensitive cultivar, AHP S15/10, as compared to the tolerant cultivar TRFCA SFS150, under water stressed condition. The induction of this gene at various levels in the tea cultivars suggest that they play significant role in signal transduction during stress.

Transcripts showing homology to galactinol synthase was also shown down regulated in the water stressed less tolerant cultivar, AHP S15/10. This probably implies that drought adaptation or tolerance of this cultivar to water stress conditions is limited, a trait that has been determine earlier through morphological studies. Galactinol 1 synthase functions as osmo-protectant in plants (Nishizawa *et al.,* 2008). It has been shown to be induced in plants subjected to drought as in *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 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 condition. The heat shock proteins (*HSP70*) were induced in water stressed tea cultivars in the present study. This can be attributed to their role as intra-cellular chaperone for other proteins and are involved in plant abiotic 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 drought tolerant Indian tea cultivars as compared to the drought sensitive cultivars (Muoki *et al.,* 2012). The results generated from the present study, corroborated this observation with the tolerant cultivar, TRFCA SFS150, showing a higher levels of HSP70-1 as compared with the less tolerant cultivar, AHP S15/10. This implies that the upregulation of heat shock proteins, *HSp70-1,* confers drought tolerance. Similar observations on the role of heat shock protein 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 low level of expression of heat shock proteins in the leaf tissues. Similar observation have also been made 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 present study. The catalase and peroxidase protein were expressed in the tolerant, TRFCA SFS150 and the susceptible, AHP S15/10, cultivars under stressed conditions. However the *SOD* was only expressed in the tolerant cultivar, TRFCA SFS150, under the same condition. This can be attributed to their role in scavenging for reactive oxygen species which damages the photosynthetic machinery in plants (Das *et al.,* 2012). Accumulation of antioxidant molecule, 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 catalyses the removal (Chaves *et al.,* 2003) and conversion of H_2O_2 into water (Rossel *et al.,* 2006) respectively. The existence of balance between *SOD* and other H_2O_2 scavenging enzyme is crucial in maintain a steady level of oxidant molecules. Expression of *SOD* has been shown to confer drought tolerance and shoot regeneration in transgenic pepper (Chatzidimitriadou *et al.,* 2009). The upregulation of *SOD* in the tolerant cultivar TRFCA SFS150 in the present study corroborated the above cited study and it suggests the role of this antioxidant molecule in drought tolerance. The absence of the

molecule in the susceptible cultivar on the other hand is the probable contributor to its sensitivity to water deficit.

5.7 Conclusions

Data from this study revealed that;

i. Increasing soil water deficit resulted in decrease in shoot water potential and leaf relative water content of tea cultivars. Shoot growth rate, reduction in stomatal conductance, transpiration and net photosynthesis of tea cultivars.
ii. Tea plants accumulate proline and glycinebetaine as a result of water deficit.
iii. Tea plants respond to water stress through a complex of gene network. 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. Proline can be used as a potential marker in clonal selection for drought tolerance in tea whereas glycinebetaine is not a good marker for selection.
ii. The identified candidate genes can 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.

REFERENCES

Ackerman W.L. (1973) Species compatibility relationships within the genus Camellia. *Heredity* **64:**356-358.

Adams M.D., Kelley J.M., Gocayne J.D., Dubnick M., Polymeropoulos M.H., Xiao H., Merril C.R., Wu A., Olde B., Moreno R.F., Kerlavage A.R., McCombie W.R., Venter J.C. (1991) Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* **252:**1651-1656.

Ahmadian A., Ehn M., Hober S. (2006) Pyrosequencing: History, Biochemistry and future. *Clinical chemistry Acta* **363:**83-94.

Ashraf M., Harris P.J.C. (2004) Potential biochemical indicators of salinity tolerance in plants. *Plant Science* **166:**3-16.

Ashraf M., Foolad M.R. (2007) Role of glycinebetaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany* **59:**206-216.

Assmann S.M., Snyder J.A., Lee Y.R.J. (2000) ABA-deficient (aba1) and ABA-insensitive (abi1-1, abi2-1) mutants of Arabidopsis have a wild-type stomatal response to humidity. *Plant Cell Environment* **23:**387-395.

Bartels D., Sunkar R. (2005) Drought and salt tolerance in plants. *Critical Reviews in Plant Science* **24:**23-58.

Bartoli C.G., Simontacchi M., Tambussi E., Beltrano J., Montaldi E., Puntarulo S. (1999) Drought and watering-dependent oxidative stress: effect on antioxidant content in *Triticum aestivum* L. leaves. *Journal of Experimental Botany* **50:**375-385.

Bates L.S., Waldren R.P., Teare I.D. (1973) Rapid determination of free proline for water stress studies. *Plant Soil* **39:**205–207.

Bausher M., Shatters R., Chaparro J., Dang P., Hunter W. (2003) An expressed sequence tag (EST) set from *Citrus sinensis* L. Osbeck whole seedling and the implications of further perennial source investigations. *Plant Science* **165:**415-422.

Benlloch-Gonzalez M., Octavio Arquero O., Jose Maria F., J. M.,, Diego Barranco D., Manuel Benlloch M. (2007) K+ starvation inhibits water-stress induced stomatal closure. *Journal of Plant Physiology* **165:**623-630.

Bezbaruah H.P. (1968) An evaluation of preparatory procedures for leaf tip chromosome spreads of the tea plant (*C. sinensis*). *Stain Techniques* **43:**279-282.

Bezbaruah H.P. (1971) Cytological investigation in the family Theacea - I. Chromosome numbers in some Camellia species and allied genera. *Carylogia* **24:**421 – 426.

Bian S., Jiang Y. (2009) Reactive oxygen species, antioxidant enzyme activities and gene expression patterns and recovery. *Science Horticulture* **120:**264–270.

Bogeat-Tribo L., Brosche M., Renaut J., Jouve L., Thiec D.L., Fayyaz P., Vinocur B., Witters E., laukens K., Teichmann T., Altman A., Hausman J.-F., Polle A., Kangasjarvi J., Dreyer E. (2007) Gradual soil depletion results in reversible changes of gene expression, protein profiles, ecophysiology and growth perfomance in *Popolus euphratica,* a popular growing in arid regions. *Plant Physiology* **143:**876-892.

Boguski M.S. (1995) The turning point in genome research. *Trends in Biochemistry Science* **20:**295–296.

Bota J., Flexas J., Medrano H. (2004a) Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress?. *New Phytology* **163:**671-681.

Bota J., Stasyk O., Flexas J., Medrano H. (2004b) Effect of water stress on partitioning of ^{14}C-labelled photosynthates in *Vitis vinifera*. *Functional Plant Biology* **31:**697-708.

Bruce W., Edmeades G., Barker T. (2002) Molecular and physiological approaches to maize improvement for drought tolerance. *Journal of Experimental Botany* **53:**13-25.

Bukowski J.F., Percival S.S. (2008) L-theanine intervention enhances human γδ T lymphocyte function. *Nutrition Review* **66:**99-102.

Burke E.J., Brown S.J., Christidis N. (2006) Modeling the recent evolution of global drought and projections for the twenty-first century with the Hadley centre climate model. *Journal Hydrometeor* **7:**1113-1125.

Carr M.K.V., Stephen W. (1992) Climate, Weather and the Yield of Tea. In: Tea: Cultivation to Consumption, in: K. C. Wilson and E. Clifford M. N. (Eds.), Chapman and Hall., London. pp. 87-135.

Chakeris D.H. (2009) Genomic,Proteomics and metabolomic approaches to study drought responses in Aquilegia. *Dissertation* 2-3.

Chakraborty U., Dutta S., Chakraborty B.N. (2002) Response of tea plants to water stress. Biologia Plantarum **45:**557-562.

Chang H.T., Bartholomew B. (1984) Camellias Basford., London.

Chatzidimitriadou K., Nianiou-Obeidat I., Madesis P., Perl-Treves R., Tsaftaris A. (2009) Expression of *SOD* transgene in pepper confer stress tolerance and improve shoot regeneration. *Electronic Journal of Biotechnology* **12:**1-9.

Chaves M.M., Maroco J.P., Pereira J.S. (2003) Understanding plant response to drought-from genes to the whole plant. *Functional Plant Biology* **30:**239–264.

Chazen O., Neumann P.M. (1994) Hydraulic signals from the roots and rapid cell-wall hardening in growing maize (Zea mays L.) leaves are primary responses to polyethylene glycol-induced water deficits. *Plant Physiology* **104:** 1385-1392.

Chen L., Zhao L., Gao Q. (2005) Generation and analysis of expresses sequenced tags from the tender shoots cDNA library of tea plant (*Camellia sinensis*). *Plant Science* 1**68:**359-363.

Cheruiyot E.K., Mumera L.M., Ng'etich W.K., Hassanali A., Wachira F. (2007) Polyphenols as Potential Indicators for Drought Tolerance in Tea (*Camellia sinensis* L. O kuntze). *Biosciences Biotechnology Biochemistry* **71:**2190-2197.

Cheruiyot E.K., Mumera L.M., Ng'etich W.K., Hassanali A., Wachira F.N. (2010) High fertilizer rates increase susceptibility of tea to water stress. *Journal of Plant Nutrition* **33:**115-129.

Cheruiyot E.K., Mumera L.M., Ng'etich W.K., Hassanali A., Wachira F.N. (2008) Shoot catechin and Epigallocatechin contents respond to water stress in Tea (*Camellia sinensis* L. O kuntze). *Biosciences Biotechnology Biochemistry* **72:**1219-1226.

Cheserek B. (2011) A report on evidence and impacts of climate change in tea growing areas in Kenya. *TRFK Quarterly Bulletin* **15:**2-3.

Crus de Carvalho M.H., Laffray D., Louguet P. (1998) Comparison of the physiological responses of phaseolus vulgaris and Vigna unguiculata cultivars when submitted to drought conditions. *Environmental and Experimental Botany* **40:**197-207.

Damatta F.M. (2004) Exploring drought tolerance in coffea: A physiological approach with some insights for plant breeding. *Brazilian journal of plant physiology* **16:**1-6.

Damayanthi M.M.N., Mohotti A.J., Nissanka S.P. (2010) Comparison of toleant ability of mature field grown tea cultivars exposed to drought stress in Passara. *Tropical Agriculture Research* **22:**66-75.

Das A., Das S., Mondal T.K. (2012) Identification of differentially expressed gene profiles in young roots of tea [*Camellia sinensis* (L.) O. Kuntze] subjected to drought stress using suppression subtractive hybridization. *Plant Molecular Biology* **30:**1088-1101. DOI: 10.1007/s11105-012-0422-x.

Fischlin A., Midgley G.F., Price J.T., Leemans R., Gopal B., Turley C. (2007) In: Ecosystems, their properties, goods and services. Climate Change 2007: Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.

Foyer C. (2001.) Prospects for enhancement of the soluble antioxidants, ascorbate and glutathione. *Biofactors* **15:**75-78.

Foyer C.H., Noctor G. (2005) Oxidant and antioxidant signaling in plants: a revaluation the concept of oxidative stress in a physiological context. *Plant Cell Environment* **28:**1056–1071.

Fray R.G., Wallace A., Grierson D., Lycett G.W. (1994) Nucleotide sequence and expression of a ripening and water stress-related cDNA from tomato with homology to the MIP class of membrane channel proteins. *Plant Molecular Biology* **24:**539- 543.

Graham H.N. (1999) Tea. In Wiley Encyclopedia of Food Science and Technology,. 2nd ed ed. John Wiley & Sons:, New York.

Grill D., Tauze M., Dekok L. (2001) Significance of glutathione to plant adaptation to the environment. Kluwer Academic Publishers, Boston, MA.

Gupta S., Bharalee R., Bhorali P., Agarwal N., Ahmed P., Saikia H., Borchetia S., Kalita M.S., Handique A.K., Das S. (2012) Identification of drought tolerant progenies in tea by gene expression analysis. *Functional intergrative genomics*. DOI: 10.1007/s10142-012-0277-0.

Hare P.D., Cress W.A., Van Staden J. (1998) Dissecting the roles of osmolyte accumulation during stress. *Plant Cell Environment* **21:**535-553.

Heath L.S., Ramakrishnam N., Sederoff R.R., Whetten R.W., Chevone B.L., Struble C.A., Jouenne V.Y., Chen D., Zyl L., Green R. (2002) Studying the functional genomics of the stress responses in Loblolly pine with the expresso microarray experiment management system. *Comprehensive functional genomics* **3:**226-243.

Ingram J., Bartels D. (1996) The molecular basis of dehydration tolerance in plants. *AMU Review of Plant Physiology and Plant Molecular Biology* **47:**377-403.

Irigoyen J.J., Emerich D.W., Sanchez-Diaz M. (1992) Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiology Plant* **84:**55–60.

Jackson R.B., Sperry J.S., Dawson T.E. (2000) Root water uptake and transport: using physiological processes in global predictions. *Trends Plant Science* **5:**482–488.

Jaleel C.A., Manivannan P., Sankar B., Kishorekumar A., Gopi R., Somasundaram R., Panneerselvam R. (2007) Water deficit stress mitigation by calcium chloride in Catharanthus roseus; effects on oxidative stress, proline metabolism and indole alkaloid accumulation. Colloids and Surfaces B: . *Biointerfaces* **60:**110-116.

Janendra W.A., Mohjotti Janaki A., A. W.M. (2007) Ecophysiology of tea. *Plant physiology*, **19:**299-332.

Jia X.-D., Han C., Chen J.-S. (2005) Tea pigments induce cell-cycle arrest and apoptosis in HepG2 cells. *World Journal of Gastroenterology* **11:**5273–5276.

Jiang Y., Huang B. (2001) Drought and heat stress injury to two cool-season turf grasses in relation to antioxidant metabolism and lipid peroxidation. *Crop Science* **41:**436-442.

Jorgensen S.T., Ntundu W.H., Ouedraogo M., Christiansen J.L. (2011) Effects of a short and severe intermittent drought on transpiration, seed yield, yield components and harvets index in four landraces of bambara groundnuts. *International journal of plant production* 5.

Karori S.M., Wachira F.N., Wanyoko J.K., Ngure R.M. (2007) Antioxidant capacity of different types of tea products. *African Journal of Biotechnology* **6:**2287-2296,.

Kavi-Kishor P.B., Hong Z., Miao G.-H., Hu C.A.A., Verma D.P.S. (1995) Overexpression of Δ1-pyrroline-5-carboxylic acid synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology* **108:**1387-1394.

Kerio L.C., Wachira F.N., Wanyoko J.K., Rotich M.K. (2013) Total polyphenols, catechin profiles and antioxidant activity of tea products from purple leaf coloured tea cultivars. *Food chemistry* **136:**1405-1413.

Khan N., Mukhtar H. (2007) Tea Polyphenols for health promotion. *Life Science* **81:**519-533.

Kin-Won L., GiJun C., Kin-Yong K., Hee C.J., Rashed Z., Sang-Hoon L. (2011) Identification of drought induced differentially expressed genes in barleyleaves using the annealing control-primer-based GeneFishing technique. *Crop Science* **5:**1364-1369.

Koech K. R., Wachira F.N., Ngure R.M., Orina I.A., Wanyoko J.K., Bii C., Karori S.M. (2013) Antifungal activity of crude tea extracts. *African Journal of Agricultural Research* **8:**2086-2089.

Kohl D.H., Kennelly E.J., Zhu Y., Schubert K.R., Shearer G. (1991) Proline accumulation, nitrogenase activity and activities of enzymes related to proline metabolism in drought-stressed soybean nodules. *Journal of Experimental Botany* **240:**831–837.

Kondo K. (1977) Chromosome numbers in the genus Camellia. Biotropica **9:**86 – 94.

Kozlowski T.T., Pallardy S.G. (2002) Acclimation and adaptive responses of woody plants to environmental stresses. *The Botanical Review* **68:**270-334.

Kristiansson E., Asker N., Forlin L., Larsson D.G.J. (2009) Characterization of the Zoarces viviparus liver transcriptome using massively parallel pyrosequencing. *BMC Genomics* **10:**345.

Lai J., Yang. W.-c., Hsiao Y. (2002) An assessment of genetic relationship in cultivated tea clones and native wild tea in Taiwan using RAPD and ISSR markers. *Botany Buletin Academic science* **42:**93-100.

Lange B.M., Wildung M.R., Stauber E.J., Sanchez C., Pouchnik D., Croteau R. (2000) Probing essential oil biosynthesis and secretion by functional evaluation of expressed sequence tags from mint glandular trichomes. Proceedings National Academy Science. U.S.A.:97.

Levitt J. (1980) (Eds.) Responses of plants to environmental stresses., Academic Press, New York.

Lin Y.S., Tsai Y.J., Tsay J.S., Lin J.K. (2003) Factors affecting the levels of tea polyphenols and caffeine in tea leave. *Journal of Agricultural Food Chemistry* **51:**1864-1873.

Liu F., Stutzel H. (2002) Leaf expansion, stomatal conductance and transpiration of vegetable Amaranth (Amaranthus sp.) in response to soil drying. *Journal of American Society of Horticulture* **127**:878-883.

Liu J., Hara C., Umeda M., Zhao Y., Okita T.W., Uchimiya H. (1995) Analysis of randomly isolated cDNAs from developing endosperm of rice (Oryza sativa L.): Evaluation of expressed sequence tags, and expression levels of mRNAs. *Plant Molecular Biology* **29:**685–689.

Mafakheri A., Siosemardeh B., Bahramnejad B., Struik P.C., Sohrabi Y. (2010) Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. *Australian Journal of crop science* **4:**580-585.

Magoma G.N., Wachira F.N., Imbuga M.O., Agong S.G. (2000) The use of catechins as biochemical markers in diversity studies in tea (*Camellia sinensis*). *Genetic Resource and Crop Evolution* **47:**107-114.

Magoma G.N., Wachira F.N., Imbuga M.O., Agong S.G. (2003) Biochemical differentiation in *Camellia sinensis* and its wild relatives by isozyme and catechin patterns. *Biochemical Systemics and Ecology* **31:**995-1010.

Mahajan S., Tujeta N. (2005) Cold, salinity and drought stresses: An overview. *Archives of Biochemistry and Biophysics* **444:**139-158.

Managua C. (2011) Future Climate Scenarios for Kenya's Tea Growing Areas, International Centre for Tropical Agriculture.

MansWeld T.J., Atkinson C.J. (1990) Stomatal behaviour in water stressed plants Wiley-Liss, New York.

Mbadi P.K., Owuor P.O. (2008) The role of Kenya Tea Development Agency Limited in the small-scale tea holder development in Kenya. *International Journal of Tea Science* **7:**7-15.

Medrano H., Escalona J.M., Bota J., Gulías J., Flexas J. (2002) Regulation of photosynthesis of C3 plants in response to progressive drought: the interest of stomatal conductance as a reference parameter. *Annals of Botany* **89:**895-905.

Mekhedov S., Martinez O., Ohlrogge J. (2000) Towards a functional catalog of the plant genome: a survey of genes for lipid biosynthesis. *Plant Physiology* **122:**389–401.

Meyer E., Aglyamova G.V., Wang S., Buchanan-Carter J., Abrego D., Colbourne J.K., Willis B.L., Matz M.V. (2009) Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx. *BMC Genomics* **10:**219.

Miko W., F., K. (2011) Does enhanced photosynthesis enhances growth. *Plant Physiology* **155:**117-124.

Mockler T.C., Chan S., Sundaresan A., Chen H., Jacobsen S.E., Ecker J.R. (2005) Applications of DNA tiling arrays for whole-genome analysis. *Genomics* **85:**1–15.

Mohammad I.A., Lin T.P. (2010) Molecular analysis of dehydration in plants. *Plant Science* **1:**21-25.

Mondal T.K. (2008) Tea Breeding. Springer, Berlin.

Monobe M., Ema K., Kato F., Maeda-Yamamoto M. (2008) Immunostimulating activity of a crude polysaccharide derived from green tea (*Camellia sinensis*) extract. *Journal of Agricultural and Food Chemistry* **56:**1423–1427.

Morozova O., Hirst M., Marra M.A. (2009) Applications of New Sequencing Technologies for Transcriptome Analysis. *Annual Review Genomics Human Genetics* **10:**135–151.

Mortazavi A., Williams B.A., McCue K., Schaeffer L., Wold B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods* **5:**621–628.

Munnik T., Meijer H. (2001) Osmotic stress activate distinct lipid and MAPK signalling pathway in plants. FEBS Lett. 498.

Muoki R.C., Paul A., Kumar S. (2012) A shared response of *thaumatin like protein, chitinase* and *late embryogenesis abundant protein3* to environmental stresses in tea (*Camellia sinensis* (L.) O. Kuntze). Functional intergrative genomics. DOI: 10.1007/s10142-012-0279-y.

Nambara E., Kawaide H., Kamiya Y., Naito S. (1998) Characterization of an *Arabidopsis thaliana* mutant that has a defect in ABA accumulation: ABA-dependent and ABA-independent accumulation of free amino acids during dehydration. *Plant Cell Physiology* **39:**853-858.

Naya L., Ladrera R., Ramos J., González E.M., Arrese-Igor C., Minchin F.R., Becana M. (2007) The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. *Plant Physiology* **144:**1104–1114.

Ngetich W.K., Stephen W., Othieno C.O. (2001) Responses of tea to environment in Kenya. III. Yield and yield distribution. *Experimental Agriculture* **37:**361-372.

Nishizawa A., Yabuta Y., Shigeoka S. (2008) Galactinol and Raffinose Constitute a Novel Function to Protect Plants from Oxidative Damage. *Plant Physiology* **147:**1251-1263.

Novaes E., Drost D.R., Farmerie W.G., Pappas G., Grattapaglia, D.,, Sederoff R.R., Kirst M. (2008) High throughput gene and SNP discovery in *Eucalyptus grandis*, an uncharacterized genome. *BMC Genomics* **9:**312.

Otieno W., Sudoi V., Wachira F., Mamati G.E., Chalo R. (2002.) A report on outbreak of root knot nematodes on tea in Kerugoya and Imenti. *TRFK Quarterly Bulletin* **7**:6-8.

Park J.S., Kim J.B., Haha B.S., Kim K.H., Ha S.H., Kim J.B., Kim Y.H. (2004) EST analysis of genes involved in secondary metabolism in Camellia sinensis (tea), using suppression subtractive hybridization. Plant Science **166:**953-961.

Parvaiz A., Satyawati S. (2008) Salt stress and phyto-biochemical responses of plants - a review. *Plant Soil Environment* **54:**89-99.

Paul A., Muoki R.C., Singh K., Kumar, S. (2012) CsNAM-like protein encodes a nuclear localized protein and responds to varied cues in tea [*Camellia sinensis* (L.) O. Kuntze]. *Gene* **502**:69-74.

Quan R., Shang M., Zhang H., Zhao Y., Zhang J. (2004) Engineering of enhanced glycinebetainesynthesis improves drought tolerance in maize. *Plant Biotechnology* **2**:477-486.

Reddy A.R., Chaitanya K.V., Vivekanandan M. (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of Plant Physiology* **61:**1189-1202.

Ricardo T.C., O'connell A.M. (2005) Genetic diversity of drought responsive genes in populations of desert forage, *Dactylis glomerata. Plant science* **168**:1327-1335.

Rossel J.B., Water P.B., Hendrickson L., Chow W.S., Poole A., Mullineaux P.M., Pogson B.J. (2006) A mutation affecting ascorbate peroxidase 2 gene expression reveals a link between responses to high light and drought tolerance. *Plant Cell Environment* **29:**269-281.

Rothberg J.M., Leamon J.H. (2008) The development and impact of 454 sequencing. *Nature Biotechnology* **26:**1117-1124.

Sakamoto A., Murata N. (2000) Genetic engineering of glycinebetaine in plants: current status and implications for enhancement of stress tolerance. *Journal of Experimental Botany* **51:**81-88.

Sannella A.R., Messori L., Casini A., Vincieri F., Bilia A.V., Majori G., Severini C. (2006) Antimalarial properties of green tea. *Biochemical and Biophysical Research Communications.* **353:**177-181.

Satoshi L., Masatomo K., Teruaki T., Masaaki N., Motoaki S., Tomohiko K., Satoshi T., Yoshitaka K., Yamaguchi- Shinozaki K., Shinozaki K. (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, key enzyme in abscisic acid biosynthesis in *Arabidopsis*. *The plant* **27:**325-333.

Schena M., Shalon D., Davis R.W., Brown P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270:**467–70.

Seki M., Narusaka M., Kamiya A., Ishida J., Satou M. (2002) Functional annotation of a full-length Arabidopsis cDNA collection. *Science* **296:**141–145.

Shakeel A.A., Xiao-yu X., Long-chang W., Muhammad F. S., Chen M., L. W. (2011) Morphological, physiological and biochemical responses of plants to drought stress. African *Journal of Agricultural Research* **6:**2026-2032.

Shao H.B., Chu L.Y., Jaleel C.A., Manivannan P., Panneerselvam R., Shao M.A. (2009) Understanding water deficit stress-induced changes in the basic metabolism of higher plants-biotechnologically and sustainably improving agriculture and the ecoenvironment in arid regions of the globe. *Critical Reviews in Biotechnology* **29:**131-151.

Sharma P., Kumar A. (2005) Differential display-mediated identification of three drought-responsive expressed sequence tags in tea [*Camellia sinensis* (L.) O. Kuntze]. *Journal of Bioscience* **30**:231-235.

Sherrard M.E., Maherali H., Latta R.G. (2009) Water stress alters the genetic architecture of functional traits associated with drought adaptation in *Avena barbata*. *Evolution.* **63:**702–715.

Shi C.-Y., Hua Y. C.-L., W., Oliver Y., Zheng-Zhu Z., Chang-Jun J., Jun S., Ye-Yun L., Qi C., Tao X., Xiao-Chun W. (2011) Deep sequencing of the Camellia sinensis transcriptome revealed candidate genes for major metabolic pathways of tea-specific compounds. *Genomics* **12:**131.

Shilpi M., Narendra T. (2005) Cold, salinity and drought stresses: An overview. *Archives of Biochemistry and Biophysics* **444:**139-158.

Shinozaki K. (2007) Regulatory metabolic networks in drought stress responses. *Current Opinion Plant Biology* **10:**296-302.

Shinozaki K., Yamaguchi-Shinozaki K. (1997) Gene Expression and Signal Transduction in Water-Stress Response. *Plant Physiology* **11**:327-334.

Shinozaki K., Yamaguchi-Shinozaki K. (2000) Molecular responses to dehydration and low temperature: differences and cross-stalk between two stress signaling pathways. *Current Opinion in Plant Biology* **3**:217-223.

Shinozaki K., Yamaguchi-Shinozaki K. (2007) Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany* **58:**221-227.

Shinozaki K., Yamaguchi-Shinozaki K., Seki M. (2003) Regulatory network of gene expression in the drought and cold stress responses. *Current opinion in Plant Biology* **6:**410-417.

Simon S.A., Zhai J., Nandety R.S., McCormick K.P., Zeng J., Mejia D., Meyers B.C. (2009) Short-read sequencing technologies for transcriptional analyses. *Annual Reviews Plant Biology* **60:**305-333.

Smirnoff N. (1993) The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytology* **25:**27–58.

Sterky F., Regan S., Karlsson J., Hertzberg M., Rohde A., Holmberg A., Amini B., Bhalerao R., Larsson M., Villarroel R., Montagu M.V., Sandberg G., Olsson O., Teeri T.T., Boerjan W., Gustafsson P., Uhle´n M., Sundberg B., Lundeberg J. (1998) Gene discovery in the woodforming tissues of poplar: analysis of 5,692 expressed sequence tags. *Proceedings National Academy of Science. U.S.A*. **95:**13330-13335.

Stone E.M., Rothblum K.N., Alevy M.C., Kuo T., M.,, Schwartz R.J. (1985) Complete sequence of the chicken glyceraldehyde-3-phosphate dehydrogenase gene. *Proceedings of National Academy of Science* **82:**1628–1632.

Sugui J.A., Deising H.B. (2002) Isolation of infection-specific sequence tags expressed during early stages of maize anthracnose disease development. *Molecular Plant Pathology* **3:**197–203.

Sun W.Q., Leopold A.C. (1997) Cytoplasmic vitrification and survival of anhydrobiotic organisms. *Components Biochemical Physiology* **117A:**327–333.

Taji T., Ohsumi. (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant Journal* 29:417-426.

Takeda Y. (1990) Cross compatibility of tea (Camellia sinensis) and its allied species in the genus Camellia. *Japan Agricultural Research Quarter* **24:**111-116.

Tanaka J., Taniguchi F. (2006) Estimation of the genome size of tea (*Camellia sinensis*), camellia (C. japonica), and their interspecific hybrids by flow cytometry. *Journal of the Remote Sensing Society of Japan* **101:**1-7.

Taniguchi F., Fukuoka H., Tanaka J. (2012) Expressed sequence tags from organ specific cDNA libraries of tea (*Camellia sinensis*) and polymorphisms and transferability of EST-SSRs across *Camellia* species. *Breeding scienc*e **62:**186-195.

TBK. (2012) Tea industry perfomance in 2011., The Tea Board of Kenya.

Tezara W., Mitchell V., Driscoll S.P., Lawlor D.W. (2002) Effects of water deficit and interaction with CO_2 supply on the biochemistry and physiology of photosynthesis in sunflower. *Journal of Experimental Botany* **53:**1781-1791.

Thevelein J.M. (1996) (Ed.)^(Eds.) Regulation of trehalose metabolism and its relevance to cell growth and function. In: The Mycota,. , Springer, Berlin. pp. Pages.

Upadhyaha H., Panda S.K., Dutta B.K. (2008) Variation of physiological and antioxidative responses in tee cultivars subjected to elevated water stress followed by rehydration recovery. *Acta Physiology Plant* **30:**457–468.

Venkatesh P., Jaiprakash M., Prasad P., Pillan B., Sadhale P.P., Sinkar P.V. (2008) Flavanoid biosynthesis in tea. *Report.*

Vera J.C., Wheat C.W., Fescemyer H.W., Frilander M.J., Crawford D.L., Hanski I., Marden J.H. (2008) Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. *Molecular Ecology* **17:**1636-1647.

Vo T.D. (2006) An assessment on genetic diversity in Vietnam tea using morphology, ISSR and microsatellites. *Dissertation*.

Voelkerding K.V., Dames S.A., Durtchi J.D. (2009) Next-Generation sequencing: From Basic research to diagnostics. *Clinical chemistry* **55:**641-658.

Volk G.M., Haritatos E.E., Turgeon R. (2003) Galactinol synthase gene expression in melon. *American Society of Horticultural Science* **128:**8-15.

Wachira F.N. (1994) Triploidy in tea (*Camellia sinensis*): Effects on yield and tea attributes. *Journal of Horticultural Sciences* **69:**53-62.

Wachira F.N. (2002) Genetic mapping of tea: A review of achievements and opportunities. Tea **23:**91-102.

Wachira F.N., Kiplangat J.K. (1991) Newly identified Kenyan polyploid tea strains. *Tea* **12**:91-102.

Wachira F.N., Waugh R. (1995) Detection of genetic diversity in tea (*Camellia sinensis*) using RAPD markers. *Genome* **38:**201-210.

Wachira F.N., Ngetich W. (1999) Dry matter production and partition in in diploid, triploid and tetraploid tea. *Journal of Horticultural Biotechnology* **74**:507-512.

Wachira F.N., Ronno W. (2004) Current research on tea in Kenya. In:. Proceedings of 2004 International Conference on O-Cha (Tea) Science; Shizouka, Japan:59-65.

Wachira F.N., Kamunya S.M. (2005) Kenyan teas are rich in antioxidants. *Tea* **26:**81-89.

Wachira F.N., Tanaka J., Takeda Y. (2001) Genetic variation and differentiation in tea (*Camellia sinensis*) germplasm revealed by RAPD and AFLP variation. *Journal of Horticultural Science and Biotechnology* **76**:557-563.

Waheed A., Hamid F.S., Shah A.H., Habib A., Khalid A., Abbasi F.M., Ahmad N., Aslam A., Sarwar S. (2012) Response of different tea (*Camellia sinensis* L.) clones against drought stress. *Journal of Material Environmental Science* **3:**395-410.

Wang W., Wang Y., Zhang Q., Qi Y., Guo D. (2009) Global characterization of *Artemesia annua* grandular trichome transcriptome using 454 pyrosequencing. *BMC Genomics* **10:**465.

Wang X.W., Luan J.B., Li J.M., Bao Y.Y., Zhang C.X., Liu S.S. (2010) De novo characterization of a whitefly transcriptome and analysis its gene expression during development. *BMC Genomics* **11:**400.

Wang Y., Jiang C.J., Zhang H.Y. (2008) Observation on the Self-incompatibility of Pollen Tubes in Self-pollination of Tea Plant in Style in vivo. *Tea Science* **28:**429-435.

Weretilnyk E.A., Bednarek S., McCue K.F., Rhodes D., Hanson A.D. (1989) Comparative biochemical and immunological studies of the glycine betaine synthesis pathway in diverse families of dicotyledons. *Plantation* **178:**342–352.

Wilkinson S., Davies W.J. (2002) ABA-based chemical signalling: the co-ordination of responses to stress in plants. *Plant Cell Environment* **25:**195-210.

Willson K.C., Clifford M.N. (1992) Tea: Cultivation to Consumption. , Chapman & Hall., London.

Wisniewki M., Bassett C., Norelli J., Macarisin D., Artlip T., Gasic K., Korban S. (2008) Expressed sequence tag analysis of the response of apple (*Malus x domestica* Royal Gala) to low temperature and water deficit. *Physiology Plant* **133:**298-317.

Wu H., Chen D., Li Jiaxian, Yu B., Qiao X., Huang H., He Y. (2012) *De novo* characterization of leaf transcriptome using 454 sequencing and development of EST-SSR markers in tea, (*Camelia sinensis*). Plant Molecular Biology Report. DOI: 10.1007/S11105-012-0519-2.

Xin Z., Browse J. (2000) Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant Cell Environment* **23:**893–902.

Xu Z.Z., Zhou G.S., Shimizu H. (2009) Effects of soil drought with nocturnal warming on leaf stomatal traits and mesophyll cell ultrastructure of a perennial grass. *Crop Science* **49:**1843–1851.

Yamada M., Morishita H., Urano K., Shiozaki N., Yamaguchi-Shinozaki K., Shinozaki K., Yoshiba Y. (**2005**) Effects of free proline accumulation in petunias under drought stress. *Experimental Botany* **56**:1975-1981.

Yamamoto K., Sasaki T. (1997) Large-scale EST sequencing in rice. *Plant Molecular Biology* **35:**135–144.

Yann S., Juan I.M. (2010) Optimization of de novo transcriptome assembly from next-generation sequencing data. *Genome Research* **20:**1432-1440.

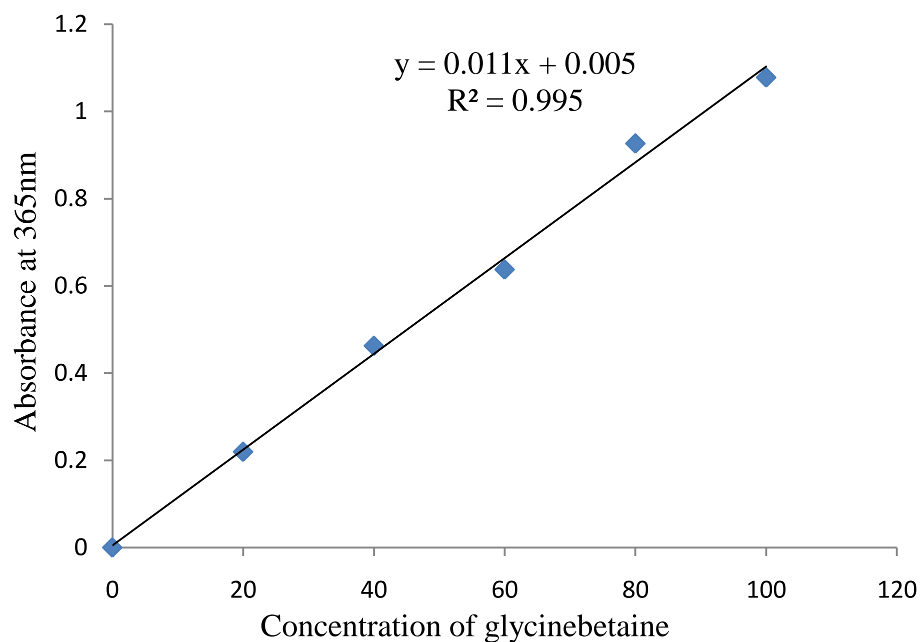
Yordanov I., Velikova V., Tsonev T. (2003) Plant response to drought and stress tolerance. *Plant Physiology* 187-206.

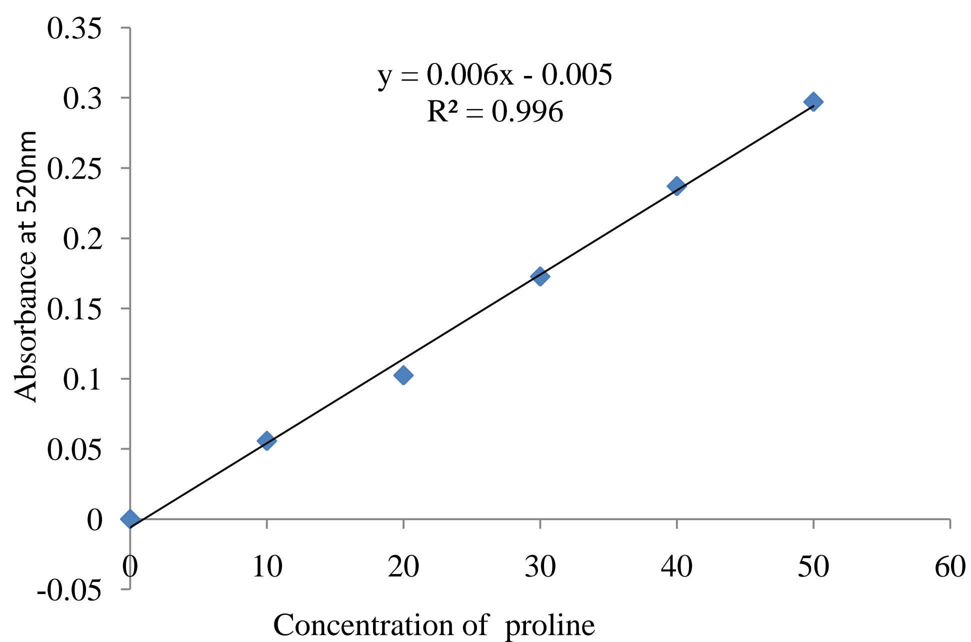
Zhou Y., Gao F., Liu R., Feng J., Li H. (2012) *De novo* sequencing and analysis of root transcriptome using 454 pyrosequencing to discover putative genes associated with drought tolerance in *Ammopipnanthus mongolicus*. *BMC Genomics* **13:**266.

Zhu J.Y. (2002) Salt and drought stress signal transduction in plants. *Annual Review Plant Biology* **53:**247-273.

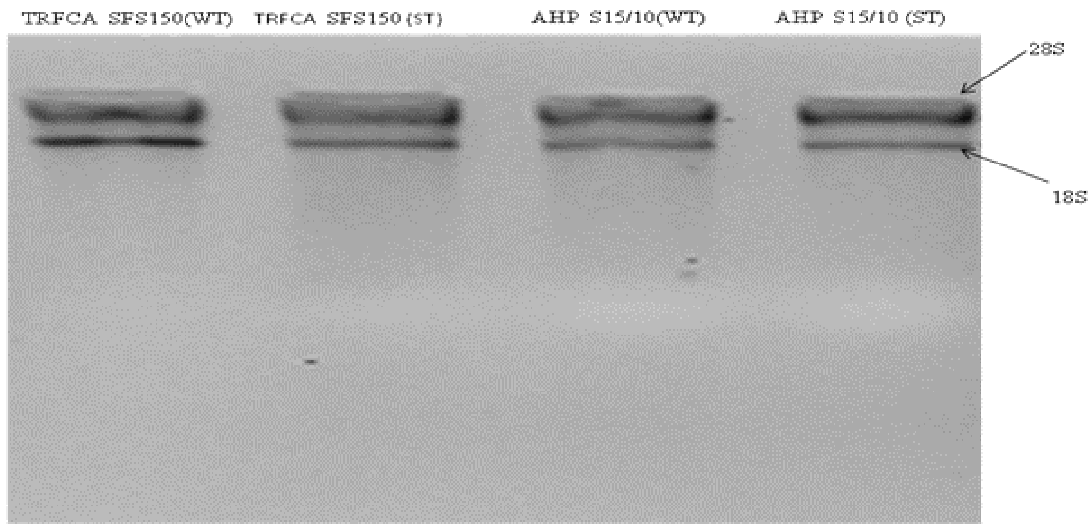
APPENDICES

Appendix 1; Standard calibration curve of glycinebetaine and proline

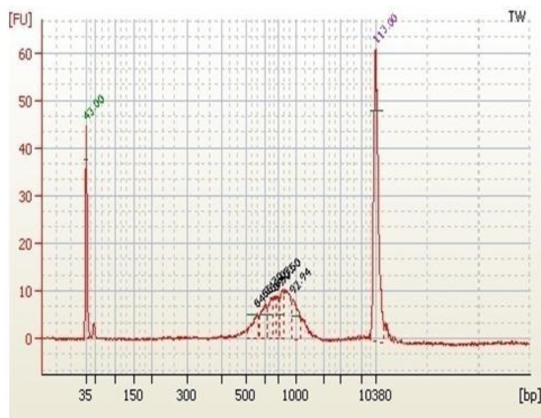


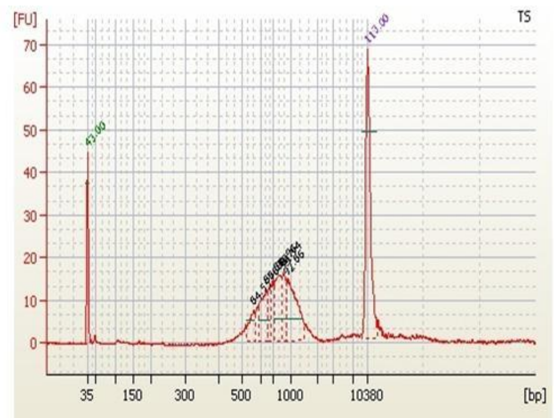


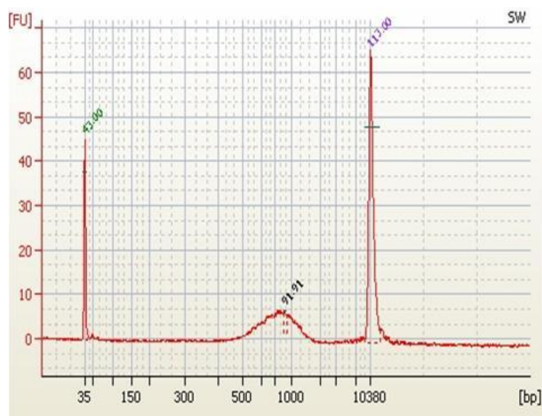
Appendix 2; Gel electrophoresis image of total RNA. ST; stressed condition, WT; watered condition

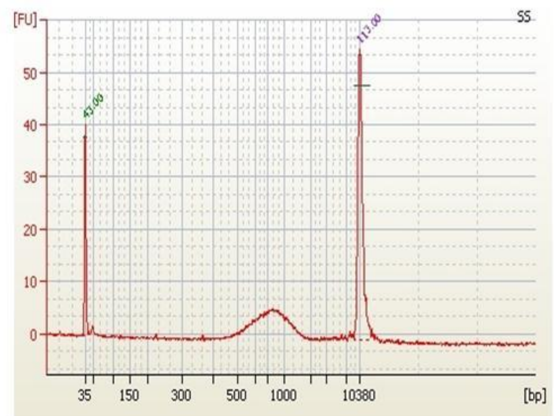


Appendix 3 ; Trace representation of cDNA library from 4 tea samples as assessed on an Agilent Bioanalyzer High sensitivity DNA chip. TW = TRFCA SFS150 (Watered), TS= TRFCA SFS150 (stressed), SW= AHP S15/10 (Watered), SS= AHP S15/10 (stressed)









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)