SEASONALVARIATIONS ON FLAVONOID BIOSYNTHETIC PATHWAY PROCESSES IN *Camellia sinensis* (L) O Kuntze OF SELECTED KENYAN CULTIVARS

BY

CHEROTICH CHEPTOT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PLANT GENETICS, UNIVERSITY OF ELDORET, KENYA

NOVEMBER, 2019

DECLARATION

This thesis is my original work and has not been submitted for academic award in any institution; and shall not be reproduced in part or full, or in any format without prior written permission from the author and / or University of Eldoret.

Ms. Cherotich Cheptot

SC/PGB/034/11

Signature……. Date……

This thesis has been submitted with our approval as University supervisors

Dr. Lexa Gomezgani Matasyoh

Department of Biological Sciences

University of Eldoret, Kenya

P.O. Box 1125-30100

Eldoret - Kenya

Signature……. Date……

Dr. Richard Chalo Muoki

Crop Improvement and Management Programme

Tea Research Institute

P.O. Box 820-20200

Kericho-Kenya

Signature……. Date……

DEDICATION

Dedicated to my nuclear family, parents and collegues at large.

ABSTRACT

Globally, production of black tea has been on an increasing trend. This has posed a challenge on the market prices due to glut in the market. Thus need for diversification of tea products. Flavonoids are good antioxidants hence health-promoting properties. They are also relevant in determining the quality of food products. The quality of beverage tea is determined by the accumulation of flavonoids which are profoundly influenced by changes in environmental parameters and germplasm. The objective of this study was to investigate the effect of seasonal variations on tea flavonoids. Five cultivars comprising green-(TRFK 31/8, TRFK 6/8, and NRIT St. 536) and purple-leafed (TRFK 306 and TRFK 91/1) grown at Tea Research Institute (TRI), Timbilil center, Kericho county and Kangaita center, Kirinyaga county were evaluated for flavonoid profiles over the hot-dry, cool-wet and warm-wet seasons in the year 2014. Stomatal density (S_D) was determined to find the effect on the biosynthesis of flavonoids. It involved scoring of leaf imprints of the 3rd tea leaf under the light microscope (Mg ×25). Results showed significant ($P< 0.05$) variation of stomatal density among the cultivars and all the interactions. Higher stomatal density was recorded in the cool-wet season in assam varieties; TRFK 31/8, 306 and 91/1. For biochemical analysis, three leaves plus apical bud were analyzed for catechins using HPLC (high performance liquid chromatography). There was significant ($P<0.05$) variation in total catechins (TC) due to cultivars and seasons. The cultivar TRFK 6/8 showed signifigant high accumulation of total catechins during the warm-wet season. EGCG (epigallocatechin gallate) which is the most abundant catechin in tea leaves was significantly higher during the warm-wet season in Timbilil. Further, three leaves plus apical bud of the purple-leafed cultivar TRFK 306 and TRFK 91/1 were sampled for anthocyanins. Total monomeric anthocyanin was determined using pH differential method of two buffers systems. Results revealed significant ($P<0.05$) variation among the cultivars and all the interactions. Anthocyanins content was significantly accumulated during the warm-wet season compared with other seasons. The hybrid TRFK 306 showed significantly higher contents compared with the parent (wild tea) TRFK 91/1. For gene expression studies, three leaves plus apical bud were analysed for eight gene transcripts; *CsPAL, CsCHS, CsCHI CF3H, CsF3'5'H, CsDFR, CsANR* and *CsANS*. The raw threshold cycle (C_t) values were normalized against a housekeeping gene encoding *CsActin* and the values used to calculate the differences in expression using the Relative Expression Software Tool (REST). Results revealed that there was differential expression of gene transcripts *CsPAL*, *CsCHI*, *CF3H* and *CsF3'5'H* in purple-leafed and green-leafed cultivars. In all the seasons, purple leafed cultivar TRFK 306 showed; up regulation of *CsCHI* and down regulation of *CsCHS*, down regulation of *CsPAL* in cool-wet and warm-wet seasons, and down regulation of *CsF3'5H* only in hot-dry season. Overall, the results of the study demonstrated that, stomatal density was higher in cool-wet season in assam cultivars; TRFK 306 accumulated higher contents of total monomeric anthocyanis during the warm-wet season. While TRFK 6/8 commercially grown for quality tea showed significantly higher total catechins during the warm-wet season. For molecular analysis, results showed differentially expression of flavonoids gene transcripts in the two selected cultivars (TRFK 306 and 31/8). The observed season-specific differences in flavonoids content in different tea cultivars are an indication of the potential for product diversification.

TABLE OF CONTENTS

DECLARATION.....i
DEDICATION.....ii
ABSTRACT.....iii
LIST OF FIGURES.....viii
LIST OF TABLES.....ix
LIST OF ABBREVIATIONS AND ACRONYMS.....xi
ACKNOWLEDGEMENT.....xiii
CHAPTER ONE.....1
INTRODUCTION.....1
1.1 Background information.....1
1.2 Statement of the problem.....6
1.3 Justification of the study.....6
1.4 Objectives.....7
1.4.1 General Objective.....7
1.4.2 Specific objectives.....7
1.5 Null hypothesis.....8
CHAPTER TWO.....9
LITERATURE REVIEW.....9
2.1 Botany of the tea plant.....9
2.2 Tea products.....9
2.3 Ecological requirements for tea plant.....10
2.4 The effect of seasons and elevation on the tea quality.....10
2.5 Effects of seasonal weather changes on stomatal density (SD).....12
2.6 Bioactive compounds of tea.....14
2.7 Tea flavonoids.....15
2.8 Variation of catechins accumulation in plants.....16
2.9 Variation of anthocyanins accumulation in plants.....17
2.9.1 Flavonoids biosynthetic pathway.....20
2.9.2 Influence of environmental cues on Flavonoid biosynthetic pathway genes.....23
CHAPTER THREE.....25
MATERIALS AND METHODS.....25
3.1 Plant materials and experimental sites.....25
3.2 Experimental design.....26

3.3 Stomatal density (SD) 26
3.4 Biochemical analysis 27
3.4.1 Sample collection 27
3.4.2 Catechins analysis 27
3.4.2.1 Sample extraction 27
3.4.2.2 Determination of catechins content 27
3.4.3 Anthocyanin analysis 29
3.4.3.1 Sample extraction and purification 29
3.4.3.2 Determination of total monomeric anthocyanin content 30
3.5 Molecular analysis 30
3.5.1 Isolation of total RNA 31
3.5.2 RNA quantification 32
3.5.3 Concentrating RNA 32
3.5.4 Evaluation of RNA integrity 32
3.5.5 First-strand cDNA synthesis 33
3.5.5.1 Removal of genomic DNA 33
3.5.5.2 First-strand cDNA synthesis using Superscript III (Invitrogen, USA) 33
3.5.6 Quantitative RT-PCR analyses 34
3.6 Data analysis 35
CHAPTER FOUR 36
RESULTS 36
4.1 Seasonal variation in stomatal density (mm^2) 36
4.3 Seasonal variations in catechins accumulation 38
4.4 Seasonal variations in caffeine accumulation 39
4.6 Seasonal changes in anthocyanin concentration 41
4.7 Gene expression patterns 41
CHAPTER FIVE 45
DISCUSSION 45
5.1 Seasonal variation of leaf stomatal density 45
5.2 Seasonal variation of catechins 46
5.3 Seasonal variation in anthocyanin accumulation 49
5.4 Gene expression patterns 50
CHAPTER SIX 52

CONCLUSIONS AND RECOMMENDATIONS 52
6.1 Conclusions 52
6.2 Recommendations 53
6.3 Suggestion for further research 53
REFERENCES **54**
APPENDICES **78**
Appendix (i): Anova for stomatal density in (mm^2) for five cultivars 78
Appendix (ii): Anova for total catechin 78
Appendix (iii): Anova for EGCG 79
Appendix (iv): Anova for EGC 79
Appendix (v): Anova for ECG 80
Appendix (vi): Anova for EC 80
Appendix (vii): Anova for C 81
Appendix (viii): Anova for GA 81
Appendix (ix): Anova for CAFF 82
Appendix (x): Anova for anthocyanins 82
Appendix (xi): Raw CT values for qPCR 83
Appendix (xii): Preparations of 6×RNA loading dye and formaldehyde agrose-gel 84
Appendix(xiii): Extraction buffer 1(EB1) 84
Appendix (xiv): Extraction of buffer 11: (EB11) SSTE (Suspension Sodium Tris EDTA) 85
Appendix (xv): preparation of 3.0 m sodium acetate, PH 4.8 85
Appendix (xvi): Means seasonal weather and tea auction prices during the year 2014 86
Appendix (xvii): Weekly environmental variation of precipitation and relative humidity 86
Appendix (xviii): Weekly environmental variation of temperatures in 2014. 87

LIST OF FIGURES

Figure (i): Flavonoids basic structure…….....16

Figure(ii): Biosynthesis of flavonoids in the tea leaf…….....23

Figure (iii): Seasonal variation of total monomeric anthocyanins…….....41

Figure(iv): Relative expression profiles of the flavonoid biosynthetic genes……43

LIST OF TABLES

Table (i):Tea shoot components……... 14

Table (ii): Primer sequences of flavonoid pathway genes…….....35

Table (iii): Seasonal variation in stomatal density (mm^2) among tea cultivars…… 37

Table (iv): % of different quality components of purple and green……..40

LIST OF PLATES

Plate(i): Photographs of the green and purple tea cultivars……..25

LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	:	Analysis of variance
ANR	:	Anthocyanidin reductase
ANS	:	Anthocyanidin synthase
ASL	:	Above sea level
C_4H	:	Cinnamate 4- hydroxylase
cDNA	:	Complementary deoxyribonucleic acid
CG	:	Catechin gallate
CG	:	Catechin gallate
CHI	:	Chalcone isomerase
CHS	:	Chalcone synthase
CIA	:	Chloro isoamyl alcohol
CoA	:	Coenyme A
CTC	:	Cut, tear, curl
CV	:	Coefficient of variation
DEPC	:	Diethylpyrocarbonate
DFR	:	Dihydroflavonolreductase
DM	:	Dry matter
DMRT	:	Duncan multiple range test
dNTP	:	Deoxynucleoside triphosphate
dT	:	Deoxynucleoside thymine
DTT	:	Dichloro diphenyl trichloroethane
EATTA	:	East African Tea Trade Association
EB I	:	Extraction buffer I
EB II	:	Extraction buffer II

EC	:	Epicatechin
ECG	:	Epicatechin gallate
EDTA	:	Ethylenediaminetetra acetic acid
EGC	:	Epigallocatechin gallate
EGCG	:	Epigallocatechin gallate
FGS	:	Flavon- 3- ol- gallate synthase
GA	:	Gallic acid
GCG	:	Gallocatechin gallate
GDP	:	Gross Domestic Product
HCl	:	Hydrochloric acid
HPLC	:	High performance liquid chromatography
ISO	:	International standards Organization
ITC	:	International Tea Committee
KTDA	:	Kenya Tea Development Authority
LAR	:	Leucoanthocyanidin reductase
MTP	:	Millenium Development Plan
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NCBI	:	National Center for Biotechnology Information
PAL	:	Phenylalanine ammonia – lyase
PCR	:	Polymerase chain reaction
PPO	:	Polyphenol oxidase
PVPP	:	Polyvinylpolypyrolidone
QPCR	:	Quantitative PCR
RCBD	:	Randomized complete block design
REST	:	Relative expression software tool

RNA	:	Ribonucleic acid
RP	:	Reverse phase
RPM	:	Revolutions per minute
RRFS	:	Relative response factors
RT- PCR	:	Real time PCR
RT	:	Room temperature
SD	:	Stomatal density
TBK	:	Tea Board of Kenya
TFs	:	Theaflavins
TRFK	:	Tea Research Foundation of Kenya
TRI	:	Tea Research Institute
TRs	:	Thearubigs
UFGT	:	Glucose flavonoid 3-0- glucocyltransferase
UV	:	Ultraviolet
βME	:	Beta -mercaptoethanol

ACKNOWLEDGEMENT

I thank our Almighty God for the gift of life and good health. My strongest appreciation goes to my supervisors Dr. L. G. Matasyoh of University of Eldoret and Dr. R. C. Muoki of Tea Research Institute (TRI) for their constant guidance about the project. My sincere gratitude goes to the management of Tea Research Institute (TRI) for funding the project and University of Eldoret for giving me an opportunity to study there. I will also not forget to thank the staff in the Tea Breeding and Genetic Improvement led by center director who is also in charge of crop improvement management programme Dr. Samson Kamunya for his encouragement. I really appreciate the work of research assistant Tony Maritim and Mr Robert Korir for their great assistance in data analysis.

I would like to take this opportunity to sincerely thank my family for their prayers, moral and financial support during the entire period of my studies. I would not also forget to acknowledge my dear friends of my former school Kapsokwony boys high school, and also in my current working station Friends College Kaimosi, Institute of Science and technology for their constant encouragement and motivation. I sincerely thank my employer Teachers' Service Commission of Kenya for granting me study leave.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Tea [*Camellia sinensis* (L.) O. Kuntze] is a widely consumed beverage which has contributed significantly to the economy of many developing countries (Chen and Zhou, 2005; Zhang *et al.,* 2014). The plant is a woody evergreen perennial crop that thrives well in moderate temperatures, acidic soils and highly humid environmental conditions (Dufresne and Farnworth, 2001). There are three distinct varieties namely: Chinery (China type), Assamica (Assam type) and Cambodia (Cambod type) (Wight, 1962; Barua, 1965). This grouping is based on the growth and morphological features such as foliar, floral (Wachira *et al.*,2001) The Assamica variety is the most commonly cultivated in Kenya.

Beverage tea is classified into four groups: green, oolong, and black and pu-erh tea. Green, Oolong, Black and Pu-erh tea are unfermented, semi-fermented fermented and post fermented (Zhao *et al.,* 2006) respectively. Kenya is the third leading producer of tea after China and India. It is the largest exporter of black Cut, Tear and Curl (CTC) tea worldwide (ITC, 2016). As the leading foreign exchange earner for Kenya, the tea subsector contributes 4% to the(GDP) gross domestic product (TBK, 2013) making it a key pillar towards poverty reduction, employment creation and infrastructural development (FAO, 2016). However, the global trends for black CTC tea markets have been on the decline calling for value addition and product diversification, important means towards sustainable tea production. There is need for appropriate interventions that will contribute to expansion of market outlets. Such interventions can be driven by product diversification and value addition (Nyirenda *et al.,* 2006;

Anon, 2016). This entails the production and branding of tea products and extracts. Such an endeavour should be preceded by the extensive characterization of tea cultivars to identify biochemical properties that can enhance their nutritional potential. In this respect, the Tea Research Institute has embarked on breeding of new varieties that are suitable for different types of tea products (Kamunya *et al.*, 2009).

The potential health benefits associated with the beverage is attributed to secondary metabolites/bioactive molecules such as polyphenols (catechins, anthocyanins, theaflavins, thearubigins, and chlorogenic acid among others), alkaloids (caffeine, theophylline and theobromine), amino acids (mainly theanine), vitamins and minerals (Xie *et al.*, 2010). Tea catechins (flavan-3-ols) are classiffied into two main categories non-epicatechins namely (-) gallocatechin (GC), (-) gallocatechin gallate (GCG) and epicatechins which includes (-) epicatechin (EC), (-) epigallocatechin (EGC), (-) epigallocatechin gallate (EGCG), (-) epicatechin gallate (ECG) (Thielecke and Boschmann, 2009). These biomolecules are synthesized through the phenylpropanoid biosynthetic pathway, a process greatly influenced by the genotype and environmental conditions (Cohen *et al*., 2012). Apart from the health aspects, tea flavonoids especially catechins also determine the tea quality (Owuor and Obanda, 2006). Auto-oxidation of leaf catechins results into complex compounds such as TRs (thearubigins) and TFs (theaflavins) that gives black tea its quality. The geographical location and the prevailing enviromental conditions where tea production occurs affects the quantity of these biochemicals in the beverage (Kowalsick *et al*., 2014). Apart from flavonoids, inorganic elements such as Aluminum, Potassium, Fluoride and Manganese are also constituents of tea (Modders and Amarakoon, 2002).

Studies on tea flavonoids are of great interest due to their potential to product diversification that would promote the health aspects of tea. Several properties have been reported on the pharmacological benefits of tea. It has potential to decrease lung cancer risk (Wang *et al.,* 2014) and reduce low density lipoprotein through oxidation (Haak *et al*., 2009). Tea flavonoids reduces incidences of cold, flu and antimicrobial effects on disease-causing pathogens (Paola *et al.*, 2005; Bukowski and Percival, 2008; Arab *et al*., 2013). Growing evidence suggests that catechins improves blood flow, eliminates alcohol and other toxins, improve resistance to diseases, relieve joint pain, clear urine and improve its flow (Thielecke and Boschmann, 2009). Overally, tea flavonoids act as good antioxidants that prevents degenerative diseases. These are caused by reactive oxygen species (ROS) due to oxidative damage of cells resulting to lipid peroxidation and oxidation of carbohydrates among others (Ciancolini *et al.*, 2013).

Stomata are valves that determine the key physiological processes of plants since they control the balance of water loss and carbon intake (Luomala *et al.,* 2005). The number of sites available for gaseous exchange per unit area of the leaf, are modified by the number of stomata (Franks and Farquhar, 2007). When open, they facilitate transpiration, release of oxygen and allow carbon (IV) oxide that enters into the leaf for photosynthesis (Hetherington and Woodward, 2003; Taylor *et al*., 2012). The process of photosynthesis determines the biosynthesis of primary metabolites which then influence accumulation of the secondary metabolites in plants (Cherotich *et al.,* 2103). The organic compounds malonyl- CoA and coumaroy –CoA which are the final products of secondary metabolism, are precursors of flavonoids (Stafford, 1990). Thus leaf morphology have an impact on products of flavonoids biosynthetic pathway. The development of stomata on the leaves of a plant is determined by the

interaction between different genes and environmental factors (Yoana, 2012). Environmental factors such as temperature and light intensity not only affect plant growth and development but also may have effects on stomatal density and biosynthesis of both primary and secondary metabolites (Hemm *et al*. 2004; Verma, N., and Shukla, S. 2015; Yan *et al.,* 2017). Therefore, no studies had been conducted involving the seasonal variations of stomatal density and it's correlationship with accumulation of tea flavonoids.

The phenylpropanoid and flavonoid pathways are important for biosynthesis of flavonoids (flavan -3 ol) and it involves several enzymes (Ashihara *et al.,* 2010). The flavonoids biosynthetic pathway produces a wide range of compounds derived from an amino acid phenylalanine. They include lignins, lignans, stilbenes, and flavonoids such as anthocyanins, flavonols, proanthocyanidins (Winkel-Shirley, 2001, 2006). These compounds have diverse functions in plants as structural components and in adapting to the environmental stimuli. Biological processes are genetically controlled by complex gene networks thus it's important to identify relevant genes that are involved (Vandesompele *et al.,* 2002). Transcriptional control is a major mechanism whereby cells or organisms regulate their gene expression. It uses sequence specific DNA-binding transcription regulators and transcription factors (Martinez, 2002), which play an essential role in modulating the rate of transcription of specific target genes. In this way, they direct the temporal and spatial gene expressions necessary for normal development and proper response to environmental or physiological stimuli. Since flavonoids biosynthesis is a biological process which entails several gene transcripts, there was no study that had been conducted to determine the effects of seasonal weather varations on flavonoids pathway genes.

Most cultivated teas in Kenya are green leafed rich in catechins suited for processing the popular black, white or green beverages (Kerio *et al.,* 2012). However, several pigmented tea cultivars rich in anthocyanin have been identified and commercialized in the world (Wei *et al.,* 2016; Lai *et al*., 2016). The different colours on the vegetative parts of the plant tissues is due to the presence of anthocyanins (Mori *et al.,* 2007; Lai *et al.,* 2016). In Kenya, a purple tea cultivar 'TRFK 306' was developed and released for processing of anthocyanin rich tea as well as extracts of the molecules (Kerio *et al*., 2012). Most plants subjected to biotic and abiotic stress also accumulates anthocyanins in their tissues (Chalker-Scott, 1999; Rani *et al*., 2012). Naturally, there are various shades of purple pigmentation in tea which may generate different amounts of anthocyanins. The leaves and stems of the tea plant accumulates large amounts of anthocyanins, during dry and hot seasons, the accumulation is inhibited affecting product quality (Kerio *et al.,* 2013). This demonstrates that seasonal variation has not been known to affect the quality of the tea products.

Tea quality and yield is influenced by various factors including developmental tissue, seasonal variation and cultivar type (Kumar *et al*., 2016). Tea cultivation in Kenya has spread to 18 counties in the eastern and western parts of the Great Rift Valley. The regions differ in climate and edaphic factors although the annual weather patterns can generally be grouped into three distinct seasons: the main dry season from mid-December to end of March; cool wet season from April to August; and a warm wet or warm dry season from September to mid-December (Ng'etich, 1995).

Understanding the impact of season and cultivar on crop quality would benefit both farmers and consumers by promoting the production of high-quality products. For the continued growth of the industry, the high quality reputation associated with Kenyan

tea needs to be maintained. However, formation on the mechanism of regulation of the quality parameters in response to seasons of harvest is unexplored. Further, a purple tea variety (cultivar TRFK 306) was recently developed for commercial utilization to target unique tea products rich in anthocyanin that would fetch premium prices for enhanced farmer livelihoods and improve consumer health (Kerio *et al*., 2012). Despite initial trails demonstrating wide adaptability of the cultivar in most tea growing regions, experiences show that the harvestable leaves of the plant loss anthocyanin pigmentation during the hot-dry season. This warrants research into the effect of environmental changes on anthocyanin loss in tea.

1.2 Statement of the problem

Tea production in Kenya is becoming unsustainable. This is due to overlying on only black tea production. Although numerous studies have reported the influence of weather variables on tea quality (Kumar *et al*., 2016), cultivar and environment interactions studies focusing on stability of quality parameters are scarce. Determining the effects of sesonal weather changes on the production of major flavonoids in tea will guide the selection of stable high quality cultivars that meet market demands. In return, consumer preference for such high-quality tea products would attract higher prices that can improve farmer's income. Further several studies have reported the molecular mechanisms underlying anthocyanin accumulation in some purple tea germplasm (Lin-Wang *et al*., 2011). However, studies on the effects in seasonal weather changes in the biosynthesis and stability of the flavonoid have not been done. During the hot-dry season, the young leaves of purple tea germplasm lose their purple pigmentation with a likely consequence of influencing the quality of the processed purple tea and bioavailability of anthocyanins.

1.3 Justification of the study

Global tea production in 2015 exceeded 5 million tonnes representing 2.1 % increase compared to 2014 with black teas representing over 65% as compared to green tea. Projections into the medium term suggest that supply and demand of black tea in the world market will be in equilibrium in 2020 at a constant price of USD 2.81 per kilogram or even lower (Kaison, 2015). This would actually be a decline as the export unit price for Kenyan tea per kilogram averaged USD 3.09 per Kilogram, in 2015 (ITC, 2016). Tea cultivation is the largest agribusiness in Kenya contributing over 26% of total foreign exchange earnings and over 4% of Gross Domestic Product (GDP). The country is the third largest producer of the commodity, supplying 22% of the world's black tea. The industry thus contributes significantly to improved rural incomes and household food security in Kenya. However, the tea is sold as a bulk raw product with little or no product diversification causing a decrease or stagnation of market prices. Japan for instance, enjoys the highest premium for their green tea exports at USD 20.89 per kg (kilogram). Domestic green tea price in China averaged USD 4.80 per kg. Interventions to expand the Kenyan tea market outlets can be driven by product diversification and differentiation. Such endeavours should be preceded by the extensive characterization of tea cultivars to identify biochemical properties that can putatively enhance their nutritional potential

1.4 Objectives

1.4.1 General Objective

To determine the effect of seasonal variations on flavonoids biosynthetic pathway processes in selected Kenyan tea cultivars.

1.4.2 Specific objectives

1. To determine the effect of seasonal variations of stomatal density on flavonoids biosynthesis in tea plant.

2. To determine the environmental stimuli that causes the accumulation of anthocyanins and catechins in tea plant.

3. To determine the candidate genes for molecular expression of the flavonoid biosynthetic pathway.

1.5 Null hypothesis

(i) Stomata density is not influenced by seasonal changes in tea plant.

(ii) The accumulation of catechins and anthocyanins in tea is not influenced by variation of environmental conditions.

(iii) Flavanoid biosynthetic genes are not differentially expressed over the different seasons.

CHAPTER TWO

LITERATURE REVIEW

2.1 Botany of the tea plant

Tea plant the most important crop belongs to the genus *Camellia,* of the family Theaceae and section Thea, has over 200 species (Chang and Bartholomew, 1984). Processed leaves of the plant are used to prepare the most popular non-alcoholic and low-cost beverage worldwide known as 'tea'. Growing wild, the tea bush is a perennial, cross-pollinated plant that can reach heights of 15 m, however under cultivation; the bushes are maintained at 60 – 100 cm for ease of harvesting (Mondal, 2004). The morphological features, biochemical and molecular properties are used to classify tea into the following varieties; Assam, China and Cambod (Wachira *et al.*, 1995). The assam variety is a tall and quick growing tree, the china variety is a dwarf and slow growing shrub, while the cambod variety is an intermediate between the two varieties (Wachira *et al*., 2001).

2.2 Tea products

There are several types of specialty tea products that include White Tea, Green Tea, Purple Tea, Yellow Tea, Black Tea, Dark Tea, Tea Extracts, Tea Oil, Organic Teas and Flavoured Teas. Processing of black tea involves oxidation of polyphenols by two endogenous enzymes polyphenol oxidases and peroxidases. This is termed as fermentation process. During this process the catechins (flavan-3ols) are partially converted into theaflavins (TFs) and thearubigins (TRs). The colour of black and Oolong teas is normally determined by these compounds (Obanda *et al*., 2004). Rupturing of the withered tea leaves using the Orthodox rollers or CTC (cut-tear-curl) machines, increases the surface area for enzymatic reaction. Thus accelerating oxidation process. For unfermented teas (green and white tea), fermentation is

prevented by inactivating the endogenous enzymes by either steaming or heating methods (Balentine,1997). The higher polyphenol contents in the assam variety has made it the most commonly cultivated tea in Kenya. These polyphenols give it the more astringency property hence suitable for black tea processing (Wachira *et al.*, 2001). The low polyphenol contents make the chinary teas popular commercial cultivar for green tea processing. Purple tea is produced from purple tea leafed cultivars rich in the highly medicinal pigment 'anthocyanin' (Lai, 2016; Wei *et al.* 2016; Zhou *et al*. 2016). Current research focuses on the development of technologies towards diversification of tea products that will lead to improved profitability.

2.3 Ecological requirements for tea plant

The quality of tea could further be influenced by variations in environmental conditions. Tea plants thrives well in a wide range of ecosystems from sea level up to 2700 m above sea level and 49ºN, 30ºS (Owuor *et al*., 2010). The conventional tea plant is evergreen perennial grown in regions with moderate temperatures, acidic soils and highly humid environmental conditions (Dufresne and Farnworth, 2001). The crop requires well distributed annual rainfall above 1200 mm, temperature range of 18-30ºC and well drained soils (Cheserek *et al*., 2015). The Kenyan tea growing areas are mostly situated in the western and eastern sites of the Great Rift Valley. The different geographical locations where tea plants are grown have varied seasonal environmental conditions. These has contributed to the variations in the levels and composition of phenolic compounds (Jayasekera *et al*., 2014).

2.4 The effect of seasons and elevation on the tea quality

Adaptation of plants to various geographical areas with variations in climate and physical features affects their rate of growth, yields and quality. Recent studies have demonstrated the major effect of environmental factors on flavonoid biosynthesis in

plant species (Carbone *et al.,* 2009; Lin-Wang *et al.,* 2011; Cohen *et al.,* 2012). Seasonal weather variation is recognized as the main source of variation in tea quality properties and thus variation in product quality. The varied geographical areas with distinct environmental conditions where the tea plant is grown has made the plant to develop various adaptation mechanisms of which has affected its quality and yields (Ahmed *et al*., 2014; Liu *et al*., 2015; Kumar *et al.*, 2016).

Early prediction of tea quality parameters is prerequisite in shortening the crop improvement programme (Owuor and Obanda, 2007). The phytonutrients or bioactive components derived from secondary metabolites determines the functional quality of tea hence consumer preference (Ahmed *et al*., 2014). Flavan-3-ols (catechins) are important in determning tea quality as the amount present in the leaf gives an indication of the potential of a cultivar to produce good quality tea (Kumar *et al*., 2016). Indeed, significant correlationship of the concentration of individual flavonols in leaves, tasters' sensory preferences and price evaluations of black teas has been observed (Obanda *et al*., 1997). Among the catechins, 3′4′5′-trihydroxylated catechins (GC, EGC, and EGCG) are most abundant flavonoids than 3′4′-dihydroxylated catechins (C, EC and ECG) in young leaves and stems (Wang *et al.,* 2014)

Geographical area of tea production influences theaflavins and thearubigins which are the quality parameters of black tea (Jayasekera *et al*., 2011). These compounds are formed from oxidized catechins (Lovedeep *et al*., 2014). TFs (theaflavins) and TRs (thearubigins) have been reported to vary with the season and elevation (Wang *et al*., 2006; Chen *et al.,* 2010; Lovedeep *et al.,* 2014). An increase in altitude/elevation leads to increased tea quality (Owuor *et al*., 2010). This is attributed mainly to slow growth rate of the shoots.

2.5 Effects of seasonal weather changes on stomatal density (SD)

Stomata are small pores, typically on the abaxial of the leaf epidermis, which are opened or closed under the control of the guard cells. These are the pivotal gates controlling the exchange of gases and water vapour in vascular plants. When open, they allow carbon IV oxide (CO_2) to enter the leaf for photosynthesis while allowing for water and free oxygen emission (Hetherington and Woodward, 2003; Taylor *et al*., 2012). The process of photosynthesis results to formation of primary metabolites which impacts directly on biosynthesis of secondary metabolites in plants (Cherotich *et al.,* 2103). The compounds malonyl- CoA and coumaroy- CoA which are the final products of secondary metabolism are precursors of the flavonoids biosynthetic pathway (Stafford, 1990). Leaf stomata per unit area is reffered to as stomatal density denoted as (S_D). It's unique to a specific plant and even among individual plants of the same species, there exists variations of stomatal density in their leaves (Al Afas *et al.,* 2006). The development of stomata on the leaves of a plant is determined by the interaction between different genes and environmental factors (Yoana, 2012). Stomatal densities are controlled by environmental conditions during leaf development (Schluter *et al*., 2003). The density of stomata progressively increases (Kofidis *et al*., 2003). Various hypotheses have explained the relationships between stomatal characteristics and elevation. Altitude affects the process of photosynthesis such that at high elevations there is reduced concentration of Carbon (IV) oxide due to decline in it's partial pressure. Therefore, plants will adjust to such climatic conditions by increasing their stomatal density in order to enhance photosynthetic capacity (Kouwenberg *et al*., 2007). Stomatal density also varies due to environmental factors such as light, air humidity, water availability and atmospheric CO_2 concentration (Woodward and Kelly 1995). An important environmental condition affecting plants is light (Pengelly *et al*., 2010). Light affects growth and development of plants by

influencing the physical appearance of a leaf as well the whole plant. Due to the fact that adaptation leads to their survival, plants can adapt to varying light intensities depending on the availability within the environment. This adaptation would be possible if the plant changes the distribution of its biomass and its morphology, for a better approach to surviving (Xue *et al.*,2011). Leaves developed under low light intensity have higher stomatal densities (Givnish 1988). The increase in stomata with increase in elevation could be associated with CO_2 assimilation and may also be as a response to low temperature stress at high altitude (Bosabalidis and Kofidis, 2002). At increased elevation, plants are exposed to lower mean temperatures and higher light intensities. Light intensity is known to affect not only plant growth and development but also the biosynthesis of both primary and secondary metabolites (Hemm *et al*. 2004; Verma and Shukla, 2015). Understanding the variation in stomatal characteristics in plants in relation to weather changes can reveal the adaptation strategies, and help predict response to future climate changes (Wang *et al.*, 2014).

During hot dry season, leaf stomata are very sensitivity to leaf water potential. Plants adjust their stomatal opening by reducing the opening in order to minimize water loss. Thus leaf water status makes plants to effectively regulate their stomata aperture and hence the stomatal density. Plants can fully open stomata and closes them when water status is unfavorable. This is an important adaptation strategy to survive during droughout (McDowell *et al*., 2008). Therefore; elevated temperatures may have effects on both stomatal size and density (Yan *et al.*, 2017). However, some previous studies have shown increased S_D during the dry season (Martinez *et al*., 2007).

2.6 Bioactive compounds of tea

Both quality and health benefits of tea are derived from the chemical constituents of the plant. Over four thousand bioactive molecules which include catechins, caffeine, gallic acid, theanine, anthocyanins, tannins, xanthines, vitamins; C, E, K, alkaloids among many others are shown in Table (i) (Xie *et al.,* 2010). The quality parameters of both green and black tea is determined by the flavonoids (polyphenols) that form a third of the total components of tea (Owuor and Obanda, 2006). The catechins (flavan-3-ol) are monomers categorised into two major groups non- gallated namely gallocatechin (GC), epigallocatechin (EGC), epicatechin (EC), catechin (C) and gallated which includes epicatechin gallate (ECG), epigallocatechin gallate (EGCG) (Kerio *et al*., 2012; Lovedeep *et al.*, 2014; Wu *et al*., 2014). Amino acids are also found in tea especially theanine which is biosynthesized from glutamic acid and ethylamine has been found to be very useful in contributing to the typical taste of green tea (Balentine *et al.,* 1998). Further, tea also accumulates inorganic elements that include Aluminum, Potassium, Fluoride and Manganese (Modder and Amarakoon, 2002.

Table (i): Tea shoot components

Component	Dry weight (g/100g)
Flavanols	
(-) - Epigallocatechin 3- gallate (EGCG)	9 -13
(-) - Epigallocatechin (EGC)	3 – 6
(-) - Epicatechin 3- gallate (ECG)	3 – 6
(-) - Epicatechin (EC)	1 – 3
Gallocatechin (GC)	1- 2
(+) - Catechin (C)	1- 2
Flavonoid and their glycosides	3 – 4
Leucoanthocyanins	2 -3
Phenolic acid : Theogallin and others	4
Total polyphenol	27 – 40
Caffeine	3 – 4
Amino acid	
Theanine	2
Others	2
Carbohydrates	4
Organic acids	0.5
Volatile compounds	0.01
Polysaccharides	4
Starch	2 – 5
Other	12
Proteins	15
Ash	5
Cellulose	7
Lignins	6
Lipids	3

Source: Modder and Amarakoon (2002)

2.7 Tea flavonoids

Flavonoids are natural products with a C6-C3-C6 carbon framework also known as phenylbenzopyran. The structure is basically a ring comprising of 15 carbons bonded to several hydroxyl groups (Figure i). Based on the position of the linkage of the

aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes: 2-phenylbenzopyrans, isoflavonoids 3-benzopyrans and the neoflavonoids 4-benzopyrans (Grotewold, 2006). The quality of beverage tea is determined by the abundance accumulation of flavan-3-ols (catechins), comprising 70 - 80% of the young tea leaf polyphenols which contribute to the sensory attributes of the beverage (Owuor and Obanda, 2007; Liu *et al.*, 2015). These are grouped based on the number of (OH) groups on B ring, stereochemistry of carbon-3 and the attachment of galloyl at the carbon three (OH) (Schijlen, 2004). Further catechins can classified into two major groups namely; dihydroxylated (ECG, EC and C) and trihydroxylated catechins (EGCG, EGC and GC) (Liu *et al*., 2012; Wang *et al.*, 2014; Wei *et al.*, 2015). This is on the basis of the number of hydroxylation at the 4′,3′ or 5′ positions in the B ring (Figure i)

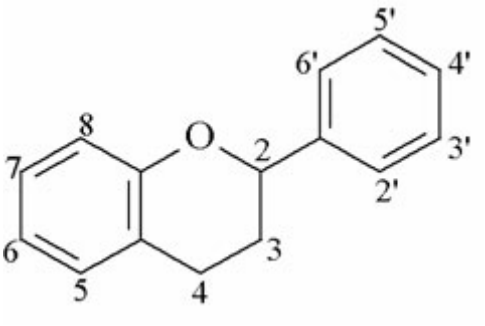


Figure (i): Flavonoids basic structure (Source: Grotewold, 2006).

2.8 Variation of catechins accumulation in plants

During the growth period of the plant, different biotic and abiotic factors affect the content of its phytochemicals (Björkman *et al.* 2011; Cui *et al*. 2013; Liu *et al.* 2015). Environmental factors such as UV irradiation, light intensity and soil moisture ((Zheng *et al.*, 2008; Cheruiyot *et al*., 2008; Hong et *al*., 2014), influences the content of various plant phytochemicals including polyphenols. During drought stress, catechin concentrations increase (Ojeda *et al*. 2002; Cheruiyot *et al*., 2008; Langat *et*

al., 2015). Green tea grown in an area with high temperatures, long sun exposure time and high rainfall have higher levels of theanine, lower levels of catechins and caffeine than those grown in areas experiencing relatively low temperatures, short sun exposure and high precipitation (Jang-Eun *et al*., 2010). The biosynthesis of the catechins in tea leaves is dependent on an amino acid called theanine (Kito *et al*., 1968). The conversion is light dependent with low light intensity translating to high accumulation of theanine and in turn lower catechins levels (Kito *et al.,* 1968). For this reason, black tea produced during the rainy season (low light intensity) have lower levels of theaflavins and thearubigins (Sud and Baru ,2000). This then implies that during the rainy season, the synthesis of major bio-constituents in the tea leaf is much affected since the plants are exposed to cloudy and rainy days (Zagoskina *et al.,* 1990). Catechins accumulation is futher affected by UV-B irradiation with short term irradiation stimulating the accumulation of major tea catechins hence increased levels of total catechins (Zheng *et al*., 2008).

2.9 Variation of anthocyanins accumulation in plants

Many studies have reported seasonal changes of environmental factors to have influence on the accumulation of anthocyanins in plant tissues (Hoch *et al.,* 2001; Steyn *et al.,* 2002). A correlationship between genetic and environmental factors shows that environmental stimuli influence the expression of both structural and regulatory genes, in turn affecting the variation in anthocyanin content. This is attributed to the fact that abiotic conditions for every season are varied (Tounekti *et al.*,2013; Martinez-Lusche*et al*.,2014; Zhang *et al*., 2014). Anthocyanins are said to be light attenuators induced in high-light conditions (Hughes *et al*., 2005; Cheynier *et al*., 2013). Light has been reported to impact on transcription factors which activate or repress the expression of structural genes that are involved in biosynthesis and accumulation of anthocyanins (Cheynier *et al*., 2013; Azuma *et al*., 2012; Jaakola,

2013). Elsewhwere, anthocyanins were accumulated under cold temperatures and repressed by higher temperatures via *myb* transcription factors (Lin-Wang *et al*., 2011). Moderate temperatures, sunlight exposure including visible and UV encourage anthocyanins accumulation (Downey *et al*., 2004; Cortell *et al*., 2007; Tarara *et al*., 2008). However, anthocyanins content may decrease under higher temperatures (Dela *et al*., 2003; Mori *et al*., 2005, 2007; Yamane *et al*., 2006). For instance, at low temperatures (18-12ºC) flavonols in tomato were highly accumulated. However, lower levels of anthocyanins were observed in grapes grown under high temperatures (30-35ºC) though flavonols accumulation was not affected (Mori *et al*., 2005; Lovdal *et al*., 2010). High temperatures alters transcript levels of activating complex hence resulting to a decrease in anthocyanin content (Lin-Wang *et al.,* 2011). Noteworthy, different sub-groups of flavonoids are differently affected by temperature depending on the plant species (Zhang *et al*., 2014). In presence of other phenolic compounds such as catechins, the enzyme polyphenol oxidase (PPO) is activated which causes rapid degradation of anthocyanins (Liu, *et al*, 2007).

Drought stress also affects the accumulation of anthocyanins. The concentration of anthocyanins and other phenolic compounds consistently increases in response to water deficits which is attributed to increased solute concentrations (Roby *et al.,* 2004). During water stress, plants accumulate solutes as an adaptation mechanism. Previous studies have shown that sugars are known to induce anthocyanins (Solfanelli *et al.,* 2006). This could be the reason for the enhanced anthocyanins content in grapevine (*Vitis vinifera* L.), which was attributed to the stimulation effect of light on gene transcript flavonoid 3-O-glucosyltransferase (UFGT) that is very sensitive to light. This enzyme is responsible for the production of endogenous sugars UDP-glucose that induces the biosynthesis of anthocyanins ((Solfanelli *et al.,* 2006).

Studies on the effects of carbon or sugars on the accumulation of phenolic compounds have been done (Koricheva *et al*., 1998). These are products of photosynthesis which form secondary metabolites in plants (Verma and Shukla, 2015).

The presence of anthocyanins in plant tissues such as flowers, fruits, leaves, and stems is developmentally controlled and affected by both biotic and abiotic factors such as nutrients (nitrogen and phosphate), sucrose, wounding, pathogen infection, methyl jasmonate, water stress, and UV, visible and far-red light (Albert *et al.,* 2009; Kerio *et al.,* 2012). Seasonal loss of anthocyanin in young leaves of the purple tea affected by environmental condition is of both economic and medicinal importance. Further, plants grown at a constant temperature of 30°C accumulate less anthocyanin than those grown at 30°C/15°C (day/night) (Mori *et al*., 2005). Plants growing at cold climates (lower temperatures), the concentration of carbon (IV)oxide is low. Thus adapt to such conditions by increasing the amount of fixed carbon and hence maintaining higher photosynthetic rates than plants growing in warmer areas. Furthermore, (Jaakola and Hohtola, 2010) reported that a combination of low temperature and light treatment induces anthocyanin biosynthesis in Arabidopsis. Similarly, anthocyanins derived from both cyanidin and delphinidin branches of the anthocyanin biosynthetic pathway in *Plantago lanceolata* were significantly increased when grown under cool-temperature conditions (15 °C 16 h day/10 °C 8 h night) compared with high-temperature (27 °C 16 h day/22 °C 8 h night). Accumulation of anthocyanins in the young tea leaves serve as a way to acclimate to low temperatures thus filtering light or a strategy to combat herbivory (Zhou *et al.,* 2016). Analysis of purple tea products identified five anthocyanin derivatives namely cyanidin, peonidin, delphinidin, petunidin, and malvidin, the latter contributing most to the final product

color (Kerio *et al*., 2012). The propositions of the individual derivatives are known to be affected by seasonal changes (Borochov-Neori *et al*., 2011).

Apart from environmental conditions, the variation of anthocyanins content could also be attributed to the various methods of storage such as refrigeration and freezing. Anthocyanins content for instance in blueberries fruits (*Vaccinium corymbosum)* differ with different storage conditions and cultivars (Connor *et al.,* 2002). This is attributed to PPO (polyphenol oxidase) enzymes and compositional factor such as chlorogenic acid (Skrede *et al*., 2000) that cause degredation. The presence of other substrates in the plant tissues can also reduce the contents of anthocyanins. For instance, phenolic compounds especially the catechins normally undergo auto-oxidation to formo-quinones which triggers the enzyme polyphenol oxidase(PPO)that degrades anthocyanins (Kader *et al*., 1998).

2.9.1 Flavonoids biosynthetic pathway

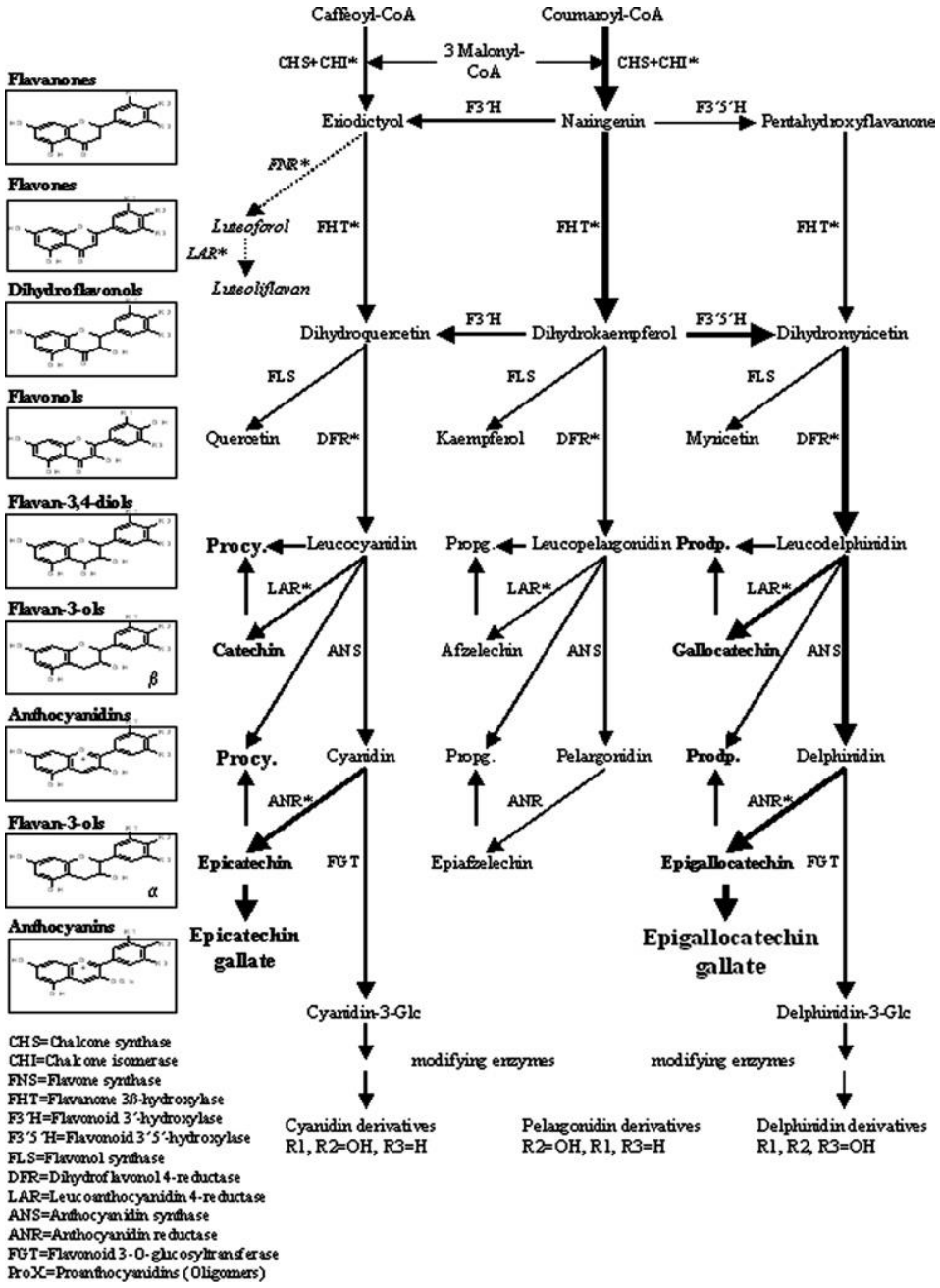
Metabolic pathways are among the various biological processes that have been studied well using the biosynthetic pathway of phenolic compounds which has become an efficient model (Winkel-Shirley 2001; Thompson *et al*., 2010). At molecular level, the biosynthesis of flavonoids is regulated via coordinated control of the structural genes; phenylalanine-ammonia-lyase (*CsPAL*), chalcone synthase (*CsCHS*), chalcone isomerase (*CsCHI*), dihydroflavanol 4-reductase (*CsDFR*), anthocyanidins synthase (*CsANS*), anthocyanidin reductase (*CsANR*) (Wu *et al*., 2014) and DNA (deoxyribonucleic acid) binding R2R3 transcription factors (Jaakola, 2013). Flavonoids biosynthesis is a process connected to many other biological pathways/cycles, such as the pentose phosphate pathway, the Calvin cycle and the Krebs cycle. Pyruvate cycle is involved in the formation of acetyl CoA which is the

key raw material for flavonoids biosynthesis. Shikimate, phenylpropanoid and flavonoid pathways are three important biosynthetic pathways that are involved in the biosynthesis of flavonoids (Tounekti *et al*., 2013; Verma and Shukla, 2015). The shikimate pathway produces chorismate, a common precursor for the tryptophan (Trp) pathway, the phenylalanine/tyrosine (Phe/Tyr) pathways, and the pathways leading to folate, phylloquinone, and salicylate (Stafford, 1990).

In the phenylpropanoid pathway, the enzyme phenylalanine ammonia-lyase (*PAL*) catalyzes deamination of the amino acid phenylalanine and converts it to coumaryl CoA an activated form of cinnamic acid (Stafford, 1990). The acetyl- CoA that originates from other biological cycles such as Krebs cycle is converted to malonyl-CoA by the enzyme acetyl -CoA carboxylase. Therefore, flavonoids are derived from condensation and decarboxylation of phenylpropanoid derivatives and malonyl-CoA units. The flavonoids pathway starts with a combination of three molecules of malonyl CoA with one molecule of coumaryl CoA in the presence of chalcone synthase (*CHS*) to form naringenin chalcone. As most plants do not accumulate chalcones, naringenin chalcone is rapidly isomerized to flavanone naringenin by the enzyme*CHI* (Lillo *et al.*, 2010). The 2S-flavanone (flavanone naringenin) is a critical intermediate for the formation of several flavonoid classes whose biosynthesis branches at this point (Ferreira *et al*., 2005). Dihydroflavonols (dihydroquercetin, dihydromyricetin) which are the precursors of anthocyanins and catechins are derivatives of flavanones.

There are two major categories of catechins namely; non-esterified catechins (C, EC, GC, EGC) and esterified catechins (ECG and EGCG). This classification is based on the presence of extra galloyl group (Graham, 1992) on carbon-3 of C - ring (Figure i).

The formation of non-esterified as well as epimerization of catechins involves reactions catalyzed by three important gene transcripts; leucoanthocyanidin 4-reductase *LAR,* anthocyanidin synthase (*ANS*) and anthocyanidin reductase *(ANR)* (Pang*et al.,* 2013; Wu *et al.,*2014; Chen*et al.,* 2017). Flavan-3-olgallate synthase (FGS) catalyzes reactions that results to formation of esterified catechins (EGCG and ECG) from respective non- esterified catechins (EGC and EC) (Ashihara *et al.,* 2010). Flavanone-3-hydroxylase (*F3H)* is an essential gene in the flavonoid biosynthetic pathway it catalyzes reactions involving hydroxylation of (2S)-naringenin and (2S)-eriodictyol to form (2R, 3R)-dihydrokaempferol and (2R, 3R)-dihydroquercetin respectively. Dihydroquercetin and dihydrokaempferol are then hydroxylated at the 3′ position or at both the 3′ and 5′ positions in the B ring (Toda *et al.,*2002). Hydroxylation is catalyzed by the enzymes flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) leading to the formation of dihydroxylated catechins(cyanidin) and (delphinidin) trihydroxylated catechins (Wei *et al.,* 2015). Thus the accumulation of dihydroxylated and trihydroxylated have some correlationship with the expression of *F3′H* and F3′5′H (Castellarin *et al.*2006). Dihydroflavonol 4-reductase (*DFR*) is another important gene in the flavonoids biosynthetic pathway. It reduces dihydroflavonols resulting to the formation of leucoanthocyanidins that give rise to various types of anthocyanins and catechins (Xie *et al*., 2004). The schematic flow diagram of flavonoids biosynthetic pathway processes is shown below (Figure ii).



Figure(ii): Biosynthesis of flavonoids in the tea leaf

(Source: Punyasiri *et al.* 2004).

2.9.2 Influence of environmental cues on Flavonoid biosynthetic pathway genes

Flavonoids in higher plants have evolved to a diverse array according to wide variety of physiological and ecological functions which are required to adapt to various growing conditions. Flavonoids play important roles in protecting plants from UV radiation, insects and pathogen attack by acting as insecticides/phytoalexins (Dixon

and Steele, 1999; Zoratti *et al*., 2014; Margaria *et al*., 2014) and providing pigments to attract animal pollinators via pigmentation of floral organs (Shang *et al*., 2011). In essence, flavonoids are vital compounds that are produced during abiotic and biotic stress which range from high-light/UV stress, cold stress, nutritional defense and pathogen attack (Winkel-Shirley *et al*., 2001; Taylor and Grotewold, 2005), low temperature and drought (Lovdal *et al.*, 2010; Martinez-Luscher *et al*., 2014). Several studies have shown that biosynthesis of flavonoids are genetically and environmentally controlled (Cheynier *et al*., 2013). The influence of environmental cues on the key genes of flavonoids biosynthetic pathway have been documented (Rani *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant materials and experimental sites

Five mature tea cultivars of over twenty years growing at Tea Research Institute (TRI), Timbilil Center (altitude 2180 m asl; 0°22´S 35°21´E), Kericho County in the west of the great Rift Valley, and Kangaita Center (altitude 2100 m asl; 0°30´S 37°16´S), Kirinyaga County in the east of the great Rift Valley, Kenya were chosen for the study in 2014. The selected cultivars comprised: (Tea Research Foundation of Kenya) TRFK 31/8, a high yielding tea cultivar with green leaves commonly used for black tea processing; TRFK 6/8, a green leafed cultivar used to process high quality black tea; TRFK 306, a purple leafed cultivar used to process anthocyanin rich purple tea; TRFK 91/1 a "wild" tea (*Camellia irrawadiensis*) with brick red pigmented leaves; and NRIT St. 536 (cultivar. Yabukita) the most popular green tea cultivar in Japan.

a) b)

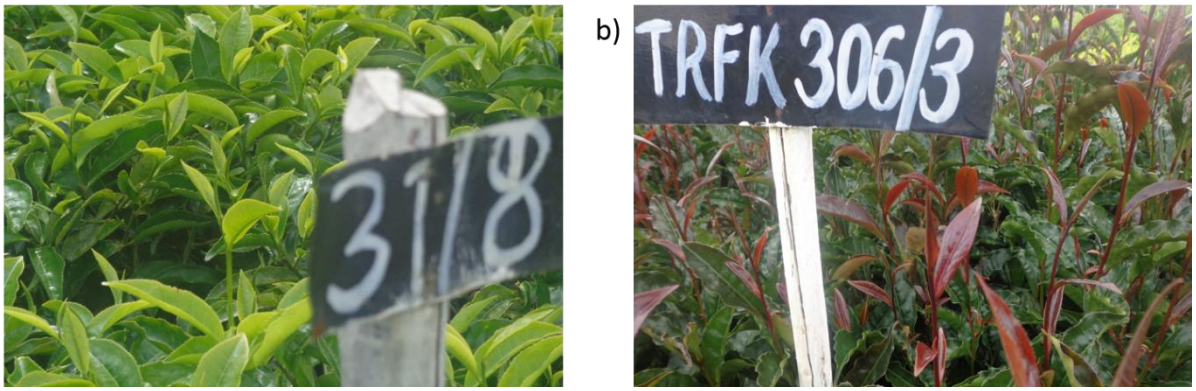


Plate (i): Photographs of the green and purple tea cultivars (Source: Author, 2014)

3.2 Experimental design

The trial plots were established in 1998 and 1999 at Timbilil and Kangaita TRI Centers, respectively for research purposes. They were laid at a spacing of 1.22m x 0.61m, with three replicates in a randomized complete block design (RCBD) and have since been maintained as per recommended agronomic practices (Anon. 2002). The following environmental data; precipitation (mm) and relative humidity (%), maximum and minimum temperatures (^{0}c) were collected daily throughout the study in weather stations situated within the two trial plots (Appendix xvii & xviii).

3.3 Stomatal density (SD)

The number of stomata per unit leaf area (stomatal density) was determined using the leaf imprints method (Radoglou and Jarvis, 1990; Coupe, 2006). Fully expanded leaves (3rd position leaf from the apical bud) were selected from the five cultivars during three different seasons (March, July, November representing the dry-hot, wet-cold, and wet-warm seasons, respectively). A thin layer of clear nail polish was applied onto the abaxial/lower epidermal surface of the middle section of the leaf, allowed to dry for 10 minutes, then a clear cellophane tape spread over the polish patch. The tape was gently pulled off thereby peeling the nail polish patch having the leaf imprints. This was then mounted on a clean microscope slide and examined under a Light Ortholux Microscope (Leitz Wetzlar, Germany) (Phaco 2, Mg ×25). Stomatal counts were taken from areas of three leaves from three separate plants of each genotype and measurements converted using conversion area factor of 0.114 mm^2. It was calculated from a calibrated graticule in the eye piece (Ng'etich and Wachira, 2003).

3.4 Biochemical analysis

3.4.1 Sample collection

Three leaves plus apical bud were harvested for three seasons of the year 2014. They were immediately frozen in liquid nitrogen and transported to the laboratory for storage at -80°C (U440 premium, Ultra-low freezer, New Brunswick Scientific, USA) until use.

3.4.2 Catechins analysis

3.4.2.1 Sample extraction

Leaf sample (0.2g) was crushed to fine powder in liquid nitrogen using a mortar and pestle. The powder was placed into 15 ml extraction tube and 5 ml preheated 70% methanol added. The tube was briefly vortexed using a vortex mixer (VM-1000, Taiwan) and heated at 70°c for 10 min with occasional shaking. The sample was left to cool to room temperature for 5 min then centrifuged (Eppendorf centrifuge, 5430R) at 3500 rotations per minute for 10 min. Supernatant was decanted into a clean extraction tube and the process repeated of adding preheated methanol until 10 ml of the combined extract was obtained.

3.4.2.2 Determination of catechins content

Analysis of catechins by HPLC (High Performance Liquid Chromatography) was done according to the ISO 14502-2-2005E procedure. Sample extract (1ml) was pipetted into separate tubes and diluted with 4 ml stabilizing solution (10% acetonitrile with 500µl/ml of EDTA and 500µl/ml ascorbic acid), the filterate was pipetted into 2ml vials and analysis was done using reverse phase HPLC. The HPLC

machine of the model (A Shimadzu L 20 AT HPLC) consisted of autosampler (SIL 20A) and a visible detector (SPD-20 UV) with a class LC10 chromatography workstation. Detection was done with UV at 278 nm using two types of columns at temperature (35°c) namely; a Gemini C18 ODS (4.0mm x 4.6 mm i.d.) column (Phenomenex Inc. Torrance CA, USA) and (4.0 mm x 3.0 mm i.d.) GeminiC6 ODS guard column (Phenomenex Inc. Torrance CA, USA). The injection volume of the mobile phase was 20µl flowing at a rate of 1ml per min. Elution involved binary gradient conditions of two mobile phases. Mobile phase A (100%) was run for 10 min then 68% for 15 min and then mobile phase B (32%) was run for10 min. Before the next injection, the column was reset to 100% with mobile phase A.

Catechins fracions were identified by comparing the retention time from sample chromatograms and absorbance of unknown peaks with those identified from peaks obtained from the mixed catechins standards under similar conditions. The reference standards (Gallic acid (GA), epigallocatechin (EGC), (+) catechin, epicatechin (EC), epigallocatechin gallate (EGCG), caffeine and epicatechingallate (ECG)) were purchased from Sigma–Aldrich (St Louis, MI, USA). Quantification of catechins was performed at 278 nm (UV- 1800, Shimadzu spectrophotometer) using caffeine external standard with a calibration curve of $R^2 = 0.9984$ together with the consensus individual catechin Relative Response Factors (RRFs) measured with respect to caffeine, calculated on dry matter basis. The total catechins content was expressed as a percentage by mass on a sample dry matter basis and given as a summation of individual catechins as:

% Total catechins = (%EGC) + (% +C) + (%EC) + (%EGCG) + (%ECG)

3.4.3 Anthocyanin analysis

3.4.3.1 Sample extraction and purification

Total monomeric anthocyanin was extracted and purified as described by Kerio *et al.* (2012). Two cultivars namely TRFK 306/3 and TRFK 91/1 were used for the analysis.

Leaf sample of (5g) was crushed to fine powder in liquid nitrogen using a mortar and pestle. The sample was placed into a 250 ml conical flask, mixed with 50 ml MeOH/ HCl (99:1 v/v) and covered with aluminum foil. Stir the mixture magnetically at 900 rpm for 4 hrs at room temperature. The resultant solution was filtered using a piece of cotton wool. The filtrate was evaporated to dryness using a Rotavapour (Buchi Rotavapour R- 300, Switzerland) at temperature 35°c under reduced pressure. The extract was then reconstituted by dissolving into 10 ml of distilled water. The resultant solution was filtered using (0.45 µM) membrane filter in a Buchner funnel and then placed on ice. Purification of the extract was done by passing it through reverse phase (RP) C18 Solid Phase Extraction cartridges (SPE, SUPELCO, Sigma-Aldrich, USA) previously activated with 10% MeOH/HCl. Acidified water with (0.01%) HCl was used to remove other phenolic and water-soluble compounds. Acidified methanol (10% formic acid/MeOH v/v) was used to recover anthocyanins that was adsorbed into the column. The cartridges were then activated by washing with ethyl acetate (Fischer Scientific, UK) to remove any other impurities adsorbed into the column other than anthocyanins. Store the purified extracts at - 10 °C till further analysis.

3.4.3.2 Determination of total monomeric anthocyanin content

Total monomeric anthocyanin content was determined in triplicate using pH differential method (Giusti and Wrolstad, 2001) using two buffer system; 0.025M potassium chloride (KCl) buffer at pH1.0 and 0.4M sodium acetate ($NaC_2H_3O_2$) buffer at pH 4.5. Anthocyanin sample (200µl) was mixed with 1.8 ml potassium chloride buffer while another similar sample was mixed with sodium acetate buffer. Absorbance for both mixtures was determined at 520nm and 700nm. The analysis was replicated three times and pigment concentration calculated using the formula:

a = (a 520nm - a 700nm) at pH 1.0 - (a 520 nm - a 700nm) at pH 4.5

This gave the difference in absorbance of the sample in the two pH ranges. Monomeric anthocyanin pigment concentration in the original sample was derived using the formula

Monomeric anthocyanin pigment concentration (mg/l) = a x mw x df x 1000/ (ε×1)

Where a - absorbance (absorbance difference in two pH ranges); mw - molecular weight of cyanidin (449.2); df - dilution factor; ε- cyanidin - 3 - glucoside molar absorbance (26,900).

3.5 Molecular analysis

The transcript levels of anthocyanin biosynthetic genes in a pigmented tea cultivar TRFK 306 and a green tea cultivar TRFK 31/8 over the three seasons were determined as described by Muoki *et al.* (2012).

3.5.1 Isolation of total RNA

Total RNA was extracted from the frozen leaves as described below:

Using a mortar and pestle,100 mg of the leaf tissue was homogenized to fine powder in liquid nitrogen. Measure 1ml of the pre-heated (65°C) extraction buffer I (Appendix xiii) into 2 ml microfuge tubes, then powdered sample was added into it. The sample was vortexed for 1 min and then incubated for 15 min at 65°C, mixed by occasional shaking. Then 1 ml of CIA (chloro isoamyl alcohol) was added to each tube and vortexed for 5 secs. The mixture was centrifuged at 13,000 rotations per minute for 10 minutes at room temperature. Thereafter supernatant was transfered into a clean 2ml microfuge tube and then addition of CIA was repeated. To the aqueous phase obtained in a clean microfuge tube, 1ml extraction buffer II (Appendix xiv) was added. The mixture was vortexed for 1 min. Then 200µl chloroform was added and vortexed for 5 secs and left for 10 minutes at room temperature. The tube was centrifuged at 13,000 rotations per minute for 10 min at 4°c. Upper aqueous phase was transferred into fresh tube (contamination with interphase was avoided). Isopropanol 0.6 volumes was added and the tube vortexed for 10 secs. The mixture was allowed to cool at room temperature for 10 min, after which it was centrifuged at 13,000 rotations per minute at temperature (4°c) for 10 min the supernatant decanted. Ethanol (70%) was used to washed RNA pellet that was formed and then air dried. Appropriate volume of diethylpyrocarbonate (DEPC)-treated autoclaved water was used to dissolve the pellet. The aqueous solution was then stored at -80°C awaiting further analysis.

3.5.2 RNA quantification

RNA was adequately diluted with DEPC-treated autoclaved water. NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, USA) was used to determine the purity and concentration of the isolated RNA. This involved determining the absorbance ratios at $A_{260/280}$ and $A_{260/230}$. Concentration, yield and purity were calculated using the following formulae:

The absorbance at 260 nm corresponds to 40 μg/ml of RNA.

Concentration of RNA (μg/ml) = A_{260} x 40 x dilution factor

Total yield (μg) = concentration x volume of stock RNA sample (ml)

RNA purity: A_{260}/A_{280} ratio

Ratio of 1.8 to 2.0 indicated pure RNA. Higher ratio represented contamination by phenol, whereas lower ratio showed polysaccharide contamination.

3.5.3 Concentrating RNA

RNA was precipitated by the addition of 4.0 volume chilled ethanol in the presence of 0.1 volume 3 M sodium acetate (pH 4.8; Appendix 15) followed by incubation at -70ºC for 3 hr. RNA was pelleted by centrifugation at 12,000 rpm for 10 min at 4ºC, rinsed with 70% chilled ethanol and finally dissolved in DEPC-treated autoclaved water. Quantification of RNA was as described in section (3.5.2).

3.5.4 Evaluation of RNA integrity

To check the integrity of RNA, approximately 2 μg of RNA was diluted with 6X RNA loading dye and incubated for 15 min at 65ºC. RNA was loaded onto 1% formaldehyde agarose-gel (preparation of 6X RNA loading dye and formaldehyde agarose-gel (Appendix xii) and electrophoresed at 70V in 1X MOPS buffer. The gel was viewed under UV transilluminator (Benchtop Variable Transilluminator,

Cambridge, UK) and captured on a gel documentation system (UVP PhotoDoc-It^{TM} Imaging Systems, Upland CA, USA).

3.5.5 First-strand cDNA synthesis

Complementary DNA (cDNA) was synthesized using total RNA (2 µg) from various treatments (section 3.5.2),1 µg of(dT) deoxynucleoside thymine (Invitrogen, USA) and 400 U of RT (Superscript III; Invitrogen, USA) after digesting with DNase I (amplification grade, Invitrogen, USA) as per manufacturer's instructions. The procedure followed is described below:

3.5.5.1 Removal of genomic DNA

The following components were added to RNase-free 0.2 ml PCR tube on ice:

Component	Volume per reaction
RNA sample (1 µg)	X
10 X DNase I reaction buffer*	1.0
DNase I (Amplification grade, Invitrogen; 1	1.0
DEPC treated autoclaved water	Y
Total	10.0

* Supplied by the manufacturer

The reaction mixture was incubated at 37°C for 30 min in a thermal cycler.

The reaction was stopped by the addition of 1 µl of 25 mM EDTA solution and the reaction was incubated at 65°C for 10 min in a thermal cycler.

3.5.5.2 First-strand cDNA synthesis using Superscript III (Invitrogen, USA)

The following components were added to RNase-free 0.2 ml PCR tube:

Component	Volume per reaction
Oligo (dT) $_{12-18}$ (500 µg/ ml;	1
dNTP mix (10 mM)*	1
DNA-free RNAfrom section 3.7.1.1	11

* Supplied by the manufacturer

The reaction was incubated at 65°C for 5 min and placed on ice for 2 min which served to remove RNA secondary structure. To the tube the following were added:

Component	Volume per reaction
5X First strand buffer*	4
100 mM DTT*	2
Superscript III (200 U)*	1

* Supplied by the manufacturer

The content was mixed, centrifuged for 10 secs, incubated at 42°C for 60 min and the reaction terminated by heating the samples at 70°C for 15 min. The reaction was stored at -80°C until further use.

3.5.6 Quantitative RT-PCR analyses

Expression analysis for selected genes was conducted to quantify the transcript levels of 8 *Camellia sinensis* representative genes involved in flavonoid biosynthesis. The genes were retrieved from NCBI database and comprised of eight genes (Table ii). Forward and reverse primers were designed using Primer 3 software (Rozen and Skaletsky, 2000). Quantitative RT-PCR was performed on a 7500 Real Time PCR System (Applied Biosystems, USA) using SYBR Green PCR Master mix (Applied Biosystems, UK). All qPCRs were run in triplicates with a no-template control to check for contamination.

Component	Volume per reaction
Sterile water	1.5
2x Brilliant III SYBR*	5.0
Forward primer (10 uM)	0.5
Reverse primer (10	0.5
cDNA (10X dilution)	2.5
Total	10.0

* Supplied by the manufacturer

Table (ii): Primer sequences of flavonoid pathway genes

S/N	Gene	Acc. No.	Reference	Primer	Sequence
1	*CsPAL*	BAA05643	Lepelley *et al*, 2012	Forward	5'-ATGAATAAAGGGACAGATAGTTATG-3'
				Reverse	5'-ATGTAAGATAGGGGGACTAGGT-3'
2	*CsCHI*	DQ904329	Lai *et al*, 2016	Forward	5'-CTTTCCGATTAAGTTGGATGAT-3'
				Reverse	5'-GTTGGAAAACACCATTACCAAT-3'
3	*CsF3H*	AAT68774	Pang *et al.* 2013	Forward	5'-GAAATAGTGACCTACTTCTCATACC-3'
				Reverse	5'-GTAGAAATTTATAACCACCTTCTGG-3'
4	*CsF3',5'H*	AAY23287	Wei *et al.* 2015	Forward	5'-TAAAATATACGGACCCATAGTCTAC-3'
				Reverse	5'-TTACTTAGTTTCCTCAGTAACTTCC-3'
5	*CsDFR*	AAT66505	Pang *et al.* 2013	Forward	5'-ACACCTATGGATTTTGAGTCTAAG-3'
				Reverse	5'-AGTCATCTTCTTCTTATTGATGAAA-3'
6	*CsANS*	AY830416	Lai *et al*., 2016	Forward	5'-AATTTGAATGGGAAGACTATTTT-3'
				Reverse	5'-CAACTTCTTTCTCTAGTTTGTTTTC-3'
7	*CsANR*	GU992402	Pang *et al.*, 2013	Forward	5'-TAATAAGAAAAACATCTCTCACCTC-3'
				Reverse	5'-ATCAAGCTGATTAATCGTTACAG-3'
8	*CsCHS*	AAT75302	Wang *et al*., 2009	Forward	5'-GATTACTAATAGCGAGCATAAGGT-3'
				Reverse	5'-CCATTCTTTATTGCCTTAGTTG-3'
9	*CsActin*	-	Muoki *et al*, 2012	Forward	5'-GCCATATTTGATTGGAATGG-3'
				Reverse	5'-GGTGCCACAACGTTGATGTT-3'

CsPAL (phenylalanine ammonia lyase), CsCHS (chalcone synthase, CsCHI (chalcone isomerase), CsF3H (flavanone 3-hydroxylase), Cs F3'5'H (flavonoid 3',5'-hydroxylase), CsDFR (dihydroflavonol 4-reductase), CsANS (anthocyanidin synthase), CsANR (anthocyanidin reductase).

3.6 Data analysis

Replicated means obtained for stomatal density, catechins, and anthocyanin contents were statistically analyzed using general statistics (GENSTAT) software. Analysis of variance (ANOVA) was performed in two factor completely randomized block design and Duncan multiple range test at ($p<0.05$) was used to separate the means. For gene expression studies, the raw threshold cycle (C_t) values were normalized against a housekeeping gene encoding *CsActin* (Muoki *et al.*, 2012) and the values used to calculate the differences in expression using the Relative Expression Software Tool (REST; Pfaffl *et al.*, 2002).

CHAPTER FOUR

RESULTS

4.1 Seasonal variation in stomatal density (mm^2)

There was significant difference ($P<0.05$) in stomatal density (S_D) among the studied cultivars and all the interactions (Appendix i). Generally, the purple leafed cultivars had higher stomatal density ($S_{D)}$ when compared to green tea cultivars (Table iii). Further, assam varieties comprising purple-leafed cultivars TRFK 91/1and TRFK 306 and green-leafed TRFK 31/8 had higher S_D as compared with the china/assam hybrid TRFK 6/8 and the chinary NRIT St.536 (Table iii). Cultivar TRFK 91/1 had the highest S_D of 254 mm^2, while TRFK 6/8 had the lowest S_D of 231mm^2 (Table iii). Stomatal density was decreased from the cool-wet season (251 mm^2), warm-wet (248 mm^2) to (235 mm^2) in the hot-dry season.

Table (iii): Seasonal variation in stomatal density (mm^2) among tea cultivars

Cultivar	Season	Site		Season means
		Kangaita	Timbilil	
TRFK 306	H-D	240	226	233
	C-W	265	215	240
	W-W	281	283	282
TRFK 91/1	H-D	239	294	267
	C-W	265	211	238
	W-W	289	224	256
TRFK 31/8	H-D	232	231	231
	C-W	284	351	318
	W-W	224	198	211
Cultivar means		*246*	*260*	*253*
TRFK 6/8	H-D	189	211	200
	C-W	216	290	253
	W-W	243	239	241
Cultivar means		*216*	*247*	*231*
NRIT St. 536	H-D	194	294	244
	C-W	206	208	207
	W-W	290	205	247
Cultivar means		*230*	*235*	*233*
Site/season means		244	245	
	H-D			235
	C-W			251
	W-W			248
LSD ($P \leq 0.05$)	Cultivar (C)			0.035
	Season (Ss)			0.087
	Site (St)			0.794
	Interactions (CSs)			0.001
	Interactions (CSt)			0.039
	Interactions (SsSt)			0.001
	Interactions (CSsSt)			0.001

NB: H-D, hot-dry season; C-W, cold-wet season; W-W, warm-wet season

4.3 Seasonal variations in catechins accumulation

Leaf accumulation of flavonoids were lowered in the main dry season from January to March when high temperatures variations, low relative humidity and precipitation were experienced, but increased remarkedly as differences in temperature variations decreased while precipitation and relative humidity increased over the cool-wet season from April to August and warm-wet season from September to December. Total catechins (TC) were higher in green-leafed cultivars compared to colored/purple leafed teas. Cultivar TRFK 6/8, a standard quality cultivar in breeding at the institute (TRI), recorded the highest amount of total catechins at 18.0% in the warm-wet season at Timbilil (Table iv). Results showed that EGCG (epigallocatechin gallate) was the most predominant catechin component, while GA (gallic acid) was the least (Table iv). During the warm-wet season, higher levels of esterified catechins (ECG and EGCG) were recorded as compared with the other two seasons of the year. The catechins formed a major component accounting for over 70% of total catechins in all the tea cultivars. EGCG content was significantly higher during the warm-wet season in Timbilil, while in Kangaita, the cool-wet season showed slightly higher EGCG content (Table iv). Overally, cultivar TRFK 31/8 recorded the highest of EGCG while TRFK 306 recorded the lowest amount with Timbilil site exhibiting significantly higher content compared to Kangaita (Appendix iii). Simple/non-esterified catechin (+C, GC, EGC and EC) varied significantly across seasons exhibiting significant interaction between seasons and cultivars (Appendix vii). Higher accumulation was observed in the warm-wet season when pigmented teas (TRFK 306 and TRFK 91/1) had the higher content compared to the green-leafed cultivars (TRFK 31/8, NRIT St. 536 and TRFK 6/8) (Table iv). Both cultivars TRFK 306 and TRFK 6/8 had significantly higher levels of EC accumulated mostly during

warm-wet and cool-wet season (Table iv) and (Appendix vi). All green-leafed cultivars had significantly higher EGC content during the warm-wet season (Table iv) and (Appendix iv). Pigmented cultivar TRFK 91/1 on the other hand recorded the lowest EGC content across sites. No significant differences were identified among cultivars, seasons and sites for ECG catechin (Appendix v). For gallic acid (GA), there was significant ($P<0.05$) variation ((Appendix viii) among the cultivars, season, sites, interactions (cultivar and season, cultivar and site). Among the cultivars, GA was significantly higher in the purple-leafed cultivars (TRFK 91/1 and TRFK 306) and lowest in the green-leafed teas (Table iv). The warm-wet season showed higher accumulation of gallic acid.

4.4 Seasonal variations in caffeine accumulation

Caffeine, a primary secondary metabolite responsible for the stimulating effect and bitter taste properties of tea, showed significant ($P<0.05$) variation across cultivars, seasons, sites and interaction between cultivar and site (Appendix ix). Based on the overall cultivars means, green tea cultivars (31/8 and 6/8) had the highest caffeine content in Timbilil and Kangaita respectively. While the cultivar TRFK 91/1 showed lowest caffeine content across the two sites. Overall, the cultivar TRFK 31/8 recorded maximum content with Timbilil recording highest caffeine accumulation. Unlike catechins, caffeine content was significant high during the hot-dry season (Table iv).

Table (iv): % of different quality components of purple and green tea

		TC		EGCG		EGC		ECG		EC		C		GA		CAFF	
Cultivar	Season	Timb	Kang	Timb	Kang	Timb	Kang	Timb	Kang	Timb	Kang	Timb	Kang	Timb	Kang	Timb	Kang
Purple tea																	
TRFK 306	H-D	8.0	8.9	2.6	2.1	1.7	2.0	2.4	2.4	1.1	2.2	0.1	0.2	0.5	0.3	2.3	1.9
	C-W	9.1	9.3	3.7	2.2	1.5	2.5	1.5	1.6	2.0	2.6	0.4	0.3	0.3	0.4	1.9	1.3
	W-W	12.3	13.3	3.8	3.6	1.9	3.0	4.4	2.1	1.2	2.4	1.0	2.1	0.6	0.5	1.6	2.0
TRFK 91/1	H-D	7.8	4.9	3.7	1.4	1.8	1.4	1.6	1.2	0.4	0.6	0.4	0.3	0.8	0.5	0.1	1.1
	C-W	11.5	7.4	6.0	2.8	1.6	1.7	2.0	1.1	1.7	1.2	0.2	0.6	0.5	0.2	1.8	1.0
	W-W	12.2	9.7	5.9	1.9	1.5	2.3	2.8	2.5	1.0	1.9	0.9	1.1	1.1	0.6	0.2	0.2
Green tea																	
TRFK 31/8	H-D	11.8	10.0	5.8	4.6	3.4	2.5	1.6	1.6	0.8	1.2	0.2	0.2	0.4	0.2	3.5	1.8
	C-W	14.3	9.6	5.6	3.4	3.8	2.8	3.0	1.8	1.1	1.2	0.8	0.5	0.3	0.2	2.3	1.6
	W-W	14.7	12.6	5.8	5.3	6.0	4.9	1.6	1.0	1.0	0.8	0.2	0.5	0.2	0.4	2.5	2.0
TRFK 6/8	H-D	8.6	12.0	3.1	3.1	3.0	5.5	1.1	1.5	1.0	1.3	0.4	0.7	0.2	0.2	1.8	2.6
	C-W	9.4	14.7	2.6	3.9	2.5	6.5	1.6	1.3	2.3	2.6	0.4	0.4	0.1	0.2	1.2	1.5
	W-W	18.0	12.9	5.5	3.3	8.0	5.5	1.7	1.4	2.2	2.1	0.6	0.6	0.3	0.4	2.1	1.4
NRIT St. 536	H-D	8.4	10.7	2.1	5.2	3.0	2.6	2.3	1.8	0.7	0.9	0.3	0.2	0.2	0.3	2.6	2.0
	C-W	14.6	9.0	5.8	3.2	3.8	2.9	2.2	1.6	2.3	0.9	0.5	0.4	0.3	0.5	2.3	1.3
	W-W	16.4	13.0	6.7	5.3	6.3	4.0	1.6	1.7	1.6	1.6	0.3	0.4	0.2	0.2	2.1	1.5
LSD (P≤0.05)	Cultivar(C)	0.007		0.038		0.001		0.122		0.001		0.240		0.001		0.001	
	Season (Ss)	0.001		0.064		0.001		0.356		0.001		0.002		0.011		0.032	
	Site (St)	0.069		0.016		0.969		0.053		0.188		0.288		0.207		0.012	
	Interaction (CSs)	0.963		0.895		0.412		0.109		0.410		0.014		0.015		0.058	
	Interaction (CSt)	0.144		0.237		0.054		0.899		0.066		0.692		0.015		0.035	
	Interaction (SsSt)	0.219		0.318		0.156		0.519		0.178		0.363		0.604		0.471	
	Interaction (CSsSt)	0.217		0.560		0.100		0.688		0.660		0.726		0.838		0.093	

TC, Total catechins; EGCG, (-) epigallocatechin gallate; ECG, (-) epicatechin gallate; EGC, (-) epigallocatechin; EC, (-) epicatechin; (+) catechin; GA, gallic acid; CAFF, caffeine; Timb, Timbilil; Kang, Kangaita; H-D, hot-dry season; C-W, cold-wet season; W-W, warm-wet season

4.6 Seasonal changes in anthocyanin concentration

In the hot-dry season, commercial cultivar TRFK 306 accumulated anthocyanin at 120.9 mg/l in Timbilil, while in the warm-wet season it showed a threefold higher accumulation to reach over 384 mg/l (Figure iii). The low rate of anthocyanin accumulation resulted in most leaves of the purple plant appearing green, while some showed low pigmentation.

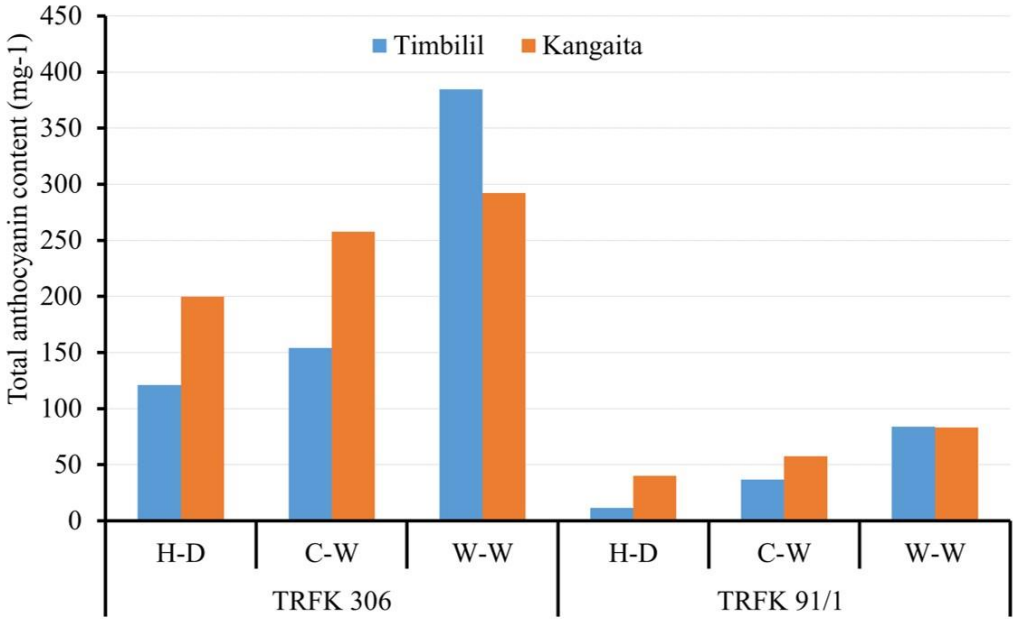


Figure (iii): Seasonal variation of total monomeric anthocyanins. H-D, hot-dry season; C-W, cool-wet season; W-W, warm-wet season.

4.7 Gene expression patterns

The variations in expression patterns of selected flavonoid pathway gene transcripts Cs*PAL, CsCHS, CsCHI, CsF3H, CsF3'5'H, CsDFR, CsANS* and Cs*ANR* were examined in leaves of green (TRFK 31/8) and purple (TRFK 306) tea cultivars over the three annual seasons (Figure iv). Except for *CsCHI* which was down-regulated, many of the flavonoid biosynthetic pathway genes from the green-leafed cultivar were slightly upregulated during the cold-wet season (Figure iv). In the same green tea

cultivar during the warm-wet season, *CsF3H* was (≥ 2 fold) down-regulated. Comparatively, the purple-leafed cultivar presented up-regulation of *CsCHI* and down regulation of *CsCHS* in all the three seasons, down-regulation of *CsPAL* in two seasons the (cold-wet and warm-wet) and down-regulation of F3'5'H in only hot-dry season when compared to the green-leafed cultivar (Appendix xi). However, genes that were not affected by the seasonal weather variation did not show expressions hence are not represented by the graphs.

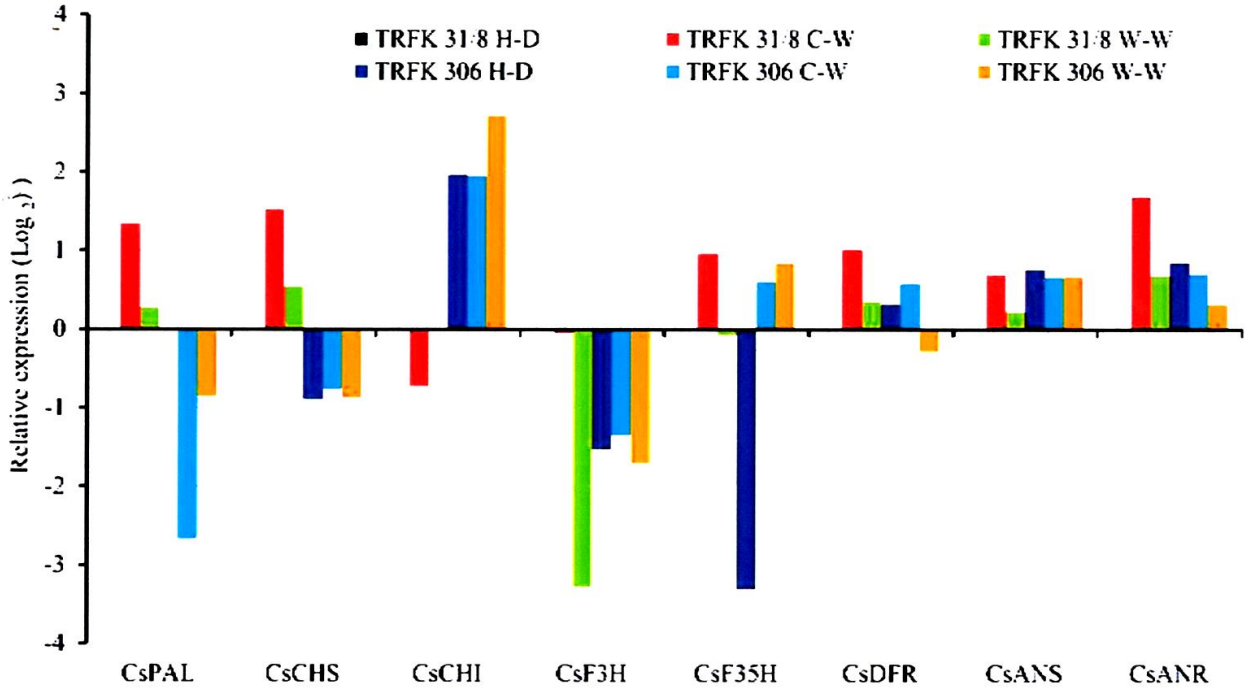


Figure (iv): **Relative expression profiles of the flavonoid biosynthetic genes** *from purple- (TRFK 306) and green- (TRFK 31/8) leafed tea cultivars over different seasons of the year 2014. H-D, hot-dry season; C-W, cold-wet season; W-W, warm-wet season; CsPAL(phenylalanine-ammonia-lyase), CsCHS(chalcone synthase), CsCHI(chalcone isomerase), CsF3H(flavanone 3-hydroxylase), Cs F3'5'H(flavonoid 3',5'-hydroxylase), CsDF(dihydroflavanol 4-reductase), CsANS(anthocyanidins synthase), CsANR(anthocyanidin reductase).*

CHAPTER FIVE

DISCUSSION

5.1 Seasonal variation of leaf stomatal density

From the current study, considerable genotypic differences were found in stomatal density of the five tea cultivars. Assamica varieties with broad leaves had higher stomatal density than the narrow leafed Chinary varieties. This suggested that the variations depend on plant species/varieties. This had earlier been reported by (Al Afas *et al.,* 2006) stomatal density is unique to a specific plant and even among individual plants of the same species. Thus the difference in stomatal density among the studied tea cultivars could be attributed to the difference in morphological; foliar, floral and growth features (Wachira *et al.,* 1995).

The results from the study indicated higher stomatal density during the cool-wet season compared with the warm-wet and hot-dry seasons respectively. It could be due to the varied environmental conditions. Normally every season has its distinct day length, rainfall, sunlight and temperature (Tounekti *et al.,* 2013). Generally, the cool-wet season is a rainy season the tea plants were exposed to cloudy and rainy days (Zagoskina *et al.,* 1990). They were subjected to low CO_2 concentration, high humidity and low light intensity (Givnish 1988). The combination of all these factors exerts pressure on plants, which becomes expressed as changes in their leaf morphology (Koffidis, *el* al., 2003). Therefore, the tea cultivars increased their leaf stomatal density so as to adapt to these enviromental conditions which are associated with the cold-wet and warm-wet seasons. Thus such changes that occurs in the leaf morphology enhances photosynthetic capacity of the plant by improving CO_2 and

light absorption (Delagrange *et al*., 2006; Peri *et al*., 2007; Valladares and Niinemets, 2008).

The results of this study also demonstrated that the tea cultivars had low stomatal density during the hot-dry season. The high temperatures cause water stress in plants by increasing soil evaporation which leads to high leaf transpiration. In such case plants adjust their number of stomata (stomatal density) in order to minimize excessive water loss (Hepworth *et al.,* 2015). Thus both stomatal size and density may be affected by variation of temperature (Yan *et al.,* 2017). Plants can effectively regulate their stomata aperture according to leaf water status. They open stomata when water status is well and close them when water status is unfavorable such a strategy is useful as an adaptation to drought (McDowell *et al.,* 2008). Therefore, tea cultivars employed this mechanism of reducing stomatal density in order to survive during water deficit. However, some studies have shown negative correlation (Yang and Wang, 2001; Martinez *et al*., 2007). From the current study there was no significant variation of stomata density between the two study sites. Despite the fact that they were at different geographical locations, Timbilil Center (altitude 2180 m above sea level; 0°22´S 35°21´E) and Kangaita Center (altitude 2100 m above sea level; 0°30´S 37°16´S). This indicates that the environmental conditions between the two sites were moreless the same. In overall the results of the present study showed that although the development of the leaf stomata is essentially controlled by different genes, the environmental conditions in which the plant is grown will have some impact (Casson and Gray, 2008).

5.2 Seasonal variation of catechins

The study showed season-specific differences in flavonoids accumulation in different tea cultivars. Variation in catechins content between cultivars, seasons and sites

indicated that the test cultivars responded differently under varying climatic conditions (Appendix ii). Leaf chemical analysis revealed significant changes in all tea quality components due to cultivar (TC, EGCG, EGC, EC and GA), seasons (TC, EGC, EC, C and GA) and sites (EGCG), cultivar and season (C and GA) and cultivar and site (GA) interactions. Green tea cultivars showed high levels of TC content compared to colored/purple teas. No significant differences were realized in ECG content in all variables. The highest TC content was exhibited by the standard quality cultivar TRFK 6/8 that is proven suitable for processing of black, green and white teas and cultivated prominently in the east of Great Rift Valley, a region reputed for high quality tea products. Catechins contribute significantly to the organoleptic characters such as color, flavor, texture and astringency of the tea products (Owuor *et al*., 2010). The results indicated that the warm-wet season (Table iv) was the most active period for the accumulation of TC in tea.

The levels of individual catechins present in the green-leaf varied significantly among cultivars, demonstrating that catechin composition was cultivar dependent. Epigallocatechin-3-gallate (EGCG), the ester of EGC and GA, is the most abundant catechin in green tea, a potent antioxidant that may have therapeutic applications in the treatment of many disorders (Lin *et al*., 2017). The content of EGCG in green tea cultivars was significantly reduced during the hot dry- compared to the other two seasons of the year. Similar studies have reported significant increase in EGCG during the warm- wet season which was attributed to stronger sun light and increased sunshine hours during the warmer months (Jaakola and Hohtola, 2010). Such plants maintain higher photosynthetic rates at lower temperatures than plants growing in hot-day seasons/areas, thus increasing the amount of fixed carbon available for secondary metabolites. Moisture deficiency associated with elevated temperatures further

reduces the synthesis of EGCG ultimately influencing the quality of made tea (Mukhopadhyay and Mondal, 2014). Epicatechin gallate (ECG) and EC are both dihydroxylated on the B-ring and compete for oxidation by polyphenol oxidase(s) to form different products but unlike ECG, EC is not esterified with the trihydroxylated moiety of gallic acid (Obanda *et al.,* 1997). Due to this difference in chemical structure, products arising from EC would be less astringent than those from ECG, translating into differences in organoleptic responses. Epicatechin (EC) content has been reported to be more abundant in wild tea trees (Li *et al.,* 2010). No significant differences were realized in ECG content in all variables showing the changes were not systematic and cannot be used to predict quality, whereas significant variation in EC indicates potential in both cultivars and seasons for less astringent specialty tea products. Results from the study revealed that the highest caffeine content was recorded in the hot-dry period as compared to the cold-wet/warm-wet seasons among all the cultivars with an exception of TRFK 91/1. This implied that some degree of stress stimulates production of this alkaloid which agreed with (Cherotich *et al.,* 2013) that maximum caffeine accumulates under water stress in teas. It's attributed to the accumulation of solutes by plants during water stress. Which stimulates the enzyme polyphenol oxidase in the phenyl-propanoid pathway. This enzyme causes hydroxylation of *p-ce* toumaric acid to caffeic acid. Further, caffeine in young leaves, fruits, and flower buds provide protection since plants are exposed to plethora of biotic stresses during the hot-dry season (Mohanpuria *et al.,* 2010). As different tea cultivars and seasons showed varying levels of caffeine in this study, there exist potential to develop special tea products targeting different consumer needs.

5.3 Seasonal variation in anthocyanin accumulation

The current study demonstrated that anthocyanins content was significantly varied amongst the cultivars. This indicated that variation of anthocynins is genotype dependent. The warm -wet season accumulated high contents of anthocyanins than the cool-wet and hot-dry seasons respectively. It agreed with (Steyn *et al*., 2002) that accumulation of anthocyanin varies with seasons. The moderate precipitation, low temperature, and average humidity (Appendix xvii & xviii) during the warm-wet season favoured biosynthesis of flavonoids in the tea plant (Cortell *et al*., 2007; Tarara *et al*., 2008). The biosynthesis of anthocyanins is genetically controlled. Therefore, such moderate environmental conditions stimulated transcription factors which caused activation or repression of structural genes that are involved in anthocyanin biosynthesis (Azuma *et al*., 2012; Cheynier *et al*., 2013; Jaakola, 2013). Enhanced anthocyanins content in the purple leafed tea cultivars during the warm-wet season, could have been attributed to moderate light that caused stimulation of gene transcript for flavonoid 3-O-glucosyltransferase (UFGT) enzyme. This enzyme is responsible for the production of endogenous sugars UDP-glucose that induces the biosynthesis of anthocyanins (Solfanelli *et al.*, 2006). However, the low contents of anthocyanins in the cultivar TRFK 306 during the hot-dry season could be due to the effects of high temperatures. Anthocyanins are sensitive to high temperatures and are easily degraded (Mori *et al*., 2005, 2007; Yamane *et al*., 2006).

5.4 Gene expression patterns

Gene transcripts underlying the biosynthesis of flavonoids in tea have been done comprehensively (Lai *et al*., 2016; Zhou *et al*., 2016; Lin *et al*., 2017). Lower accumulation of flavan-3-ols and anthocyanin could be caused by one or a combination of factors that include reduced synthesis, increased degradation or increased photo-oxidation of the phenylpropanoid intermediates (Lin-Wang *et al*., 2011). In all the seasons, purple leafed cultivar TRFK 306 showed up regulation of *CsCHI*. Its is an upstream gene that plays a key role in specifically generating (2S)-flavanones from chalcones which is a critical intermediate for formation of several flavonoid classes whose biosynthesis branches at this point (Ferreira *et al*., 2005). From the current study, it implies there was variation in flavanones in the three seasons. Anthocyanidins (cyanidin and delphinidin) and catechins are derived from dihydroflavonols (dihydroquercetin, dihydromyricetin) whose precursors are the flavanones.

Comparative analysis showed the expression of transcripts *CsPAL*, *CsCHI*, *CsF3H* and *CsF3'5'H* were differentially expressed (≥ 2.0 folds) in purple- and green-leafed cultivars supporting seasonal changes in flavonoid contents. Phenylalanine ammonia-lyase (*PAL*) catalyzes the deamination of phenylalanine (Phe) to give cinnamic acid, which is the first step in the phenylpropanoid pathway and an important regulation point between primary and secondary metabolism. Similar expression levels of *PAL* genes during the hot-dry season in both purple- and green-leafed cultivars imply responsiveness to hot-dry stress conditions (Huang *et al*., 2010). As most plants do not accumulate chalcones, naringenin chalcone is rapidly isomerized to flavanone naringenin by *CHI* (Lillo *et al*., 2010). Regulation of *CHI* was cultivar dependent with purple-leafed tea clearly demonstrating that it has evolved a special type of regulation

that enables the accumulation of flavanones. These flavanones are then converted to dihydroflavonols by a hydroxylation in position 3 catalyzed by flavanone 3-hydroxylase (*F3H*). In purple-leafed tea, the gene showed an expression pattern similar to that of *CHS*. Flavonoid 3'5'-hydroxylases (*F3'5'H*s) competitively controls the synthesis of delphinidin and cyanidin, the precursors of blue and red anthocyanins (Falginella *et al*., 2010), thus its down regulation during the hot-dry season in the purple-leafed cultivar is consistent with low anthocyanin accumulation. Notably, anthocyanin concentration was considerably affected by seasonal changes, with maximum concentration accumulated in the warm-wet season.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From the study, stomatal density was found to be significantly higher during the cool wet season among the assam tea cultivars. However, there was no correlationship between the accumulations of tea flavonoids with stomatal density of the tea plant.

Biochemical analysis revealed that total catechins (TC) were significantly higher during the warm-wet season. Green tea cultivars showed higher levels when compared with purple leafed tea cultivars. The highest TC content was exhibited by the cultivar TRFK 6/8 that is proven suitable for processing of black, green and white teas. The varying levels of caffeine with cultivar and season will be an advantage to pharmaceutical industry to venture into formulation of drugs or food additives with predetermined levels of caffeine which will be of great beneficial to consumers. The present study showed the cultivar TRFK 306 accumulated the highest levels of anthocyanins during the warm-wet season.

Results from the study demonstrated that the four gene transcipts; *CsPAL*, *CsCHI*, *CsF3H* and *CsF3'5'H* were differentially expressed in purple and green leafed cultivars supporting seasonal changes in flavonoid contents. In all the seasons, purple leafed cultivar TRFK 306 showed; up regulation of *CsCHI* and down regulation of *CsCHS,* down regulation of *CsPAL* in only two seasons (cool-wet and warm-wet) and *CsF3'5H* in only hot-dry season.

In overall, the observed season-specific differences in flavonoid content in different tea cultivars is an indication of the potential for product diversification.

6.2 Recommendations

The following recommendations were derived from this study:

1. There is no correlationship between stomatal density and the accumulation of tea catechins and anthocyanins.

2. Tea leaves harvested during the warm-wet season are suitable for the processing of flavonoid-rich tea products. As breeding for increased flavonoid content in tea is difficult, processors should take advantage of their differential accumulation with seasons and cultivars.

3. This study recommends that in the purple leafed cultivar TRFK 306, regulation of the gene transcript *CsCHI* should be utilized as a biomarker in breeding for anthocyanin rich-tea.

6.3 Suggestion for further research

The current study only investigated on the differential expression of eight gene trancripts in the three seasons. However, there's need to research on the correlationship between differential expression of genes and accumulation of tea flavonoids. This will be usefully to develope biomarkers for production of tea rich in flavonoid content.

REFERENCES

Ahmed, S., Stepp, J. R., Orians, C., Griffin, T., Matyas, C., Robbat, A., Cash, S., Xue, D., Long, C., Unachukwu, U., Buckley, S., Small, D., Kennelly, E. (2014). Effects of Extreme climate events on tea (*Camellia sinensis*) functional quality validate indigenousfarmer knowledge and sensory preferences in tropical China. *PLoS ONE*, 9: 109-126.

Anon. (2016). *Institute strategic plan 2016-2020*. Tea Research Institute.

Anon. (2002). *Tea Grower's Handbook* (5th Ed.). Tea Research Foundation of Kenya.

Albert, N.W., Lewis, D.H., Irving, L., J. (2009). Light induced in vegetative Anthocyanin in Petunia. *Journal of experimental Botany,* 60: 2191-2202.

Al Afas, N, Marron, N., Ceulemans, R. (2006). Clonal variation in stomatal Characteristics related to biomass production of 12 poplar (*Populus*) clones in a short rotation coppice culture. *Enviromental and Experimental Botany*, 58: 279-286.

Arab, L., Khan, F., Lam, H., (2013). Tea consumption and cardiovascular disease Risk. *Am, J. Clin. Nutr,* 98: 1651S-1659S.

Ashihara, H., Deng, W.W, Mullen, W., Crozier, A. (2010). Distribution and biosynthesis of flavan-3-ols in *Camellia sinensis* seedlings and expression of genes encoding biosynthetic enzymes. *Phytochemistry*, 71:559–66.

Azuma, A., Yakushiji, H., Koshita, Y., Kobashi, S. (2012). FLavonoid biosynthesis related genes in grape skin are differentially regulated by temperature and light by conditions. *Planta,* 236:1067-1080.

Balentine, D. A., Harbowy, M. E., Graham, H. N. (1997, 1998). In G. A. *Spiller (Ed.),* Caffeine, (pp. 35–72). Boca Raton: CRC Press.

Balentine, D. (1992). Manufacturing and chemistry of tea. In: Ho, C.T., Osawa, T., Huang, M.T., Rosen, R.T. (Eds), phenolic compounds in food and their effects on health. *American chemical society*, Washington, DC, (pop.102-117).

Barua, D.N. (1965). Classification of tea plant species hybrid. *Two and a bud*, 12:1327.

Bhatia, I. S. (1963). Chemical aspects of green tea leaf processing. *Two and a bud*, 10: 28-33.

Biswas, A. & Sarkar, A.R. (1973). Biological and chemical factors affecting the valuations if North-East Indian plain teas.lll statistical evaluation of the biochemical constituents and their effects in colour, brightness and strength of black teas. *Journal. Sci.Food Agri*, 24:1457-2477.

Björkman, M., Klingen, I., Birch, A.N., Bones. A.M., Bruce, T.J., Johansen, T.J., Stewart, D. (2011) Phytochemicals of Brassicaceae in plant protection and human health influences of climate, environment and agronomic practice. *Phytochemistry*, 72:538–556.

Borochov-Neori, H., Judeinstein, S., Harari, M., Bar-Ya'akov, I., Patil, B. S., Lurie, S., Holland, D. (2011). Climate effects on anthocyanin accumulation and composition in the Pomegranate (*Punicagranatum* L.) fruit arils. *Journal of Agricultural and Food Chemistry,* 59*:* 5325-5334.

Bukowski, J.F. & Percival, S.S. (2008). L.theanine interventionenhances human Yò T lymphocyte function. *Nutr. Rev*, 66:96- 102.

Bryant, J.P., Chapin, F.S.111., Klein, D.R. (1983). Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory *Oiks*, 40: 357- 368.

Carbone, F., Preuss, A., De Vos, R. C., D'Amico, E., Perrotta, G., Bovy, A. G.,

Casson, S. & Gray, J.E. (2008). Influence of environmental factors on stomatal

Castellarin, S.D., Gaspero, G.D., Marconi, R., Nonis, A., Peterlunger, E., Paillard, S. (2006.). Colour variation in red grapevines (*Vitis vinifera* L.): genomic organisation, expression of flavonoid 3'-hydroxylase, flavonoid 3',5'-hydroxylase genes and related metabolite profiling of red cyanidin-/blue delphinidin-based anthocyanins in berry skin. *BMC Genomics.*7:12.

Catala, R., Medina, J., Salinas, J. (2011). Integration of low temperature and light

Chalker-Scott, L. (1999). Enviromental significance of anthocyanins in plant stress responses. *Photochemistryand photobiology,* 70:1-9.

Chang, H.T., Bartholomew, B. (1984). Portland, Oregon, USA: Timber Press

development. *New Phytologist*, 178: 9-23. Martens, S., Rosati, C. (2009) Developmental, genetic and environmental factors affect theexpression of flavonoid genes, enzymes and metabolites in strawberry fruits. *Plant, Cell Environment*, 32:1117-1131.

signaling during cold acclimation response in Arabidopsis. *Proceedings of the National Academy of Sciences*, 108:16475-16480.

Chen, Y., Jiang, Y., Duan, J., Shi, J., Xue, S., Kakuda, (2010). Variation in catechin

contentin relation to quality of' Huang Zhi Xiang' Oolong tea (*Camellia sinensis)* at various growing altitudes and seasons: *Food Chem*,119: 648-652.

Chen, C., Wei, K, Wang, L., Ruan, L., Li, H., Zhou, X., Lin, Z., Shan, R., Cheng, H.

(2017). Expression of structural genes of the phenypropanoid pathway associated with catechin epimerization in tea cultivars. Front. *Plant.sci*, 8:702.

Chen, L.&Zhou, Z.X. (2005). Variations of main quality components of tea genetic resources [*Camellia sinensis* (L.) O. Kuntze] preserved in the China nationalgermplasm tea repository. *Plant Food Hum. Nutr*. 60: 31-35.

Cheserek, B.C., Elbeheri, A., Bore, J. (2015). Analysis links between climate variables and tea production in the recent past in Kenya. Donnish *Journal and Research in Environ*, 2: 5-7.

Cheynier, V., Comte, G., Davies, K.M., Lattanz, V., Martens, S. (2013). Plant phenolics: Recent advances on their biosynthesis, genetics and ecophysiology. *Plant physiol Biochem*, 72: 1-20.

Cherotich, L., Kamunya, S.M., Alakonya, A., Msomba, S. W., Uwimana, M.A., Wanyoko, J. K., Owuor, P.O. (2013). Variation in catechin composition of popularly cultivated tea Clones in East Africa (Kenya). *American Journal of Plant Sciences,* 4:628-640.

Cheruiyot, E. K., Miners, L. M., Ng'etich, W. K., Hassanali, A., Wachira, F.N., Wanyoko, J. K. (2008). Shoot epic epicatechin and epigallocatechin contents response to water stress in tea [*Camellia sinensis* (L.) O. Kuntze]. *Bioscience, Biotechnology and Biochemistry*, 72: 1219-1226.

Ciancolini, A., Alignan, M., Pagnotta, M.A., Miquel, J., Vilarem, G., Crinò, P. (2013). Morphological characterization, biomass and pharmaceutical compounds in Italian globe artichoke genotypes. *Ind Crops Prod*, 49:326–333.

Cohen, S. D., Tarara, J. M., Gambetta, G. A., Matthews, M. A., Kennedy, J. A. (2012). Impact of diurnal temperature variation on grape berry

development, proanthocyanidin accumulation, and theexpression of flavonoid pathway genes. *Journal of Experimental Botany,* 63*:*2655–2665.

Cordell, S., Golden, G., Muller- Dombois, D., Webb, D., Vitousek, P.M. (1998). Physiological and variation in *Metrosideros polymorpha,* a dominant Hawaiian tree species, along an altitudinal gradient. *The role of plasticity Oecologia,*113: 188- 196.

Cortell, I. M, Halbleib, M., Gallagher, A.V., Righetti, T.l., Kennedy, J.A. (2007). Influence of vine vigor on grape (*Vitis Vinifera, cv Pinot Noir*) anthocynins .1. Anthocynins concentration and composition in fruit. *Journal of agriculture and food chemistry,* 5j:6575-6584.

Coupe, S.A., Palmer, B. G., Lake, J.A., Overy, S.A., Woodward, F.I., Gray, J. E. and Quick, W.P. (2006). Systemic signaling of environmental cues in Arabidopsis leaves. *Journal of Experimental Botany,* 57: 329-341.

Connor, A. M., Luby, J. J., Hancock, J.F., Berkheimer. S., Hanson, E.J. (2002c). Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage. *J Agric Food Chem,* 50:893–8.

Cui, G., Zhang, W., Zhang, A., Mu, H., Bai, H., Duan, J., Wu, C. (2013). Variation in antioxidant activities of polysaccharides from Fructus Jujubae in South Xinjiang area. *Int J Biol Macromol* 57:278–284.

Delagrange, S., Montpied, P., Dreyer, E., Messier, C., Sinoquet, H. (2006). Does shade improve light interception efficiency? A comparison among seedlings from shade-tolerant and-intolerant temperate deciduous tree species. *NewPhytol* 172:293–304.

Dela, G., Or, E., Ovadia, R., Nassion-levi, A., Weiss, D., Oren-Shamir, M. (2003).

Changes in anthocyanin concentration and composition in 'Jaguar' rose flowers due to transient high-temperature conditions. *Plant Science*, 164: 333–340.

Dixon, R. A. & Steele, C. L. (1999). Flavonoids and isoflavonoids—a gold mine for metabolic engineering, *Trends Plant Sci* 4: 394-400.

Downey, M.O., Harvey, J.S., Robinson, S.P. (2004). The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grapes. Australian *J Grape Wine Res,*10:55–73.

Dufresne, C.J. & Farnsworth, E.R. (2001). A review of the latest research findings on health promotion properties of tea. *J. Nut. Bioch*, 12: 404-421.

FAO (2016). Overview of the Kenya tea industry and 2014/2015 performance. Inter Governmental Group on tea 22nd session Naivasha, Kenya.

Falginella, L., Castellarin, S. D., Testolin, R., Gambetta, G. A., Morgante, M., Gaspero, G.D. (2010). Expansion and subfunctionalisation of flavonoid3', 5'-hydroxylases in the grapevine lineage. *BMC Genomics,* 11*:* 562.

Franks, P. J., Farquhar, G. D. (2007). The mechanical diversity of stomata and its significant gas-exchange control. *Plant Physiol.* 143 78–8.

Ferreira, D., Slade, D., Marais, J.P.J., (2005). In O Andersen, K.R Markham Eds.), *The Flavonoids: Advances in Research*, CRC Publishers, London in press.

Gay, A.P. & Hurd, R.G. (1974). The influence of light on stomatal density of a tomato. *New Phytol*, 75:37-46.

GENSTAT software Version 15.1.8035 (VSN International Ltd, UK)

Givnish, T.J. (1988). Adaptation to sun and shade: a whole-plant perspective.

Australian Journal of Plant Physiology, 15: 63-92.

Graham, T.L. (1998). Flavonoid and flavonol glycoside metabolism in Arabidopsis. *Plant Physiol Biochem,* 36:135–144.

Graham, H.N. (1992). Green tea composition, consumption and polyphenolchemistry. *Prev.Med*, 21:334-350.

Gravano, E., Giulietti, V., Desotgiu, R., Bussoti, F., Grossini, P., Gerosa, G., Tani, C.2003). Foliar response of an *Ailanthus altissima* clone in two sites with different levels of ozone.

Grotewold, E. (2006). The genetics and biochemistry of floral pigments, *Annu Rev Plant Biol in* Press.pollution. *Environmental pollution*, 121: 137-146.

Giusti, M M. & Wrolstad, R.E. (2001). Unit F1.2: Anthocyanins characterization and measurement with UV- visible spectroscopy. In R.E (Ed.), current protocols in *food analytical chemistry*, (pp. 1-3). New York John Wiley and sons.

Haak, L., Raes, K., Smet, S.D. (2009). Effect of plant phenolics, tocopherol and ascorbicacid on oxidative stability of pork pathies. *J. Sci.Food Agri,* 89:1360-1365.

Harbowy, M. E. & Balentine, D. A. (1997). Tea chemistry. *Critical Review Plant Science*, 16:415–480.

Harbowy, M.E. & Balentine D.A. (2001). Tea Chemistry, *Crit. Rev. Plant Sci*, 16: 415-480.

Haselgrove, L., Blotting, D., Van Heeswijck, R., Hoj, P.B., Dry, P.R., Ford, C., Iland, P.G. (2000). Canopy microclimate and berry composition: the effects of bunch exposure on the phenolic composition of *Vitis vinifera*L. cv Shiraz grape berries. Australian *journal of Grape and Wine Research*, 6:141:149.

Hetherington, A. M., Woodward, F. I. (2003). The role of stomata in sensing and driving environmental change. *Nature* 424 901–908.

Hemm, M.R., Rider, S.D., Ogas, J., Murry, D.J., Chapple, C. (2004) Light induces Phenylpropanoid metabolism in Arabidopsis roots. *Plant J*,38:765-778.

Hoch, W. A., Zeldin, E.L., McCown, B.H. (2001). Physiological significance of anthocyanins during autumnal leaf senescence. *Tree Physiol*, 21:1–8

Hong, G.J., Wang, J., Zhang, Y., Hochstetter, D., Zhang, S.P., Pan, Y., Shi, Y.L., Xu, P., Wang, Y.F. (2014). Biosynthesis of catechin components is differentially regulated in darktreated tea (*Camellia sinensis* L.). *Plant Physiol. Biochem,*78: 49-52.

Hughes, N. M., Neufield, H. S., Burkey, K. O. (2005). Functional role of anthocyanins in high light winter leaves of the evergreen herb *Galax urceolata*. *New Phytologist,* 168: 575–578.

Hilton, P.J.& Palmer-Jones, R. (1973). Relationship between the flavonoid composition of fresh tea shoots and the theaflavin content in manufactured tea. *Journal of science Food agriculture*, 50: 9-17.

Huits, H. S. M., Gerats, A. G. M., Kreike, M. M., Mol, J. N. M. Koes, R. E. (1994). Genetic control of dihydroflavonol 4-reductase gene expression in *Petunia hybrida*, *Plant J*, 6: 295-310.

ISO 14502- 2- 2005E. Determination of substance characteristic of green and black tea: Method using high performance liquid chromatography.

International Tea Committee, (2016). World tea production and consumption 1995-2015.

Jaakola, L. (2013). New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends in plant science,* 18:477-483.

Jaakola, L. & Hohtola, A. (2010). Effect of latitude on flavonoid biosynthesis in

plants. *Plant, Cell and Environment,* 33:1239–1247.

Jang-Eun, L., Bum, -Jin, Bum-Jin, l., Jin-Ch. C., Jeong-Ah, H., Sang- Juh, L., Cheri-HO, L., Young-Shick, H. (2010). Geographical and climatical dependencies of green tea (*Camellia sinensis*) metabolites A^1H NMR based metabolomics study. *J. Agric. Food Chem,* 58: 446-729.

Jayasekera, S., Kaur, l., Molan, L., Garg, M.L., Moughan, P.S. (2014). Effects of season and plantation on phenolic contentment of unfermentated and fermentated fermentated Sri Lanka tea. *Food Chem,* 152:546-551.

Jayasekera, S., Molan, A.L., Garg, M., Moughan, P.J. (2011). Variation in antioxidant potential and total polyphenol content of fresh and fully fermented Sri Lanka tea. *Food Chem,* 125: 536-541.

Jiang, L., Shen, X., Shoji, T., Kanda, T., Zhou, J., Zhao, L. (2013). Characterization and activity of anthocyanins in Zijuantea (*Camellia sinensis* var. *kitamura*). *Journal of Agricultural and Food Chemistry*, 61, 3306–3310.

Kader, F., Rovel, B., Girardin, M., Metche, M. (1997). Mechanism of browning in fresh highbush blueberry fruit (*Vaccinium corymbosum L*). Role of blueberry polyphenol oxidase, chlorogenic acid and anthocyanins. *J Sci Food Agric,* 74:314.

Kader, F., Haluk, J.P., Nicolas, J.P., Metche, M. (1998). Degradation of cyanidin 3-glucoside by blueberry polyphenol oxidase: kinetic studies and mechanisms. *J AgricFood Chem,* 46:3060–5.

Kaison.C. (2015). World Tea Production and Trade: Current and Future Development.

Kamunya, S. M., Wachira, F. N., Pathak, R. S., Muoki, R. C., Wanyoko, J. K.,

Ronno, W.K., et al. (2009). Quantitative genetic parameters in tea (Camellia sinensis (L.) O. Kuntze): I. combining abilities for yield, drought tolerance and quality traits.

African Journal of Plant Science, 3, 93–101.

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

Kerio, L.C., Wachira, F.N., Wanyoko, J.K. and Rotich, M.K. (2012). Characterization of anthocyanins in Kenyan teas: Extraction and identification. *Food Science*, 131: 31-38.

Kerio, L.C., Bend, J. R., Wachira, F.N., Wanyoko, J.k. and Rotich, M.K. (2011). Attenuation of t- Butylhydroperoxide induced oxidative stress in HEK 293 WT cells by tea catechins and anthocyanins. *Journal of Taxicology and enviromental Health Sciences*, 3: 367-375.

Kito, M., Kokura, H., Izaki, J., Sasaoka, K. (1968). Theanine, a precursor of the phloroglucinol nucleus of catechins in tea plants. *Phytochemistry*, 7: 599–603.

Koffidis, G., Bosabadilis, A.M., Moustakas, M. (2003). Contemporary seasonal and altitudinal variations of leaf structural features in Oregano *(Origanum vulgare* L.). *Annals of botany*, 92: 635-645.

Kouwenberg, R., Kurschner, M., McElwain, C. (2007) Stomatal frequency change over altitudinal gradients: prospects for paleoaltimetry. *Reviews in Mineralogy and Geochemistry,*66:215-241.

Koricheva, J., Larsson, S., Haukioja, E., Keinanen, M. (1998). Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta- analysis *Oikos*, 83: 212-226.

Kumar, A., Chawla, V., Sharma, E., Mahajan, P., Shankar, R., Yadav, S. K. (2016). Comparative transcriptome analysis of chinary, assamica and cambod tea (*Camellia sinensis*) types during development and seasonal variation using RNA-seqtechnology. *Scientific Reports,*6*:*37244.

Koes R, Quattrocchio R, Mol J (1994). The flavonoid biosynthetic pathway in plants: function and evolution. *BioEssays*, 16: 123–132.

Kowalsick, A., Ahmed, S., Orians, C., Griffins, T., Cash, S.B., Stepp, J.R. (2014). Metaboilte profiling of Camellia sinensis by automated sequential multidimensional gas chromatography/ mass spectrometry reveals strong monsoon effects on tea constituents. *J. Chromatogr.A*, 1370:230-239.

Lai, Y-S., Li, S., Tang, Q., Li, H-X., Chen, S-X., Li, P-W, Xu, J-Y., Xu, Y., Guo, X. (2016). The dark-purple tea cultivar 'Ziyan' accumulates alarge amount of delphinidin-related anthocyanins. *Journal of Agricultural and Food Chemistry,* 64*:*2719–2726.

Langat, C., Gathaara, M. H., Cheruiyot, R. (2015). Comparative response of catechin levels in drought tolerant and drought susceptible tea clones (*Camellia sinensis*) (L) O. Kuntze under different moisture regime. *African Journal of Plant Science,* 8: 334-338.

Luomala E. M., Laitinen K., Sutinen S., Kellomäki S., Vapaavuori E. (2005). Stomatal density, anatomy and nutrient concentrations of Scots pine needles are affected by elevated CO2 and temperature. *Plant Cell Environ.* 28: 733–749.

Lillo, C. (2010). Synergic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomatoes. *Phytochemistry*, 71:605- 613.

Li, J., Li, G., Gao, S., Martinez, C., He, G., Zhou, Z, et al. (2010). *Arabidopsis* transcription factor ELONGATED HYPOCOTYL 5plays akey role in the feedback regulation of phytochrome a signalling. *Plant Cell*, 22:3634-3649.

Liu, Y., Gao, L., Liu, L., Yang, Q., Luz, *et. al*. (2012). Purification and characterization of anovel galloyl transferase involved in catechin galloylation in the tea plant (*Camellia sinensis*). J. *Biol.Chem,*287:44406- 44417.

Liu, M., Tian, H-L., Wu, J-H., Cang, R-R., Wang, R-X., Qi, X-H., Xu, Q., Chen, X-H. (2015). Relationship between gene expression and the accumulation of catechin during spring and autumn in tea plants (*Camellia sinensis* L.). *Horticulture Research,* 2: 15011.

Liu, Y., Chenyun, Q., Sihui, D., Xulan S., Wanxia, Y., Shengzuo, F. (2016). Effect of light regime and provenance on leaf characteristics, growth and flavonoid accumulation in *Cyclocarya paliurus* (Batal) Iljinskaja coppices *Bot Stud,* 57:28.

Liu, L., Gao, S., Xie, B., Sun, Z., Wu, J. (2007). Degradation of cyanidin 3-rutinoside in the presence of (-)-epicatechin and litchi pericarp polyphenol oxidase. *Journal of Agricultural and Food Chemistry*, 55: 9074–9078.

Lin, J., Wilson, I. W., Ge, G., Sun, G., Xie, F., Yang, Y., Wu, L., Zhang, B., Wu, J., Zhang, Y., Qiu, D. (2017). Whole transcriptome analysis of three leaf stages in two cultivars and one of their F1 hybrids of *Camellia sinensis* L. with differing EGCG content. *Tree Genetics and Genomes,*13:13.

Lin-Wang, K., Micheletti, d., Palmer, J., Volz, R., Lozano, L., Espley, R., Hellens, R. P., Chagne, D., Rowan, D.D., Troggio, M.*et al.*(2011). High temperature reduces apple fruit colourmodulation of anthocyanin regulatory complex. *Plant cell Environ,* 34:1176-1190.

Lovdal, T., Olsen, K.M., Slimestad, R., Verheul, M., Lillo, C., (2010). Synergetic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomato. *Phytochemistry,* 71: 605-613.

Lovedeep, K., Jayasekera, S., Paul J. M. (2014). Antioxidant Quality of Tea (Camellia sinensis) as Affected by Environmental Factors.pp121-129.

Lo, S.C.& Nicholson, R.L. (1998). Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls. Implicatios for a compensatory role on the defense response. *Plant physiol*, 116: 979-989.

Margaria, P., Ferrandino, A., Caciagli, P., Kedrina, O., Schubert, A., Palmano, S. (2014). Metabolic and transcript analysis of the flavonoid pathway in diseased andrecovered Nebbiolo and Barbera grapevines (*Vitis vinifera* L.) following infectionby Flavescence doree phytoplasma. *Plant Cell Environ,* 37: 2183-2200.

Martinez-Luscher, J., Torres, N., Hilbert, G., Richard, T., Sanchez-Diaz, M., Delrot, S., Aguirreolea, J., Pascual, I., Gomes, E. (2014). Ultraviolet-B radiation modifies thequantitative and qualitative profile of flavonoids and amino acids in grape berries. *Phytochemistry,*102: 106-114.

Martinez., J.P., Silva, H., Ledent, J.F., Pinto, M. (2007). Effect of drought stress

stress on theosmotic adjustment, cell wall elasticity and cell volume of six cultivars of common beans (*Phaseolus vulgaris).* European *journal of Agronomy,*26:30 – 38.

Mahotti, A.J. & Lawlor, D.W. (2002). Diurnal variation of phosynthesis and photoinhibition in tea: effects of irradiance and nitrogen supply during growth in the field. *Journal of experimental Botany,* 53: 313-322.

McDowell N., Pockman W. T., Allen C. D., Breshears D. D., Cobb N., Kolb T., et al. (2008). Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought. *New Phytol.* 178: 719–739.

Modder, D. & Amarakoon, K. (2002). Tea and health, Sri-Lanka.Mukhopadhyay, M., & Mondal, T. K. (2014). The physio-chemical responses of camellia plants to abiotic stresses. *Journal of Plant Science & Research, 1,*105.

Mohanpuria, P., Kumar, V., Joshi, R., Gulati, A., Ahuja, P. S., and Yada, S. K. (2009). Caffeine biosynthesis and degradation in tea [*Camellia sinensis* (L) O. Kuntze] is developmental and seasonal regulation. *Molecular Biotechnology,* 43:104 -111.

Mohanpuria, P. & Yadav, S.K. (2010). Characterization of novel small RNAs from tea (*Camellia sinensis* L.). *Mol. Biol. Rep*., 39: 3977-3986

Mondal, T. K., Bhattacharya, A., Laxmikumaran, M., Ahuja, P. S. (2004). Recent advances in tea biotechnology. *Plant Cell Tissue and Organ Culture*, 76 195-254.

Mori, K., Goto-Yamamoto, N., Kitayama, M., Hashizume, K. (2007). Loss of anthocyanins in red-wine grape under high temperature. *Journal of Experimental Botany, 58,* 1935-1945.

Mori, K., Suaya, S., Gemma, H. (2005). Decreased anthocyanin biosynthesis in grape berries grown under elevated night temperature condition. *Sci.Horti*, 10: 319-330.

Muoki, R.C., Paul, A., Singh, K. and Kumar, S. (2012). An improved protocol for the isolation of RNA from roots of tea (Camellia sinensis (L.) O. Kuntze.*Molecular Biotechnology*, 52: 82-88.

Mukhopadhyay, M.& Mondal, T. K. (2014). The physio-chemical responses of camellia plants to abiotic stresses. *Journal of Plant Science & Research*, 1:105.

Ng'etich, W.K. (1995). An evaluation of the responses of some clones to Environment in Kenya. PhD Thesis, Cranfield University, Silsoe, U.K.

Nicoue, E., Savard, S., Belkacemi K. (2007). Anthocyanins in wild blueberries of Quebec: extraction and identification. *J Agric Food Chem,* 55:5626-35.

Nicholson, R. L. & Hammerschmidt, R. (1992), Phenolic compounds and them role in disease resistance, *Ann Rev Phytopathol*, 30: 369-389.

Nyirenda, H.E., Apostolides, Z., Mphangwe, N. (2006). Diversification of the tea product through value adding and business viability. Tea Research Foundation of Central Africa News.

Obanda, M., Owuor, P. O., Taylor, S. J. (1997). Flavanol Composition and Caffeine Content of Green Leaf as Quality Potential Indicators of Kenyan Black Teas. *Journal of the Science of Food and Agriculture,* 74:209-215.

Obanda, M., Owuor, P.O., Mang'oka, R. (2004). Changes in thearubi-gen fractions and theaflavins levels due to variations in processing conditions and their effects on black tea liquor brightness and total colour. *Food Chem*,85:163-173.

Ohl, S., Hedrick, S.A., Chory, J., Lamb, C.J. (1990). Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis. Plant Cell,* 2*:* 837-848.

Ojeda, H., Andary, C., Kraeva, E., Carbonneau, A., Deloire, A. (2002). Influence of pre- and postvéraison water deficit on synthesis and concentration of skin phenolic compounds during berry growth of *Vitis vinifera cv. Shiraz*. *Am. J. Enol. Viti*. 53:261-267.

Owuor, P. O. & Obanda, M. (2006). The use of green tea (*Camellia sinensis*) leaf flavan-3-ol composition in predicting plain black tea quality potential. *Food Chemistry,* 100: 873-884.

Owuor, P.O. & Obanda, M. (2007). The use of green tea (*Camellia sinensis*) leaf flavan-3-ol composition in predicting plain black tea quality potential. *Food Chem,* 100:873-884.

Owuor, P.O., Obanda, M., Nyirenda, H.E., Mandala, W.L. (2008). Influence of region of production on clonal black tea chemical characteristics. *Food chemistry,* 108: 263-271.

Owuor, P. O., & Obanda, M. (2007). The use of green tea (*Camellia sinensis*) leaf flavan-3-ol composition in predicting plain black tea quality potential. *Food Chemistry,*100: 873-884.

Owuor, P.O., Wachira, F.N., Ng'etich, W.K. (2010). Influence of region of production on relative clonal plain tea quality parameters in Kenya. *Food chemistry,* 119:1168-1178.

Paola, R.D., Mazzon, E., Muia, C., Genovese, T., Menegazi, M., Zaffini, R., et al. (2005). Green tea polyphenols attenuate lung injury Carragean-induced pleurisy. *Respiratory Research,* 6:66.

Pang, Y., Abeysinghe, I.S.B., He, J., *et al. (2013). Functional characterization of*

proanthocyanidin pathway enzymes from tea and their application for metabolic engineering. *Plant Physiol*,61: 1103-1116.

Pengelly. J.J., Sirault, X.R., Tazoe, Y.J.R., Furbank, R.T., and Von Caemmerer, S (2010). "Growth of the C4 dicot Flaveria bidentis: photosynthetic acclimation to low light through shifts in leaf anatomy and biochemistry", *Journal of Experimental Botany*61(14): 4109–4122.

Peri, P.L., Lucas, R.J., Moot, D.J. (2007). Dry matter production, morphology and nutritive value of *Dactylis glomerata* growing under different light regimes. *Agrofor Syst*,70:63-79.

Pfaffl, M.W., Horgan, G.W., Dempfle, L. *(*2002*).* Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res30, 1–10.*

Punyasiri, P.A., Abeysighe, I.S.B., Kumar, V., Treutter, D., Duty, D., Gisch, C. (2004). Flavonoid biosynthesis in the tea plant *Camellia sinensis:* properties of enzymes of prominent epicatechin and catechin pathways. *Archives of biochemistry and biophysics*, 431: 22-30.

Puupponen, R., Nohynek, L., Meier, C., K¨ahk¨onen, M., Heinonen, M., Hopia, A., Oksman, Caldentey, K.M. (2001). Antimicrobial properties of phenolic compounds from berries. *J Appl Microbiol*, 90:494–507.

Quarrie, S.A., &Jones, H.G. (1977). Effects of abscisic acid and water stress on development and morphology of Wheat. *Journal of Experimental Botany*, 28: 192 -203.

Rani, A., Singh, K., Ahuja, P. S., Kumar, S. (2012). Molecular regulation of catechins biosynthesis in tea [*Camellia sinensis* (L.) O. Kuntze]. *Gene*,495: 205-210.

Ravichandran, R. and Parthiban, R. (1998). Changes in enzyme activities

(polyphenol oxidase and phenylalanine ammonia lyase) with type of tea leaf and during black tea manufacture and the effect of enzyme supplementation of dhoolon black tea quality. *Food Chem*, 62:277-281.

Radoglou, K.M and Jarvis, P.G. (1990). Effects of CO2 enrichment on four poplar clones. II. Leaf surface properties. *Annals of Botany*, 65: 627-632.

Roby, G., Harbertson, J.F., Adams, D.O., Matthews, M.A. (2004). Berry size and vine water deficits as factors in winegrape composition: Anthocyanins and tannins. Aust. *J. Grape Wine Res*. 10:100-107.

Rozen, S. &Skalesky, H. (2000). Primers 3 on the www for general users and for biologist programmers. Methods *Mol.Biol,*132: 365 - 86.

Sanchez-Rodriguez, E., Moreno, D.A., Ferreres, F., Rubio-Wilhelmi, M.D., Ruiz, J.M. (2011). Differential responses of fIve cherry tomato varieties to water stress: changes on phenolic metabolites and related enzymes. *Phytochemistry*, 72:723-729.

Schmidt, S., Zietz, M., Schreiner, M., Rohn, S., Kroh, L.W., Krumbein, A. (2010) Genotypic and climatic influences on the concentration and composition of flavonoids in kale (*Brassica oleracea var. sabellica*). *Food Chem*, 119:1293–1299.

Schlüter, U., Muschak, M., Berger, D., Altmann, T. (2003). Photosynthetic performance of Arabidopsis mutant with elevated stomatal density under different light regimes. *J Exp Bot*; 54:867-74.

Schijlen, E. G., Ric de Vos, C. H., van Tunen, A. J. and Bovy, A. G., (2004). Modification of Flavonoid biosynthesis in crop plants, *Phytochem*,65: 2631-2648.

Shang, Y.J., Venail, J., Mackay, S., Bailey, P.C., Schwinn, K.E., Jameson, P.E.,

Martin, C.R., Davies, K.M. (2011). The molecular basis for venation patterning of pigmentation and its effect on pollinator attraction in flowers of Antirrhinum. New *Phytol*, 189: 602- 615.

Shi, C-Y., Yang, H., Wei, C-L., Yu, O., Zhang, Z-Z., Jiang, C-J., Sun, J., Li, Y-Y., Chen, Q., Xia, T., Wan, X-C. (2011). Deep sequencing of the Camellia sinensistranscriptome revealed candidate genes formajor metabolic pathways of tea-specificcompounds. *BMC Genomics*,12:131.

Skrede, G., Wrolstad, R.E., Durst, R.W. (2000). Changes in anthocyanins and polyphenolics during juice processing of highbush blueberries (*Vaccinium corymbosum* L.). *J. Food Sci*, 65:357–64.

Solfenelli, C., Poggi, A., Loreti, E., Apli, A., Perata, P. (2006). Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. *Plant physiol*, 140: 637-646.

Stafford, H, A. (1990). Flavonoid metabolism. *Boca Raton, Fla*: CRC Press.

Steyn, W., Holcroft, D.M., Jacobd, G. (2000). Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. *New phytologist*, 155: 349-361.

Steyn, W. J., Wand, S. J. E., Holcroft, D. M., Jacobs, G. (2002). Anthocyanins in vegetative tissues: A proposed unified function in photoprotection. New Phytologist, 155: 349-361.

Sud, R.G. & Baru, A. (2000). Seasonal variations in theaflavins, thearubi-gins, total colour and brightness of Kangra orthodox tea (*Camellia sinensis (*L) O Kuntze) in Himachal Pradesh. *J.Sci. Food Agric.* 80:1291-1299.

Taylor, L. P.& Grotewold, E. (2005). Flavonoids as developmental regulators, *Curr Op Plant Biol*, 8: 317-323.

Taylor, S.H., Franks, P.J., Hulme, S.P., Spriggs, E., Christin, P.A, *et al.* (2012). Photosynthetic pathway and ecological adaptation explain stomatal trait diversity amongst grasses. *New Phytologist,* 193:387–396.

Tarara, J.M, Lee, J.M., Spayd, S.E., Scagel, C.F. (2008). Berry temp and solar radiation alter acylation, proportion and concentration of anthocyanins in Merlot grapes, *American journal of Enology and viticulture,*59*:235*-247.

Thielecke, F. & Boschmann, M. (2009). The potential role of green tea catechins in the prevention of the metabolic sundrome- A review. *Phytochemistry,* 70: 11-24.

Toda K, Yang D, Yamanaka N, Watanabe S, Harada K, Takahashi R. A single-base deletion in soybean flavonoid 3′-hydroxylase gene is associated with gray pubescence color. *Plant Mol Biol.*2002; 50:187-196.

Thompson, E.P., Davies, J.M., Glover, B.J. (2010). Identifying the transporters of different flavonoids in plants. *Plant Signal Behav,* 5: 860–863.

Tounekti, T., Joubert, E., Hernandez, I., Munne-Bosch, S. (2013). Improving the polyphenol content of tea. *Crit. Rev. Plant Sci*,32: 192-215

Upandyay &Panda, S.K. (2004). Responses of (*Camellia sinensis*) to drought and rehydration.*Biol. Plant,* 48: 577- 600.

Valladares, F.& Niinemets, Ü. (2008). Shade tolerance, a key plant feature of complex nature and consequences. *Annu Rev Ecol Evol Syst,* 39:237–257.

Vaknin, H., Bar-Akiva, A., Ovadia, R., Nissim-Levi, A., Forer, I., Weiss, D., Oren-Shamir, M. (2005). Active anthocyanin degradation in *Brunfelsiacalycina* (yesterday--today--tomorrow) flowers. *Planta,*222: 19-26.

Verma, N., & Shukla, S. (2015). Impact of various factors responsible for fluctuation in plant secondary metabolites. *J.App. Res.Med. arom. Plants* 2:105-113.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology,* 3: 111.

Wachira, F.N., Tanaka, J., Takeda, Y. (2001). "Genetic Varia-tion and Differentiation in Tea (*Camellia Sinensis*) Germ- plasm Revealed by RAPD and AFLP Variation," *Journal of Horticultural Sciences and Biotechnology,* 5:557-563.

Wachira, F, N., Waugh. R., Hachet, C.A., Powel, W. (1995). Detection of genetic diversity among (*Camellia sinensis*) using RAPD markers. *Genome,* 38: 201- 210.

Wachira, F, N., Waugh. R., Hachet, C.A., Powel, W. (1997). Assessment of genetic diversity among*Camellia sinensis* L. (cultivated tea) and its wild relatives based on randomly amplified polymorphic DNA and organelle specific STS. Hereditary, 78: 603 – 611.

Wang, Y-S., Xu, Y-J., Gao, L-P., Yu, O., Wang, X-Z., He, X-J., Jiang, X-L., Liu, Y-J., and Xia, T. (2014). Functional analysis of Flavonoid 3′, 5′-hydroxylase from Tea plant (*Camellia sinensis*): critical role in the accumulation of catechins. BMC *Plant Biology,* 14:347.

Wang, X. (2011). Structure, function, and engineering of enzymes in isoflavonoid biosynthesis. *Funct. Integr. Genomics*,11: 13-22.

Wang, H.F., Tsai, Y.S., Lin, M.L., Ou, A.S.M. (2006). Effects of different variety,

production area and season on the major chemical constituents in Taiwan GABA tea. *Taiwanese J. Agric. Chem Food Sci*, 44: 90–99

Wesche-Ebeling, P., Montgomery, M. W. (1990). Strawberry polyphenol oxidase: Its role in anthocyanin degradation. *Journal of Food Science*, 55: 731-734.

Wei, K., Zhang, Y., Wu, L., Li, H., Ruan, L., Bai, P., Zhang, C., Zhang, F., Xu, L., Wang, L., and Cheng, H. (2016). Gene expression analysis of bud and leaf color in tea. *PlantPhysiology and Biochemistry*, 107: 310-318.

Wei, K., Wang, L., Zhang, C., Wu, L., Li, H., Zhang, F., Cheng, H. (2015). Transcriptome analysis reveals key Flavonoid 3′-Hydroxylase and Flavonoid 3′, 5′-Hydroxylase genes in affecting the ratio of dihydroxylated to trihydroxylated catechins in *Camellia sinensis*. *PLoS ONE*, 10: e0137925.

Wei, K., Wang, L.Y., Zhou, J., He, W., Zeng, J.M., Jiang, Y.W., Cheng, H. (2011). Catechin contents in tea (*Camellia sinensis*) as affected by cultivar and environment and their relation to chlorophyll contents. *Food Chem*, 125, 44 - 48.

Winkel-Shirley. (2001). Flag biosynthesis: a colour model for genetics, biochemistry, cellbiology, and biotechnology. *Plant physiology*, 126: 485.

Wu, Z.J., Li, X.H., Liu, Z.W., Xu, Z.S., Zhuang, J. (2014). *De novo* assembly and transcriptome characterization: novel insights into catechins biosynthesis in *Camellia sinensis*. *BMC Plant biology*,14:277

Xie, D.-Y., Sharma, S. B., and Dixon, R. A. (2004), Anthocyanidin reductases from *Medicago truncatula andArabidopsis thaliana*, *Arch Biochem Biophys*, 422: 91-102.

Xie, J.H., Xie, M.Y., Shen, M.Y., Nie, S.P., Li, C., Wang, Y.X. (2010).

Optimisation of microwave assisted extraction of polysaccharides from *Cyclocarya paliurus*(Batal.) Iljinskaja using response surface methodology. *J Sci Food Agric*,90:1353-1360.

Xue, W., Li, X, Y., Zhu, J.T., Lin, L.S., Wang, Y.J. (2011) "Effects of shading on leaf morphology and response characteristics of photo-synthesis in Alhagi *sparsifolia",* Chinese *Journal of Plant Ecology* 35(1), 82-9035.

Xu, Z. Zhou, G. (2008). Responses of Leaf Stomatal Density to Water Status and Its Relationship with Photosynthesis in a Grass. *Journal of Experimental Botany*, 59, 3317-3325.

Yamane, T., Jeong, S.T., Goto-Yamamoto, N., Koshita, Y., Kobayashi, S. (2006). Effects of temp on antho-cyanins biosynthesis in grape berry skin. *America*, 57:54-59.

Yan, W., Zhong, Y., Shangguan, Z. (2017). Contrasting responses of leaf stomatal characteristics to climate change: a considerable challenge to predict carbon and water cycles. *Glob. Change Biol.* 104145-157.

Yoana, P. (2012). The effect of light intensity on the stomatal density of lavender, *Lavandulaangustifolia. Young Scientists Journal Issue,* 12:89-93.

Zhang, Y., Zhu, X.J., Chen, X., Song, C.N.A., Zou, Z.W., Wang, Y.H., Wang, M.L., Fang, W.P., Li, X.H. (2014). Identication and characterization of cold-responsive microRNAs in tea plant (*Camellia sinensis*) and their targets using high. *BMCPlant Biol*, 14.

Zhao, J.W., Chen, Q.S., Huang, X.Y. (2006). Qualitative identification of tea categories by infrared spectroscopy and support vector machine. *Journal of pharmaceutical and biomedical analysis*, 41: 1198-1204.

Zheng, X.Q., Jin, J., Chen, H., Du, Y.Y., Ye, J.H., *et al*. (2008). Effect of

Ultraviolet irradiation on accumulation of catechins in tea (*Camellia sinensis* (L) O. Kuntze). *Afr J Biotechnol,*7: 3283-3287.

Zhou, Q., Sun, W., Lai, Z. (2016). Differential expression of genes in purple-shoot tea tender leaves and mature leaves during leaf growth. *Journal of the Science of Food and Agriculture,* 9:1982-1989.

APPENDICES

Summary tables for analysis of variance (ANOVA) results of means for the stomatal density, biochemical and molecular parameters of tea plant *(Camellia sinensis*L.) O. Kuntze.

Appendix (i): Anova for stomatal density in (mm^2) for five cultivars

variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	4	9440.8	2360.2	2.76	0.035
season	2	4348.5	2174.3	2.55	0.087
site	1	58.8	58.8	0.07	0.794
cultivar. season	8	60605.5	7575	8.87	<.001
cultivar. site	4	9212.3	2303.1	2.70	0.039
Season.site	2	17607.6	8803.8	10.31	<.001
cultivar. season. site	8	35282.0	4410.3	5.17	<.001
Residual	60	51229.3	853.8		
Total	89	187784.9			

Appendix (ii): Anova for total catechin

variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	1	5.204	5.204	0.75	
cultivar	4	118.592	29.648	4.30	0.007
season	2	194.971	97.485	14.13	<.001
site	1	24.538	24.538	3.56	0.069
cultivar.season	8	16.154	2.019	0.29	0.963
cultivar.site	4	51.335	12.834	1.86	0.144
season.site	2	22.065	11.032	1.60	0.219
cultivar.season.site	8	80.294	10.037	1.46	0.217
Residual	29	200.010	6.897		
Total	59	713.161			

Appendix (iii): Anova for EGCG

variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	4	35.144	8.786	2.92	0.038
season	2	18.244	9.122	3.03	0.064
site	1	19.757	19.757	6.56	0.016
cultivar.season	8	10.272	1.284	0.43	0.895
cultivar.site	4	17.690	4.422	1.47	0.237
season.site	2	7.176	3.588	1.19	0.318
cultivar.season.site	8	20.711	2.589	0.86	0.560
Residual	29	87.290	3.010		
Total	59	216.296			

Appendix (iv): Anova for EGC

variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	4	96.087	24.022	17.69	<.001
season	2	30.776	15.388	11.33	<.001
site	1	0.002	0.002	0.00	0.969
cultivar.season	8	11.592	1.449	1.07	0.412
cultivar.site	4	14.353	3.588	2.64	0.054
season.site	2	5.383	2.692	1.98	0.156
cultivar.season.site	8	20.560	2.570	1.89	0.100
Residual	29	39.375	1.358		
Total	59	222.232			

Appendix (v): Anova for ECG

variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	4	5.9476	1.4869	1.99	0.122
season	2	1.5968	0.7984	1.07	0.356
site	1	3.0240	3.0240	4.05	0.053
cultivar.season	8	10.9908	1.3738	1.84	0.109
cultivar.site	4	0.7859	0.1965	0.26	0.899
season.site	2	1.0016	0.5008	0.67	0.519
cultivar.season.site	8	4.1826	0.5228	0.70	0.688
Residual	29	21.6328	0.7460		
Total	59	50.2422			

Appendix (vi): Anova for EC

variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	4	9.0896	2.2724	7.58	<.001
season	2	6.1756	3.0878	10.31	<.001
site	1	0.5453	0.5453	1.82	0.188
cultivar.season	8	2.5657	0.3207	1.07	0.410
cultivar.site	4	2.9760	0.7440	2.48	0.066
season.site	2	1.0985	0.5493	1.83	0.178
cultivar.season.site	8	1.7625	0.2203	0.74	0.660
Residual	29	8.6895	0.2996		
Total	59	33.4595			

Appendix (vii): Anova for C

variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	4	0.9326	0.2332	1.46	0.240
season	2	2.4542	1.2271	7.69	0.002
site	1	0.1870	0.1870	1.17	0.288
cultivar.season	8	3.8418	0.4802	3.01	0.014
cultivar.site	4	0.3588	0.0897	0.56	0.692
season.site	2	0.3354	0.1677	1.05	0.363
cultivar.season.site	8	0.8351	0.1044	0.65	0.726
Residual	29	4.6281	0.1596		
Total	59	13.5959			

Appendix (viii): Anova for GA

variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	4	1.09248	0.27312	10.94	<.001
season	2	0.26222	0.13111	5.25	0.011
site	1	0.04161	0.04161	1.67	0.207
cultivar.season	8	0.59449	0.07431	2.98	0.015
cultivar.site	4	0.36664	0.09166	3.67	0.015
season.site	2	0.02560	0.01280	0.51	0.604
cultivar.season.site	8	0.10195	0.01274	0.51	0.838
Residual	29	0.72374	0.02496		
Total	59	3.28649			

Appendix (ix): Anova for CAFF

variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	4	16.7154	4.1789	17.18	<.001
season	2	1.8892	0.9446	3.88	0.032
site	1	1.7272	1.7272	7.10	0.012
cultivar.season	8	4.2665	0.5333	2.19	0.058
cultivar.site	4	2.9168	0.7292	3.00	0.035
season.site	2	0.3760	0.1880	0.77	0.471
cultivar.season.site	8	3.7620	0.4702	1.93	0.093
Residual	29	7.0547	0.2433		
Total	59	39.1585			

Appendix (x): Anova for anthocyanins

variation	d.f.	s.s.	m.s.	v.r.	F pr
cultivar	3	601386.3	200462.1	526.04	<.001
season	2	74524.3	37262.2	97.78	<.001
site	1	1.1	1.1	0.00	0.957
cultivar. season	6	30901.5	5150.2	13.51	<.001
cultivar. site	3	49167.5	16389.2	43.01	<.001
season. site	2	8746.6	4373.3	11.48	<.001
cultivar. season. site	6	33387.9	5564.7	14.60	<.001
Residual	24	9145.9	381.1		
Total	47	807261.2			

Appendix (xi): Raw CT values for qPCR

Relative expression software tool (rest) analysis (Log 2 transformed)

20 v 20	20 v 1	20 v 2	20 v 3	20 v 4	20 v 5	20 v 6	20 v7	20 v 8	20 v 9	20 v 10	20 v 11
Myb	0	1.06	1.34	1.89	0.36	-1.74	-1.10	1.20	0.24	0.22	-0.81
ANR 2	0	1.13	1.13	1.36	0.88	1.02	1.91	-0.69	0.23	1.11	1.09
ANR 1	0	-1.33	-0.33	1.02	0.62	1.06	-0.11	-1.25	-0.36	1.09	1.98
ANS	0	-1.19	-1.83	-1.38	-5.27	1.16	-1.62	-1.56	-0.51	-1.87	-3.84
C4H	0	1. 39	2.15	1.49	3.24	1.54	1.51	0.22	2.80	1.51	3.04
CHI	0	1.68	0.58	0.31	0.07	1.72	1.40	0.54	0.89	-2.57	0.73
CHS	0	1.37	0.96	1.18	0.71	1.40	1.03	0.39	1.48	1.47	1.16
DFR	0	0.55	0.30	0.78	0.31	1.35	0.96	-0.08	0.35	1.08	0.32
F3H	0	1.03	-0.73	0.56	0.56	1.35	0.72	-0.61	0.51	1.54	0.69
F2’5’H	0	1.02	1.49	2.50	2.49	1.22	0.98	-0.14	1.38	1.05	2.20
PAL	0	0.37	-0.66	0.21	0.55	1.06	0.79	-1.17	1.08	1.38	1.06

20 v 13	20 v14	20 v 15	20 v 16	20 v 17	20 v 18	20 v 19	20 v 20	20 v 21	20 v 22	20 v 23	20 v 24
-1.07	-1.15	-1.08	-0.19	1.25	-0.46	0.26	1.65	0.36	0.36	-0.11	1.12
1.34	1.86	1.24	1.37	1.22	1.43	0.06	1.18	1.57	-1.57	0.91	1.81
1.48	1.44	1.11	1.52	1.04	1.50	0.57	1.84	1.84	1.22	0.80	1.49
-2.04	-3.04	-1.13	-3.84	-1.37	-4.57	-1.90	-4.72	-4.72	-1.90	-0.10	-1.21
1.78	1.60	1.34	-0.24	1.96	1.00	0.81	3.34	3.34	1.70	1.30	-2.29
1.69	-0.15	1.57	1.65	1.04	1.69	1.30	1.09	1.09	1.33	1.26	1.25
1.60	1.30	0.87	1.50	1.94	2.17	1.00	1.98	1.98	1.73	1.72	2.00
1.30	1.41	0.99	0.54	0.46	1.01	0.98	1.24	1.24	0.98	1.47	1.19
1.17	1.10	1.01	1.37	0.95	2.37	1.94	0.96	0.96	1.39	1.33	1.79
2.19	1.74	-2.47	2.43	-1.14	1.02	1.40	2.39	1.39	1.57	1.23	0.94
-0.02	0.41	0.09	-0.40	1.61	1.73	1.89	136	1.36	1.68	0.45	0.78

N/B: Cultivar 20 was the reference cultivar (TRFK 6/8); TRFK 306/3was represented by numbers 1&10(hot-dry), 22& 15(cold-wet), 7&19(warm-wet) while TRFK 31/8; 2 &11(hot-dry), 5&16(cold-wet), 21&18(warm-wet) seasons inTimbilil and Kangaita sites

Appendix (xii): Preparations of 6×RNA loading dye and formaldehyde agrose-gel

- 8 µl saturated aqueous Orange G solution
- 40 µl 0.5 M EDTA, pH 8.0
- 360 µl 37% formaldehyde
- 1 ml 100% glycerol
- 1542 µl formamide
- 2 ml 10 x FA Gel Buffer
- RNase-free water to 5 ml

Appendix (xiii): Extraction buffer 1(EB1)

It consisted of 2% (w/v) cetyltrimethylammonium bromide (CTAB), 2% (w/v) polyvinylpolypyrrolidone (PVPP), 100mM (hydroxymethyl)aminomethane [Tris-HCI (pH8.0) (used from a stock of1M Tris-HCI prepared in DEPC (diethylpyrocarbonate dissolved into autoclaved water solution adjusted to pH 8.0)], 25mM ethylenediaminetetra acetic acid (EDTA) (used from a stock of 0.5M; pH 8.0)], and 2M sodium chloride [NaCI (used from a stock of 5M)], Spermidine 0.05g in 100 mls. The mixture was autoclaved. Before using was added Beta - mercaptoethanol (βME) (2%, concentration)

Appendix (xiv): Extraction buffer 11: (EB11) SSTE (Suspension Sodium Tris EDTA)

It consisted of autoclaved 1 M NaCl (1µl/200 µl of 5 M stock in 788ul DEPC); Autoclaved 10 mM Tris, (10 µl of 1 M Tris stock); Autoclaved EDTA 1mM, (2µl of 0.5 M EDTA stock). Other reagents and solutions included; chloroform: isoamyl alcohol (C.I.A) [24: 1 (v/v)]; 70% ethanol; 10MLiCl (lithium chloride); DEPC treated autoclaved water

Appendix (xv): preparation of 3.0 m sodium acetate, PH 4.8

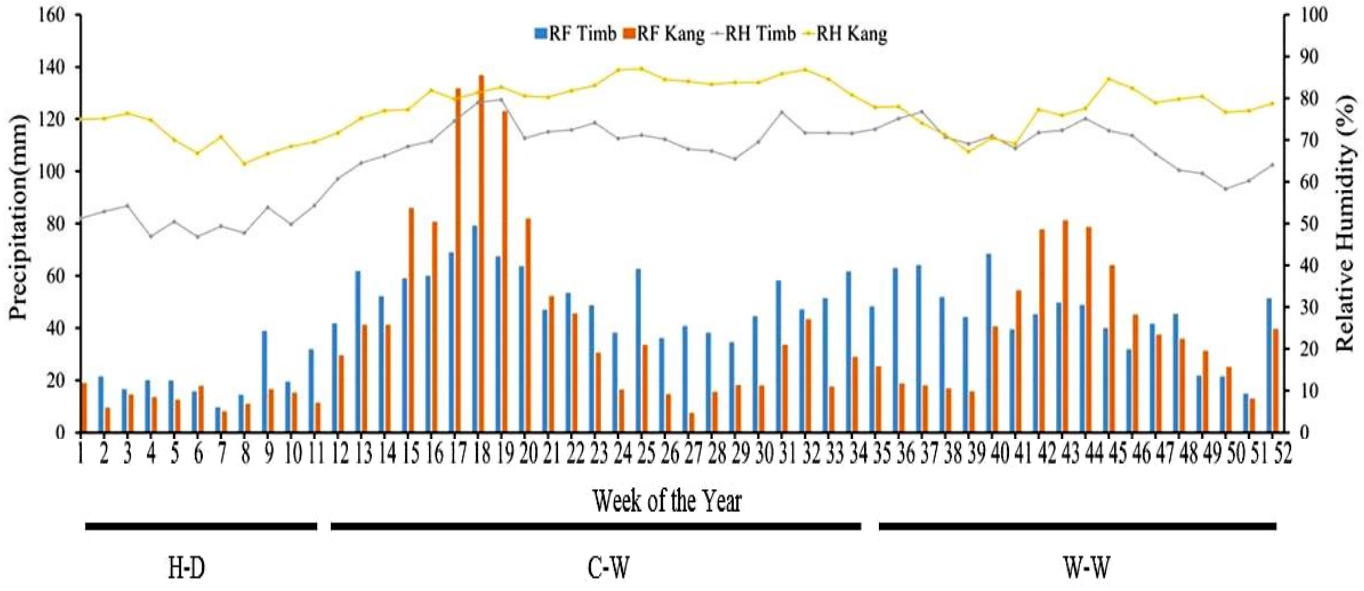
To make one liter: 408.1 g NaAce (trihydrate; gets cold in soln) about 700 mL H20 adjust pH with glacial acetic acid (takes a lot); Measure tru pH by dilution with water; range will be between 4.8 and 5.5.

Appendix (xvi): Means seasonal weather and tea auction prices during the year 2014

	Precipitation (mm)		Temp Max (°C)		Temp Min (°C)		Relative Humidity (%)	
Season	Timbilil	Kangaita	Timbilil	Kangaita	Timbilil	Kangaita	Timbilil	Kangaita
H-D	221	150	25.8	22.6	9.0	10.0	50.7	70.7
C-W	1218	1130	23.3	19.0	9.2	11.5	70.7	81.9
W-W	793	721	23.5	20.6	9.3	10.8	68.9	76.5

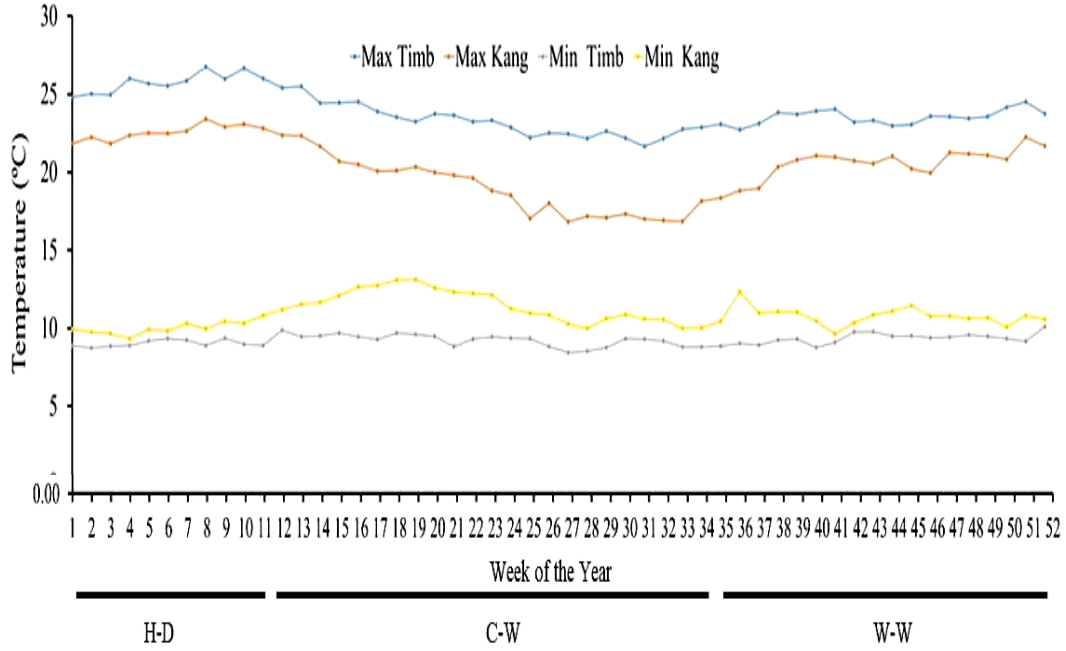
Temp Max, maximum temperature; Temp Min, minimum temperature; H-D, hot-dry; C-W, cold-wet; W-W, warm-wet

Appendix (xvii): Weekly environmental variation of precipitation andrelative humidity



NB: Precipitation and relative humidity, RF, rainfall/precipitation; RH, relative humidity; Timb, Timbilil; Kang, Kangaita; H-D, hot-dry season; C-W, cold-wet season; W-W, warm-wet season.

Appendix (xviii): Weekly environmental variation of temperatures in 2014.



NB: H-D (hot-dry), C-W(cool-wet), W-W(warm-wet)